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Progressive Screening of Thermostable Yeasts for Phytase Production

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Abstract Biotechnological phytase preparations are commercially available and are currently used in animal feeding. However, thermostability constraints, low yields, and the high cost of the enzyme have limited its use. This study represents a new perspective for the food enzyme market. The research screened thermostable yeast strains for their ability to produce phytase. The screening was carried out with a gradual increase in temperature (30-48°C). Sixteen strains (1 strain identified as *Saccharomyces cerevisiae*) maintained the ability to produce phytase at 48°C and their phytase activity was confirmed using 2 phytase assay methodologies. The yeast strains tested in this study seem to be potential efficient producers of phytase, indicating a possible new source of thermostable phytase of commercial interest, particularly that from *S. cerevisiae.*

Keywords: phytase, thermostable, screening, yeast, *Saccharomyces cerevisiae*

Introduction

Phytases (EC 3.1.3.8 and EC 3.1.3.26) are enzymes that release phosphate groups from phytic acid that represents the main storage form of phosphorus in cereal grains, legumes, pollens, and oilseeds (1). The phosphate in phytate is unavailable and passes undigested through the digestive tract of monogastric animals and as consequence; feed for pig, poultry, and fish must be supplemented with inorganic phosphate to supply nutritional requirements for phosphorus (2,3). The phosphorus from phytate that is not used is excreted in the animal feces, and consequently turns up in the soil particularly in areas of intensive monogastric livestock production (4,5), where it can reach the water sources causing eutrophication and affecting the amount of oxygen in the water, leading to the death of fish (6).

Another alternative for the reduced availability of phosphorus is the addition of enzymes, which can increase the nutritional value of the food due to phytate hydrolysis during its digestion in the stomach or during food processing. The improvement of the protein digestibility and availability of phosphorus and of other minerals usually chelated by phytate, such as iron and zinc (7), diminishes the anti-nutritive properties of phytate and environmental pollution. Phytases are produced by a wide range of sources (1,8). Phytase activities have been described for a number of plants, animals, fungi, and bacteria but, many possible new sources of phytases continue unexplored (9).

The most frequently desired characteristics for new phytases are thermostability, in order to survive feed manufacturing processes, and acid stability, that would make the enzyme stable in monogastric stomachs, since commercial phytases are generally relatively heat and acid stable (10), and according to Selle and Ravindran (11), the ideal enzyme should have high catalytic specificity, resistance to proteolysis and good stability at room temperature.

On a commercial scale, phytase production is derived from mutated fungal strains or the use of recombinant DNA technology. Three phytases commonly used for application in animal feeding are derived from *Aspergillus niger*, which is a 3-phytase fungus, or from *Peniophora lycci,* and *Escherichia coli,* which are 6-phytase organisms (11,12), to improve phosphorus and mineral availability. However, no corresponding approved enzyme is available for human consumption. The use of yeasts may be an alternative to improve the mineral state in vulnerable human populations (13).

Phytase from baker's yeast has been studied in some detail (14). The phytase from the yeast *Saccharomyces cerevisiae* is generally recognized as safe (GRAS, as defined by U.S. Food and Drug Administration) for food production (15). It would thus be an ideal candidate for use as a bioavailability improving food additive, if it demonstrate significant phytase activity (13). Some studies about other phytases produced by yeasts are available (16-19). The process based on recombinant yeast strains expressing different fungal phytases has currently become a subject of interest (20-24).

Yeasts have capacity to adapt their metabolism to anaerobic and to facultative anaerobic conditions (25). These microorganisms are extremely stable presenting fast proliferation, resistance to infection and relatively simple fermentative process. The aim of the current research was to study and select yeast strains that could be considered as potential producers of thermostable phytase. This study was innovative since it considered yeast screening with a gradual increase in temperature, which is an important parameter, since thermostability is an important property in the application of these enzymes in animal feeding and food processing.

Materials and Methods

Isolation and identification of microorganisms Soil samples were collected from different areas in the states of São Paulo and Rio Grande do Sul. Ten mL aliquots of

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sterilized water were added to 1 g of soil sample and incubated in a rotary shaker (TECNAL TE 421; Piracicaba, SP, Brazil) at 30°C (100 rpm for 24 hr). The cultures were inoculated into petri plates containing yeast malt agar (YMA) and incubated at 28°C for 48 hr for yeast isolation and growth, which was visually identified as yeast growth. The purified yeast strains were maintained in assay vials containing YMA and stored in the refrigerator at 4°C until used.

Screening for potential hydrolyzing phytate yeasts using progressive temperature increase The yeast strains isolated (total of 140 strains), were tested for phytase production in solid medium. The screening was carried out on agar plates containing calcium phytate as sole phosphorus source (26). The phytase screening medium (PSM) agar was composed as follows (g/L): glucose, 15.0; calcium phytate, 1.0; $(NH_4)_2SO_4$, 5.0; $MgSO_4\ 7H_2O$, 0.5; KCl, 0.5; $FeSO_4\ 7H_2O$, 0.01; $MnSO_4\ 1H_2O$, 0.1; and agar 15.0; and made up to volume with distilled water. The pH was adjusted to 5.5 using 0.1 N NaOH and then autoclaved at 121°C and 1 atm for 15 min. The inoculated plates were incubated at 30°C for 120 hr. During this time, colony growth and phytase production were observed in the plates.

This primary screening in solid media was carried out with a gradual increase in temperature, and then all positive strains were reinoculated in PSM agar and incubated at 35°C for 5 days. This process was repeated at 38, 40, 42, 45, and 48°C in order to screen for the thermostable yeast strains able to produce phytase. The strains screened at 48°C were maintained in YMA tubes and stored at 4°C until assayed for phytase. Calcium phytate was acquired from Sigma-Aldrich (Steinheim, Germany).

Preinoculum preparation and culture conditions for phytase production The preinoculum was prepared suspending the spores from YMA culture in 2.5 mL of sterilized water. This suspension was used to inoculate the culture medium. The culture medium (g/L) was composed of sucrose, 10.0; sodium phytate, 0.5; $(NH_4)_2SO_4$, 3.0; $MgSO_4\ 7H_2O$, 0.5; KCl, 0.5; $FeSO_4\ 7H_2O$, 0.001 $MnSO_4\ H_2O$, 0.0075, and $CaCl_2$ 0.1, the pH adjusted to 4.5 and autoclaved at 121°C and 1 atm for 15 min. A sample of 15 mL of culture medium and 1 mL of preinoculum were added to conical flasks. The cultures were incubated in a rotary shaker at 35°C, (150 rpm) for 96 hr. After culture development, the tubes were centrifuged (7,100×g) at 10°C for 15 min in a Beckman Coulter Allegra X-22R Centrifuge (Fullerton, CA, USA), and the culture supernatant used directly for the enzyme assay. The phytase assay was repeated after 24, 48, 72, and 96 hr. Sodium phytate was acquired from Sigma-Aldrich.

Phytase assay Phytase activity was determined by the method proposed by Stockamnn *et al.* (23) using a synthetic substrate for phosphatases. This reaction was based on the hydrolysis of 4-nitrophenylphosphate to phosphate and *p*-nitrophenol. The substrate (1,000 µL of 5 mM 4-nitrophenylphosphate disodium salt hexahydrate) was added to a mixture of 500 µL of 0.8 M sodium acetate buffer (pH 5.0) and 500 µL of enzyme preparation. The tubes were incubated at 37°C for 10 min and the reaction was stopped by the addition of 2,000 µL of 0.1 N NaOH. The *p*-nitrophenol liberated was determined colorimetrically at 410 nm in a Beckman Coulter DU640 Spectrophotometer (Fullerton). A calibration curve was plotted for *p*-nitrophenol concentrations of from 0.0719 to 7.1891 µM. Natuphos was used to adapt the methodology.

Phytase activity was also measured by following the liberation of inorganic phosphate in the presence of sodium phytate. Free inorganic phosphate was assayed in the culture supernatant based on the phosphate concentration released after hydrolysis of the sodium phytate by phytase (27). Each assay tube contained 600 µL of 2 mM sodium phytate in 0.1 M Tris-HCl (pH 7.0) supplemented with 2 mM of $CaCl_2$ and 150 µL of enzyme solution, and was incubated at 37°C for 30 min. After incubation, the reaction was stopped by the addition of 750 µL of 5% trichloroacetic acid and then, 1.5 mL of the color reagent was added producing phosphomolybdate. The inorganic orthophosphate (Pi) was determined colorimetrically at 700 nm in a Beckman Coulter DU640 Spectrophotometer. The color reagent was prepared daily by mixing 4 volumes of 1.5%(w/v) ammonium molybdate solution with 5.5%(v/v) sulfuric acid and 1 volume of 2.7%(w/v) ferrous sulfate solution. The results were compared with a standard curve prepared with inorganic phosphate (K_2HPO_4 concentrations of 0.0448 to 2.8706 µM). Natuphos was used to adapt the methodology. One unit of phytase activity (U) was defined as the amount of enzyme capable of releasing 1 µmol of inorganic phosphate/min under the defined reaction conditions. All analyses were carried out in triplicate and the results presented as mean±standard deviation (SD). The Tukey test was performed to determine significant differences between the mean values for the fermentation times ($p \leq 0.05$).

Synthetic substrate for phosphatases was purchased from Sigma-Aldrich and *p*-nitrophenol was acquired from Vetec (São Paulo, SP, Brazil). The commercial phytase Natuphos was kindly donated by Basf.

Results and Discussion

Screening for potential hydrolyzing phytate yeasts using progressive temperature increases A total of 140 yeast strains were screened for their ability to hydrolyse calcium phytate in solid media with a gradual increase in temperature. The insolubility of calcium phytate in aqueous media gives a white turbidity to an agar plate, and the appearance of a clear area or disappearance of the precipitate is a visual indication of phytase production (26). Currently there are still few screening studies about yeast strain phytase producers, but the present results were interesting when compared to the few available in the literature. It is notable that no such research with a gradual increase in temperature was found. The vast majority of the yeast strains already presented growth (Fig. 1a) after 24 hr at all the temperatures tested (data not presented), and less than 5% of the total strains evaluated in the present study were unable to grow on solid phytase screening media. Furthermore, in the study carried out by Sano *et al.* (18), the majority of the strains tested (about 1,200 yeasts) did not grow on medium containing sodium phytate as the sole source of phosphate and carbon.

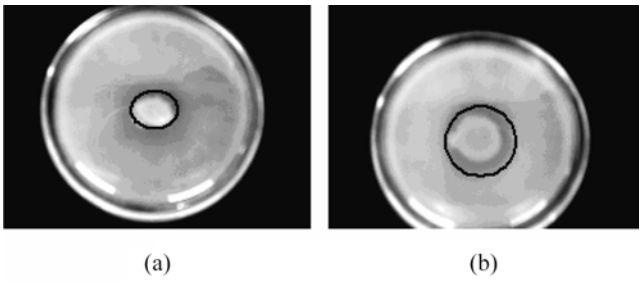

Fig. 1. Screening for potential hydrolyzing phytate yeasts in solid media. (a) Plates showing growth, (b) translucent halo of hydrolysis originated by phytase production.

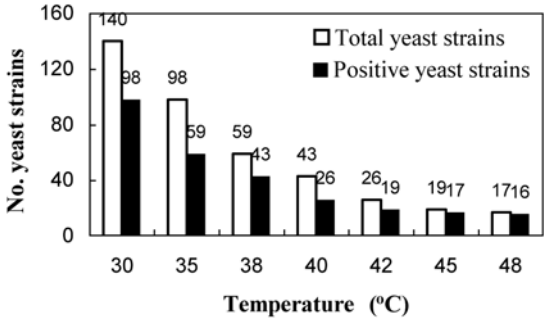

Fig. 2. Yeast strains tested and those positive for phytase production at different temperatures.

The absence of yeast growth on solid medium containing phytate has already been evidenced by other authors (18,28) and can be explained by one or more of the following reasons, depending on the characteristics of each yeast strain: very high temperature, inadequate pH, high phytate concentration in the medium or even the absence of phytase production and consequent lack of available nutrients and important minerals for microbial growth.

Phytase production in solid media can be visualized in Fig. 1b, showing a clear halo of phytate hydrolysis around the growth. Similar to growth, the vast majority of the strains hydrolyzed the phytate after 24 hr, but no relationship between the gradual increase in temperature and the time required for medium hydrolysis could be established. While some strains lost the capacity or required more time to hydrolyse the phytate with increase in temperature, other yeasts produced the enzyme in a shorter time when submitted to high temperatures, evidencing an increase in metabolism. In general, considering all the strains producing phytase at the experimental temperatures, the number of positives strains decreased as the temperature increased (Fig. 2). About 11% of the yeasts (16 strains) maintained the ability to produce phytase at the highest temperature (48°C). Amongst these, 87.5% of the strains hydrolyzed phytate after 24 hr.

Lambrechts *et al.* (28) observed the phytase production by 21 yeast strains in solid medium with 0.5% calcium phytate during 5 days at 28°C. Four strains presented a weak transparent halo around the colonies and 8 showed very limited growth and absence of the characteristic hydrolysis halo. Only 9 strains (43% of the total) hydrolyzed phytate and they produced a translucent zone 2 times smaller than the diameter of the colonies. This percentage was small when compared to that found in the present study, where about 70 and 94% of the yeasts showed phytase production at 30 and 48°C, respectively.

In another study on phytase producing fungi (29), although 2 strains presented translucent zones around the colonies in solid medium, no activity was detected in liquid medium. According to Chelius and Wodzinski (30), in this case this was due to acid production by the *Aspergillus*, with the formation of a translucent zone around the colonies.

The last parameter analyzed in the screening was the phytate hydrolysis intensity as evidenced by the dimension

Table 1. Phytase production and phytate hydrolysis at 48°C[1)]

Strain	24 hr	48 hr	72 hr	96 hr	120 hr
03	+	+	+	+	+
S. cerevisiae	++	++	++	+++	+++
17	+	+	+	+	+
34	+	+	+	+	+
38	+	+	+	+	+
40	+*	+	+	+	+
44	-	+	+	+	+
109	+	+	+	+	+
113	+	+	+	+	+
119	++	++	++	++	++
124	+	+	+	+	+
128	+	+	+	+	+
146	+	+	+	+	+
163	-	-	-	-	-
164	+	+	+	+	+
166	-	-	-	+	+
167	+*	+	+	+	+

[1)]-, absence of hydrolysis; +*, small sign of hydrolysis under the colony; +, hydrolysis diameter ≤2.0 cm; ++, 2.0 cm < hydrolysis diameter < 3.0 cm; +++, hydrolysis of practically all of the medium; ++++, hydrolysis of all of the medium.

Table 2. Phytase activity with 4-nitrophenylphosphate disodium salt hexahydrate substrate

	Phytase activity (U/mL)[1)] in different incubation periods			
Yeasts strains	24 hr	48 hr	72 hr	96 hr
03	0.04 (<0.01)[a]	0.03 (<0.01)[b]	0.03 (<0.01)[b]	0.03 (<0.01)[b]
S. cerevisiae	0.06 (0.01)[a]	0.04 (<0.01)[a]	0.07 (0.01)[a]	0.04 (0.01)[a]
17	0.03 (0.01)[a]	0.03 (<0.01)[a]	0.04 (0.01)[a]	0.04 (0.01)[a]
34	0.05 (<0.01)[a]	0.05 (0.01)[a]	0.05 (0.01)[a]	0.05 (0.01)[a]
38	0.06 (0.01)[a]	0.08 (0.01)[a]	0.08 (0.01)[a]	0.08 (0.03)[a]
40	0.06 (0.01)[a]	0.08 (0.01)[a]	0.05 (0.01)[a]	0.06 (0.02)[a]
44	0.04 (0.01)[a]	0.05 (<0.01)[a]	0.05 (<0.01)[a]	0.04 (<0.01)[a]
109	0.03 (0.01)[a]	0.04 (0.01)[a]	0.06 (0.01)[a]	0.05 (0.01)[a]
113	0.02 (<0.01)[a]	0.03 (<0.01)[ab]	0.04 (<0.01)[b]	0.03 (0.01)[ab]
119	0.06 (0.01)[a]	0.13 (0.01)[b]	0.08 (<0.01)[ab]	0.09 (0.02)[ab]
124	0.03 (0.01)[a]	0.04 (<0.01)[a]	0.06 (0.01)[a]	0.05 (0.01)[a]
128	0.03 (0.01)[a]	0.04 (0.01)[a]	0.04 (0.01)[a]	0.05 (0.01)[a]
146	0.03 (0.01)[a]	0.03 (0.01)[a]	0.03 (0.01)[a]	0.03 (0.01)[a]
164	0.06 (0.01)[a]	0.05 (0.01)[a]	0.04 (0.01)[a]	0.04 (<0.01)[a]
166	0.03 (0.01)[a]	0.05 (<0.01)[a]	0.07 (0.01)[b]	0.05 (0.01)[a]
167	0.03 (0.01)[a]	0.04 (0.01)[a]	0.04 (0.01)[a]	0.05 (<0.01)[a]

[1)]Mean and SD presented in brackets; Different superscript letters in same line indicate significant difference between means ($p \leq 0.05$).

of the transparent halo produced, and a possible relationship with enzyme concentration. Table 1 shows the intensity of the hydrolysis produced by the strains at 48°C, being indicated by signs going from (-) to (++++), representing the absence of hydrolysis and hydrolysis of the entire medium, respectively. Fourteen strains displayed a translucent zone whose diameter was narrower than 2.0 cm, and 1 strain showed greater hydrolytic capacity than the others and a particularly vigorous growth at all the temperatures tested. This strain was identified by molecular taxonomy as *S. cerevisiae* strain zi (EU188613). This strain is deposited in CBMAI (Coleção Brasileira de Microrganismos de Ambiente e Indústria) collection. *S. cerevisiae* shows useful properties, as resistance against high sugar and alcohol (31), and a higher growth rate with higher culture temperature (32).

Culture and phytase activity Assays for phytase activity include hydrolysis using organic and synthetic substrates, such as sodium phytate and 4-nitrophenylphosphate (33). Although 4-nitrophenylphosphate has been described as a phytase substrate, research on selectivity has shown phytate to be the preferred substrate (27,34,35). Phytase activity was assayed using both sodium phytate and 4-nitrophenylphosphate as substrates for the 16 yeast strains selected at 48°C. The results are presented in Table 2 and 3, respectively. The enzymatic activity of all the yeast strains tested that produced phytase at 48°C was confirmed by both methodologies at all the times tested, except for Strain 3. No phytase activity was detected for this strain when sodium phytate was used as the substrate.

The vast majority of the yeast strains tested showed best enzyme activities with sodium phytate as the substrate. According to previously observed for the phytase produced by *Schwanniomyces castelli* (36). Particularly, a clear substrate preference for sodium phytate was shown in the tests with *S. cerevisiae*, the activities determined in the medium with 4-nitrophenylphosphate being about 2.8 times smaller.

The phytase activities were determined after 24, 48, 72, and 96 hr. It was not possible to establish a direct relationship between the culture time in the shaker and phytase activity. Some yeast strains presented their highest activity after 24 hr while others presented it after 96 hr, but the majority of the strains showed no significant differences in their phytase activities between the 4 incubation times.

There is no international unit to express phytase activity, once this depends on the enzyme assay used, such as the substrate concentration, temperature, and pH, it being difficult to compare the efficacy of different phytase sources (11). In the present study, one phytase activity unit (U) was defined as the amount of enzyme to release 1 μmol of inorganic phosphate/min under the reaction conditions defined.

The yeast strain in which the highest enzymatic activities were detected in sodium phytate was *S. cerevisiae* (0.15 U/mL at 24 and 96 hr) and the Fig. 3 shows the levels of phytase activity determined in the 2 substrates at all the fermentation times for this strain.

S. cerevisiae also presented particularly vigorous growth in the solid medium containing phytate, and a high capacity for phytate hydrolysis. The relative extension of the translucent halo produced by hydrolysis was estimated and correlated with phytase activity. Sano *et al.* (18) had already established a similar relationship for phytase production by *Arxula adeninivorans*.

The enzymatic activities determined for *S. cerevisiae* were higher than the initial rate of phosphate released in the presence of sodium phytate of 0.138 mM by the phytase from *S. castelli* (0.025 μmol/min/mL). They also showed that the phytase from *S. castelli* hydrolyzed phytic acid in other substances. The initial phosphate release rates from wheat bran and cotton flour were 0.024 and 0.026 μmol/min/mL, respectively. The hydrolysis was performed

Table 3. Phytase activity with sodium phytate as the substrate

Yeast strains	Phytase activity (U/mL)[1] after different incubation periods			
	24 hr	48 hr	72 hr	96 hr
03	UD[2]	UD	UD	UD
S. cerevisiae	0.15 (<0.01)[a]	0.13 (<0.01)[a]	0.13 (0.01)[a]	0.15 (0.01)[a]
17	0.04 (0.01)[a]	0.02 (0.01)[a]	0.03 (0.01)[a]	0.04 (0.01)[a]
34	0.07 (0.01)[a]	0.11 (0.01)[a]	0.06 (<0.01)[a]	0.08 (0.02)[a]
38	0.06 (0.01)[a]	0.06 (0.01)[a]	0.06 (0.01)[a]	0.06 (<0.01)[a]
40	0.06 (<0.01)[a]	0.07 (<0.01)[a]	0.06 (<0.01)[a]	0.07 (<0.01)[a]
44	0.06 (0.01)[a]	0.07 (0.01)[a]	0.06 (<0.01)[a]	0.06 (0.01)[a]
109	0.06 (0.01)[a]	0.06 (<0.01)[a]	0.06 (0.01)[a]	0.06 (<0.01)[a]
113	0.06 (<0.01)[a]	0.05 (0.01)[a]	0.06 (0.01)[a]	0.06 (<0.01) [a]
119	0.09 (0.01)[a]	0.11 (0.01)[a]	0.12 (0.03)[a]	0.11 (0.01)[a]
124	0.05 (0.01)[a]	0.05 (<0.01)[a]	0.05 (0.01)[a]	0.06 (0.01)[a]
128	0.06 (<0.01)[a]	0.05 (0.01)[a]	0.06 (0.01)[a]	0.06 (0.01)[a]
146	0.07 (<0.01)[a]	0.06 (<0.01)[a]	0.06 (<0.01)[a]	0.06 (<0.01)[a]
164	0.06 (0.01)[a]	0.05 (0.01)[a]	0.07 (<0.01)[a]	0.06 (<0.01)[a]
166	0.07 (0.01)[a]	0.06 (<0.01)[a]	0.07 (<0.01)[a]	0.06 (0.01)[a]
167	0.06 (0.01)[a]	0.07 (<0.01)[a]	0.07 (0.01)[a]	0.06 (<0.01)[a]

[1]Mean and SD in brackets; Different superscript letters in the same line indicate significant difference between the means ($p \leq 0.05$).
[2]Undetectable level.

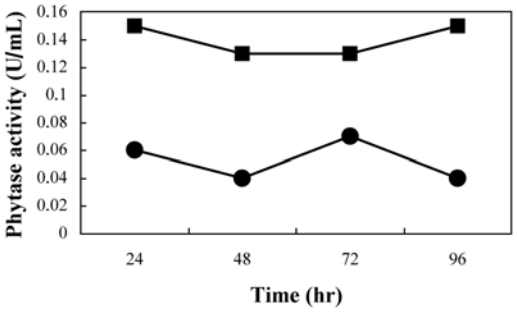

Fig. 3. *Saccharomyces cerevisiae* phytase activities (U/mL) in the different substrates. -●-, 4-Nitrophenylphosphate; -■-, sodium phytate.

at pH 4.4 and 70°C, which were the optimal conditions of the previously studied phytase from *S. castelli* (36).

Considering the phytase activities measured by Sano *et al.* (18) at 42°C for 11 yeasts, only 1 yeast strain (*Arxula adeninivorans* CBS 8335) showed enzyme levels superior to those detected for *S. cerevisiae* in the present research. Moreover, when compared to phytase producing fungi (29), the enzyme levels detected for *S. cerevisiae* were less than 60% of the phytase activities obtained from *Aspergillus* spp. when tested at pH 5.5. In study of phytase properties from *Bifidobacterium animalis*, the cells did not grow in shaking culture (37).

Other research on *S. cerevisiae* phytase activity was carried out in the context of yeast degrading inositol hexaphosphate (IP6), and showed the importance of the study of this promising phytase source. Andlid *et al.* (13) studied *S. cerevisiae* phytase activity as well as the phosphatase (PHO) system and IP6 as the substrate. The PHO system seemed to be aimed at yeast growth in the absence of inorganic phosphate. The yeast genes were all expressed as *A. niger*. The experiments showed that *S. cerevisiae* was well adapted to use extracellular IP6 as the phosphorus source and that repression of the IP6-degrading enzymes was not only dependent on the extracellular concentration of inorganic phosphate, but also on the pH and medium composition.

Veide and Andlid (15) investigated different strategies to increase extracellular phytase activity in a *S. cerevisiae* strain, and assessed the relative IP6 degradation capacities of different improved strains using only *S. cerevisiae* genes, by modifications of the PHO system. In this study, the most effective strain was YD80 (pPHO5) (deletion of the negative regulator PHO80 combined with over expression of PHO5), which showed a 9-fold increase in biomass specific phytate degradation as compared to the de-repressed wild-type.

In the future, a high-phytase *S. cerevisiae* strain, with corresponding modifications and preferably without the use of any heterologous DNA, might be a suitable organism for food applications such as traditionally fermented foods or for producing new kinds of low-phosphate (low-IP6) foods (15). *S. cerevisiae* was selected due to its performance and promising results, to continue the studies on the characterization and optimization of phytase production.

Considering that the enzymatic activities measured for *S. cerevisiae* after 24 and 96 hr did not differ significantly, the phytase production will be carried out at 24 hr. This has special significance in the fermentative process in order to reduce costs and increase enzyme production. Obtaining phytase from *S. cerevisiae* is of particular interest, since this yeast is recognized as safe for food production and potential applications.

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