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Enhanced and Targeted Expression of Fungal Phytase in *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. In order to express a high level of fungal phytase in *Saccharomyces cerevisiae*, various expression vectors were constructed with different combinations of promoters, translation enhancers, signal peptides, and terminator. Three different promoters fused to the phytase gene (*phyA*) from *Aspergillus niger* were tested: a galactokinase (*GAL1*) promoter, glyceraldehyde-3-phosphate dehydrogenase (*GPD*) promoter, and yeast hybrid *ADH2-GPD* promoter consisting of alcohol dehydrogenase II (*ADH2*) and a *GPD* promoter. The signal peptides of phytase, glucose oxidase (*GO*), and rice amylase 1A (*RAmy1A*) were included. Plus, the translation enhancers of the Ω sequence and UTR70 from the tobacco mosaic virus (TMV) and spinach, respectively, were also tested. Among the recombinant vectors, pGphyA-6 containing the *GPD* promoter, the Ω sequence, *RAmy1A*, and GAL7 terminator expressed the highest phytase activity in a culture filtrate, which was estimated at 20 IU/ml. An intracellular localization of the expressed phytase was also performed by inserting an endoplasmic reticulum (ER) retention signal, KDEL sequence, into the C-terminus of the phytase within the vector pGphyA-6. It appeared that the KDEL sequence directed most of the early expression of phytase into the intracellular compartment yet more than 60% of the total phytase activity was still retained within the cell even after the prolonged (>3 days) incubation of the transformant. However, the intracellular enzyme activity of the transformant without a KDEL sequence was as high as that of the extracellular one, thereby strongly suggesting that the secretion of phytase in *S. cerevisiae* appeared to be the rate-limiting step for the expression of a large amount of extracellular recombinant phytase, when compared with other yeasts.

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Phytate (*myo*-inositol hexakisphosphate), which is also known as phytic acid, is a storage form of phosphorus in plants, including soybeans, cottonseeds, and other legumes and cereals. It also plays other important roles in plants; such as 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-nutritive part of human and animal diets.

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. Accordingly, a simple feeding of inorganic phosphate as a feed additive can no longer be afforded due to its environmental impact [23]. As an alternative, the enzymatic treatment of phytate has been suggested and Nelson *et al.* [11] demonstrated that when phytase is fed to poultry, the phytin-P present in soybean meal and corn is made available and deposited in the bones of the chick. Furthermore, many countries have approved the use of phytase as a feed additive and the FDA also approved a GRAS (Generally Recognized as Safe) petition related to the use of phytase in food [23].

Although plant seeds, such as wheat, bean, rice, corn, and maize, have some phytase activity, microorganisms are a more feasible source of the enzyme. Several microbial

phytases have already been characterized and evaluated for biotechnological purposes. Among these, phytase A from *Aspergillus niger* is known to be the most active enzyme. It is an extracellular glycoprotein, and the mature enzyme has a mass of approximately 80 kDa. Several studies on the heterologous expression of fungal phytases have also been conducted. Among these, the expression of a consensus phytase gene using *Hansenula polymorpha* as the host gave a yield of 13.5 g/l, which would appear to be the highest amount of a heterologously expressed gene of interest from *H. polymorpha* [10].

The efficiency of phytase as a feed additive depends on the pH- and thermo-stability of the protein with regards to the passage through the stomach acid and the sterilization step during the feed process, respectively [26]. Thus, it is preferable if the expressed protein resides within the intracellular compartment. In addition, the oral administration of exogenous proteins appears to be more effective when they are present within the cell, due to the protective role of the host cell wall [24].

Food yeast, *Saccharomyces cerevisiae*, has been used as a host strain to express many foreign gene products. It has several advantages over other expression systems in that it consists of simple eukaryotes, the expressed products can be secreted, and it is a GRAS organism with a long history of applications for the production of a rich biomass in high quality proteins and vitamins. Consequently, it is used in livestock feeds for fish (salmonids), poultry, and fur-bearing animals, and as a food supplement for human consumption. The heterologous expression of phytase in *S. cerevisiae* was previously conducted by Han *et al.* [5]. However, the yield of recombinant phytase, when compared with those of *H. polymorpha* and *Pichia pastoris*, was significantly low [9, 10]. Accordingly, the current study attempted to improve the expression of phytase in *S. cerevisiae* using a combination of several promoters, 5' UTR, and leader sequences, and also an intracellular localization of the expressed phytase was performed by inserting an endoplasmic reticulum (ER) retention signal.

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, U.S.A.), Difco Laboratories (Detroit, MI, U.S.A.), or Boehringer Mannheim (Mannheim, Germany), respectively.

Strains and Culture Conditions

The plasmids were maintained and propagated in *E. coli* HB101 or DH5α according to Sambrook *et al.* [17]. *A. niger* NRRL 3135 was used for the *phyA* cloning, and *S. cerevisiae* 2805 (*MATα pep4::HIS3 prb1-δ Can1 GAL2 his3 ura3-52*) used as the recipient cell for phytase production [19].

For phytase expression, *A. niger* was incubated at 30°C in a medium as described previously [21]. *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 for screening the transformants at 30°C. The primary inoculum was prepared from 5 ml of the uracil selective medium cultured for 24 h and 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 filtrates were assayed for phytase activity. The induction of phytase expression was performed by supplementing with 1% galactose or 1% ethanol, according to the procedure described previously [12, 18].

Vector Construction

Total RNA was extracted from *A. niger* grown under phytase expression conditions according to the procedure described previously [8]. The *phyA*-containing sequence from the translational start codon to 7 bp downstream of the translational stop codon was amplified by polymerase chain reactions using reverse transcriptase (RT-PCR) and cloned into a pGEM-T vector according to the manufacturer's manual (Promega, Madison, WI, U.S.A.). For the construction of the yeast expression recombinant vectors, an episomal shuttle vector YEp352 was used. The *phyA* was placed between various promoters and the galactose-1-P uridyl transferase (*GAL7*) terminator. The galactokinase (*GAL1*) promoter, *GPD* (glyceraldehyde-3-phosphate dehydrogenase) promoter, and a yeast hybrid (*AG*) promoter consisting of the upstream activating sequence (UAS) of *ADH2* (alcohol dehydrogenase 2) and the *GPD* TATA element were described previously [12]. The *GAL7* terminator was kindly provided by Dr. S. K. Rhee, Korea Research Institute of Biotechnology and Biochemistry. The signal peptides of glucose oxidase from *A. niger* and amylase 1A from rice were amplified by a PCR using primers forward 5'-ATGCAGACTCTCCTTGTGAG-3', reverse 5'-TCCT-GATGTAGTGTGGCAGG-3', and forward 5'-GGATCC-GCATGCAGGTGCTGA-3', reverse 5'-GGCGGGGACT-GCCAGCCCGGCTGTCAAGTTGGAGGA-3', respectively. The intrinsic *phyA* signal peptide was replaced with that of either glucose oxidase (*GO*) from *A. niger* or amylase 1A (*RAmy1A*) from rice, using an overlap extension method [4]. The translational enhancers, such as the sequence from the tobacco mosaic virus (TMV) [3] and UTR70 from spinach [13], were also obtained using a PCR, and an overlap extension was conducted to insert a translational

enhancer between the promoter and the signal peptide. For the intracellular localization of the expressed phytase, the ER retention signal of KDEL was added to the C-terminal end of the phytase.

The different recombinant vectors were introduced into *S. cerevisiae* 2805 according to the lithium acetate procedure [7]. The stability of the introduced plasmids in yeast was measured as follows: samples grown in the nonselective medium YEPD were serially diluted with sterile H_2O to an expected 50 colony forming units (CFUs) per plate, plated on a ura⁻ selective and nonselective plate, and then the relative number of CFUs determined.

Enzymatic Assay

The phytase activity was measured according to the modified protocol of Heinonen and Lahti [6] and the corresponding cell density was also estimated using a hemacytometer. Briefly, 4 µl of 0.1 M sodium phytate was diluted with 350 µl of 0.1 M sodium acetate buffer (pH 5.5) and added to a tube containing 50 µl of the culture filtrate. The reaction mixture was further incubated at 58°C for 30 min. After the incubation, 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), vortexed for 10 sec, and then 80 µl of 1 M citric acid was added. After the reaction, the color change was measured using a Packard Spectra Count™ colorimeter (Packard Instrument Co., Downers Grove, IL, U.S.A.) at a wavelength of 405 nm. 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 µmole of phosphate per min at 58°C. The measurements were performed in triplicate and all experiments were carried out at least three times with similar results.

In order to measure the intracellular phytase activity, a cell-free crude extract of yeast was prepared as follows: the cells were grown for three days, harvested, washed twice with an extraction buffer (50 mM Tris-HCl, 2 mM EDTA), and ground three times in a bead beater (Biospec Products Inc., Bartlesville, OK, U.S.A.) for 1 min. The lysate was centrifuged (10 min at 10,000 ×g), and the supernatant was saved to measure the enzyme activity. All preparation steps were performed in a cold room (4°C).

A plate assay was modified from the method of Yoon *et al.* [27] as follows: cells were streaked on a plate and grown for 3 days in a modified PSM medium [27], 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 incubating at 30°C, an opaque precipitation around the colony was observed.

Biochemical Characteristics of Recombinant Phytase

In order to measure the thermal stability and pH optimum of the expressed phytase, the culture filtrates were pre-incubated for 1 h at an appropriate temperature and pH, and then the residual activity was determined. The thermostability from 10°C to 80°C with 10°C increments and the pH optimum from pH 1.5 to pH 8.5 with pH 1.0 increments were measured.

RESULTS AND DISCUSSION

Plate Assay of Phytase Expression

Ten to twenty transformants of *S. cerevisiae* for each recombinant plasmid were selected on a ura⁻ medium, and then these transformations were confirmed by plasmid extraction followed by back transformation into *E. coli*.

A further improvement of the recombinant strain was observed using mutagenesis followed by an efficient screening method [12]. Thus, the establishment of an efficient screening method for the expression of phytase in yeast was desirable. However, we found that the plate assay using a medium previously described for bacteria was unsuccessful in detecting the phytase expression from *S. cerevisiae*. Accordingly, several changes of medium components were tested (data not shown) and it appeared that the presence of $(NH_4)_2SO_4$ inhibited the growth of yeast. Therefore, when $(NH_4)_2SO_4$ was replaced with $(NH_4)_2NO_3$, a better growth

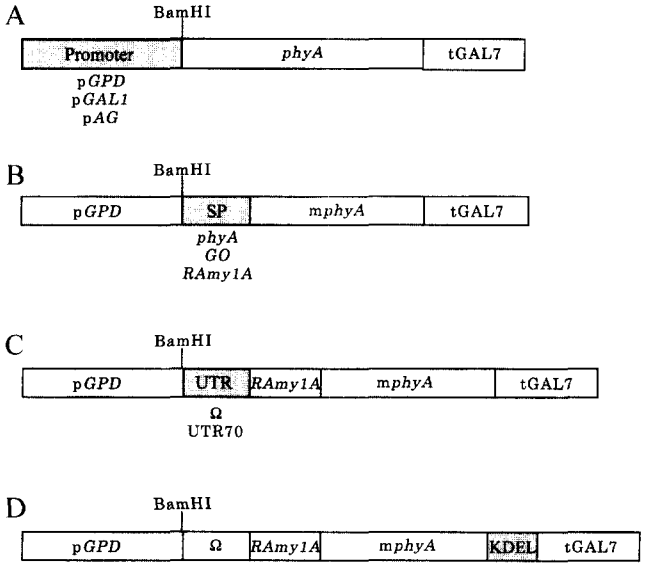

Fig. 1. Schematic diagram of transforming plasmids with various promoters (A), signal peptides (B), UTRs (C), and targeting signal KDEL (D).

The boxes represent the gene or their corresponding functional domains. The shaded box in each construct is the one compared with other substitutes. p*GPD*, p*GAL1*, and p*AG* represent the promoters of glyceraldehyde-3-phosphate dehydrogenase, galactokinase, and hybrid, respectively. *phyA*, *GO*, and *RAmy1A* are signal peptides of phytase, glucose oxidase, and amylase 1A, respectively. Ω and UTR70 represent the translation enhancers of the Ω sequence of TMV and the UTR70 from spinach, respectively.

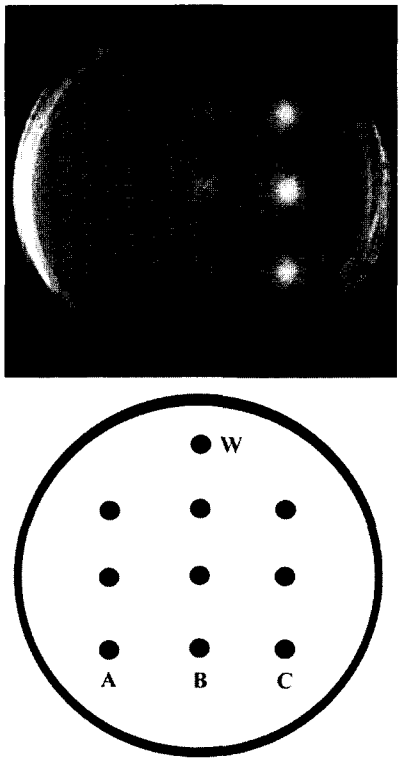

Fig. 2. Plate assay for phytase expression.
Left column (A) represents *GPD*-mediated phytase expressing yeasts, middle (B) for *GPD::GO*-mediated phytase expressing yeasts, and right (C) for *GPD::RAmy1A::*Ω-mediated phytase expressing yeasts. The recipient strain is marked as (W).

of yeast was observed, which resulted in opaque zones around the colonies expressing phytase (Fig. 2). The plate assay in this study was sensitive enough to discern differences between the transformants showing 5 IU/ml differences (refers to section on expression of recombinant phytase) (Fig. 2). The plate assay revealed no differences in the phytase activity among the recombinant yeasts transformed by the same plasmid. The plasmid of the selected transformant was stable enough, so that more than 80% of the plated cells harboured the plasmids up to 72 h after liquid cultivation (data not shown).

Expression of Recombinant Phytase

The culture filtrates of the transformant using different promoters were examined for phytase expression (Fig. 1A). In order to induce the *GAL1* promoter, 1% of galactose was supplemented in the culture media [12]. For all transformants using the three different promoters, the highest activity of *phyA* expression was obtained in the stationary phase, which occurred 3 days after cultivation. The maximum yields were 2 IU/ml, 6 IU/ml, and 8 IU/ml for the *GAL1*, *AG*, and *GPD* promoters, respectively (Fig. 3). No growth defect in the transformant was observed due to the expression of phytase (data not shown). The strength

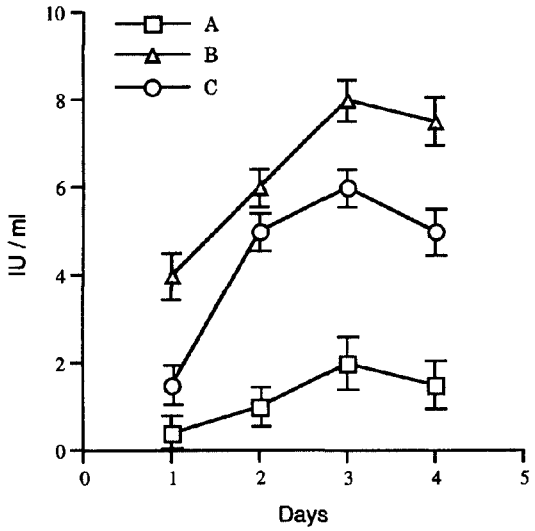

Fig. 3. Comparison of phytase expression using different promoters.
The different promoters are indicated under the shaded box of Fig. 1A. A; phytase production with *GAL1* promoter. B; phytase production with *GPD* promoter. C; phytase production with *AG* promoter. Data are means and standard deviations of three replicates.

of the promoters in this study differed slightly from previous ones [12], in that the hybrid promoter gave the highest expression level of the heterologous gene. This could have been due to the gene of interest rather than intrinsic differences in the promoter strengths.

The efficacy of the signal peptides of *GO* and *RAmy1A* was compared to that of a phytase (Fig. 1B). The signal peptide of *RAmy1A* resulted in the highest phytase activity among the three tested signal peptides (Fig. 4). The highest phytase activity was again from a 3-day culture medium and was 18 IU/ml of the culture filtrate. Meanwhile, the signal peptide from *phyA* exhibited the least enzyme activity (8 IU/ml) and that from *GO* was 13 IU/ml. Besides the signal peptide of *phyA*, signal peptides of both *GO* and *RAmy1A* have already shown an efficient secretion of target proteins in *S. cerevisiae* [12, 16]. Furthermore, the efficacy of the signal peptide of *GO* is as good as an a-factor [12], one of the most commonly used signal peptides in *S. cerevisiae* and the one used in other yeasts for phytase expression [9]. Signal peptides in eukaryotic organisms show a divergence in amino acid residues with a conserved hydrophobic core region of 6–8 amino acids. They work well in other organisms as long as they can maintain the conserved core [22]. However, it is not yet clear whether the lower efficacy in the current study was due to an intrinsic difference in the secreting capability between the signal peptides of *GO* and *RAmy1A* or a specific incidence in this study.

In order to increase the translational efficiency, two translational enhancers, Ω and UTR70, were compared

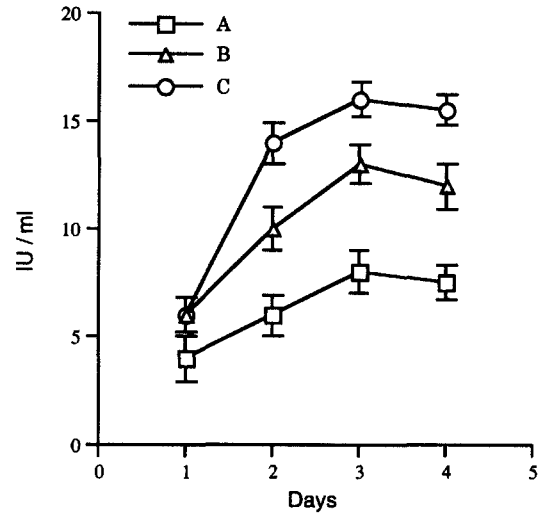

Fig. 4. Comparison of phytase expression using different signal peptides.
The different signal peptides are indicated under the shaded box of Fig. 1B. A; phytase production with intrinsic signal peptide of *phyA*. B; phytase production with signal peptide of *GO*. C; phytase production with signal peptide of *RAmy1A*. Data are means and standard deviations of three replicates.

(Fig. 1C). No difference in the phytase activity was observed when using UTR70 from spinach. In contrast, the presence of the W sequence from TMV increased the phytase activity and reached 20 IU/ml of enzyme activity, which was the highest value from all the transformants tested (Fig. 5). Although the heterologous expression level of phytase in this study was still considerably lower than those of other studies using different yeasts such as *H. polymorpha* [10] and *P. pastoris* [9], the expression of phytase in this study was higher than in previous studies using *S. cerevisiae* when more than 7-fold increase of productivity was obtained [5].

Targeted Expression of Phytase

The intracellular expression of phytase was examined with the recombinant yeasts transformed by a vector containing an ER retention signal KDEL sequence (Fig. 1D). The enzyme activity of an intracellular compartment was observed from the beginning of cultivation, which then increased as the cultivation proceeded. However, the enzyme activity from the culture filtrate started to appear after two days of cultivation, although the extracellular activity never reached more than 30% of the total activity when compared with that of the intracellular compartment (Fig. 6). Thus, it is suggested that the ER retention signal KDEL sequence can actively direct the expressed phytase inside the cell and the delayed extracellular phytase activity would seem to come from the lysed cells rather than the actively secreted enzyme. As a comparison, the intracellular activity of the recombinant yeasts transformed by the corresponding vector without a KDEL sequence (Fig. 1C) was also examined, and surprisingly the intracellular activity was as high as that of the extracellular activity, which resulted in a total of 38 IU/ml of enzyme activity. Unlike the transformant with a KDEL sequence, the extracellular enzyme activity of the transformant was observed from the very beginning of cultivation. In addition, the temporal

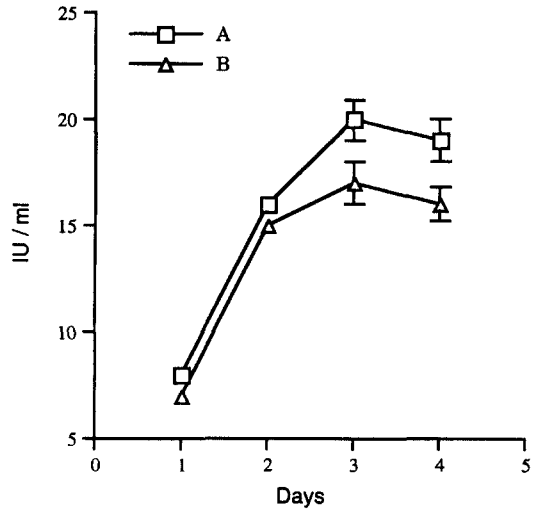

Fig. 5. Comparison of phytase expression using different 5' UTRs.
The different 5' UTRs are indicated under the shaded box of Fig. 1C. A; phytase production with Ω sequence of TMV. B; phytase production with UTR70 from spinach. Data are means and standard deviations of three replicates.

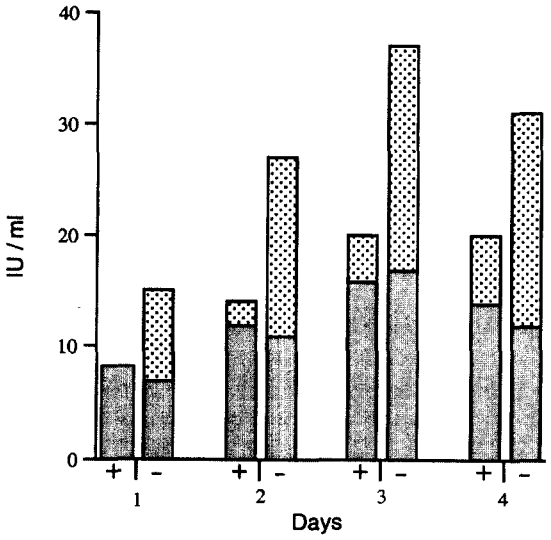

Fig. 6. Comparison of extracellular and intracellular phytase activities depending on either the presence or absence of a retension signal KDEL sequence.
The shaded and dotted bars represent the intracellular and extracellular phytase activities, respectively. The + or - indicates the presence or absence of KDEL, respectively.

expression pattern of the intracellular activity was similar to that of the extracellular activity; i.e., the intracellular activity without a KDEL sequence increased and then decreased in concordance with the extracellular activity. When compared with other studies where most of the expressed foreign proteins with appropriate signal peptides were secreted [1, 2], the present study indicated that there was still a large amount of phytase (~50% of total phytase) present within the cells. These results implied that the rate-limiting step for the expression of a large amount of extracellular phytase using recombinant *S. cerevisiae* appeared to be the secretory machinery to exit from ER, therefore, the expression of phytase was likely to further increase as the secretion capacity improved. Although the secretion pathway in yeast is generally considered as a default pathway, several studies have demonstrated that the secretory mechanism is rather an active mechanism that can enhance the transcription and/or translation of the gene of interest as a return [15, 20]. Within the ER, the correct folding of secretory proteins is required for export competency, hence, the cellular proteins involved in these events are likely to be important for efficient secretion. In addition, several genes taking part in correct folding and promoting the secretion of a target protein have been identified [15, 25]. These include BiP, PDI, and PPI encoding a binding protein, protein disulfide isomerase, and peptidyl prolyl *cis-trans* isomerase, respectively, which are currently being investigated.

As reported previously [5], the recombinant phytase showed two pH optima (2 to 2.5 and 5 to 5.5) and a temperature optimum between 55°C and 60°C (data not shown). Although the productivity lower than other expression systems was cumbersome, the biochemical characteristics of the recombinant phytase and advantages of using the GRAS organism of *S. cerevisiae* as an expression host still make this system worthy of challenge. Accordingly, this study showed that an increased phytase activity and the suggested rate-limiting step for the overexpression of extracellular phytase appeared to be very promising.

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