Reduction of Phytate Content in Wheat Bran and Glandless Cotton Flour by *Schwanniomyces castellii*

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After verification that the phytase from *Schwanniomyces castellii* was able to hydrolyze the phytic acid in soft wheat bran and in glandless cotton flour, the ability of the strain to develop in a medium containing one of these two substances as sole phosphate source was tested. Growth was satisfactory in both cases, and 90–95% of phytic acid was hydrolyzed.

INTRODUCTION

Phytic acid [*myo*-inositol-hexakis(dihydrogen phosphate)] salts, or phytates, are known as the major storage form of phosphate in plants (Graf, 1986). However, this large source of phosphorus is not metabolized by monogastric animals. Furthermore, phytic acid is considered to be an antinutritional factor since it reduces the bioavailability of proteins and minerals by chelation (Cheryan, 1980). Hydrolyzing phytic acid is therefore a useful way of increasing the nutritional value of many plant foodstuffs. The enzymes that catalyze the conversion of phytic acid to inositol and inorganic phosphate are known as phytases. Phytase-producing microorganisms include bacteria (Powar and Jagannathan, 1982), fungi (Gibson and Ullah, 1990), and yeasts (Nayini and Markakis, 1984).

Schwanniomyces castellii was chosen after a survey of different strains of yeasts for phytase production (Lambrechts *et al.*, 1992). Biosynthesis (Lambrechts *et al.*, 1993) and properties (Segueilha *et al.*, 1992) of the phytase from *S. castellii* had been studied previously. The present work was undertaken to evaluate the ability of the strain to hydrolyze phytic acid from two plant products with high phytate contents: soft wheat bran and glandless cotton seed flour.

MATERIALS AND METHODS

Organism. The strain used is listed at the Centraal bureau voor schimmelculture (Delft) as *S. castellii* Capriotti CBS 2863. Under the new classification system it is included in the species *Schwanniomyces occidentalis* Klöcker (Kreger Van Rij, 1984).

Growth Media and Culture Conditions. The MSA medium had the following composition per liter: $(NH_4)_2SO_4$, 3 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; KCl, 0.5 g; $CaCl_2$, 0.1 g; $MnSO_4 \cdot H_2O$, 7.5 mg. Mineral salts were autoclaved separately at 120 °C for 30 min. Vitamins, trace elements, and $FeCl_3$ were sterilized by filtration through an HA 0.45-μm Millipore membrane and added at the same concentrations as in G medium described by Galzy (1964). Soft wheat bran and glandless cotton seed flour (obtained by direct hexane extraction) were not sterilized. A 100 g L^{-1} soluble starch (Prolabo) solution was autoclaved separately for the culture with wheat bran. Cultures were performed in a Braun fermenter with a useful capacity of 2.5 L. The pH was adjusted at 4.0 by the addition of NaOH (2 N) or H_2SO_4 (2 N), and the temperature was maintained at 30 °C. The biostat was aerated at 4 vvm (volumes of air/(volume liquid) × min) and agitation maintained at 1000 rpm.

Analytical Methods. *Growth.* This was monitored by hematimeter cell count.

Inorganic Phosphate. Free inorganic phosphate was assayed in the culture supernatant using the ammonium molybdate method (Heinonen and Lahti, 1981) as modified by Lambrechts *et al.* (1992). Phosphate concentrations from 0.10 to 5 mM were measured.

Phytic Phosphate. The phytic phosphate content of the culture medium was assayed by determination of the phosphate concentration released after hydrolysis of phytate by the phytase of *Aspergillus ficuum* (Sigma) as described by Lambrechts *et al.* (1992). A calibration curve was plotted for sodium phytate (Sigma) concentrations from 0.01 to 0.5 mM.

Inositol Phosphates. Inostiol tri- (IP3), tetra- (IP4), penta- (IP5), and hexaphosphate (IP6, phytic acid) were separated by ion-pair C_{18} reversed-phase HPLC (Merck), using the method of Sandberg and Ahderinne (1986) as modified by Segueilha *et al.* (1992). Only inositol hexaphosphate was determined quantitatively (from 0.1 to 10 mM) because the other compounds were too expensive for a calibration curve to be established.

Inositol phosphates were extracted from wheat bran and cotton flour by agitation with 0.50 M HCl (20 mL/0.5 g of substrate) for 3 h at ambient temperature. The mixture was centrifuged at 12000*g* for 15 min and the supernatant evaporated to dryness under vacuum at 40 °C. The dry extract obtained was resuspended in 15 mL of 0.025 N HCl and loaded on anion-exchange resin (SAX, Extra Sep, 500 mg) that was then washed with the same volume of 0.025 N HCl (0.75 mL min^{-1}). Inositol phosphates were eluted from the resin using 25 mL of 2 N HCl (0.75 mL min^{-1}). The resulting extract was evaporated to dryness, resuspended in 2 mL of double-distilled water, and filtered at 0.45 μm being injection into the chromatographic system.

Carbohydrates. Residual carbohydrate contents were assayed in the culture supernatant.

During culture with cotton flour, glucose, fructose, melibiose, and raffinose were separated and quantified by HPLC using a carbohydrate column (Waters, Part 84038). The eluent was a mixture of acetonitrile (80%) and water (20%). Flow rate was 2 mL min^{-1}. Detection was performed by refractometry. Sample carbohydrate contents had to be between 1 and 10 g L^{-1}.

During culture with starch, total carbohydrates were assayed after enzymatic hydrolysis. A 0.9-mL measured amount of sample containing a maximum of 10 g L^{-1} starch was mixed with 0.1 mL of a 1/25 solution of amyloglucosidase (Amigase from Rapidase). After 2 h of reaction at ambient temperature, the glucose released was assayed according to the enzymatic method described by Bergmeyer *et al.* (1974).

Enzyme Preparation and Assay. Crude enzymatic extracts were prepared as described by Lambrechts *et al.* (1992). Two fractions were obtained: ultrafiltered culture supernatant and ultrafiltered crushing supernatant. Phytase activity was assayed in both fractions at 37 °C using the method of Ullah and Gibson (1987). One phytase activity unit is defined as the amount of enzyme that releases 1 μmol of phosphate/min.

Purified extract of phytase was obtained as described by Segueilha *et al.* (1992).

RESULTS

Determination of Substrate Phytic Acid Contents. Phytic acid (IP6) content per gram of substance determined by HPLC was 45 μmol (±2) in wheat bran and 61

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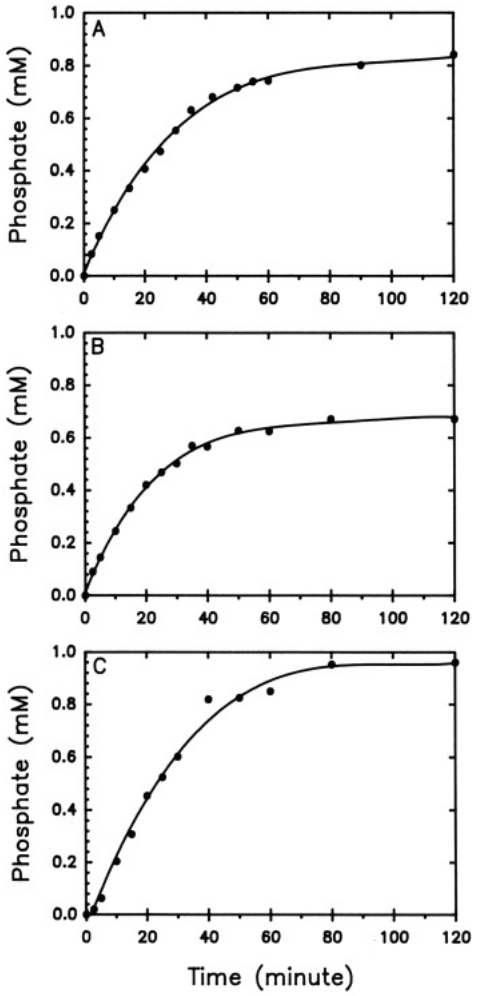

Figure 1. Kinetics of phosphate release during hydrolysis of different substrates by phytase (0.025 unit mL^{-1}) from *S. castellii*, pH 4.4, T = 70 °C: (A) sodium phytate (0.138 mM); (B) soft wheat bran (2.5 mg/mL^{-1}); (C) glandless cotton flour (2.5 mg mL^{-1}).

μmol (±3) in cotton flour. In both cases, chromatographic analysis revealed small amounts of inositol pentaphosphate (IP5). The IP5 peak area was less than 10% of that of the phytic acid peak. Tri- and tetraphosphate inositol were not detected.

***In Vitro* Hydrolysis of Phytic Acid by Purified Extract of Phytase of *S. castellii* (Figure 1).** The study was performed by monitoring phosphate release during hydrolysis of wheat bran and cotton flour phytates by purified extract of phytase of *S. castellii*. Hydrolysis was performed at pH 4.4 and 70 °C; these are optimal conditions for enzyme functioning (Segueilha *et al.*, 1992). The medium contained 0.025 unit mL^{-1} (enzyme unit measured at 70 °C) phytase and 2.5 mg mL^{-1} wheat bran (i.e., 0.112 mM phytic acid) or cotton flour (i.e., 0.153 mM phytic acid). One hydrolysis was also realized in the presence of 0.138 mM sodium phytate.

The initial rate of phosphate release in the presence of sodium phytate (Figure 1A) was 0.025 μmol min^{-1} mL^{-1}. The 0.84 mM final phosphate content corresponds to the release of 6 mol of phosphate/mol of phytic acid, that is to say, complete hydrolysis of the latter. With wheat bran (Figure 1B) and cotton flour (Figure 1C), the initial phosphate release rates of 0.024 and 0.026 μmol min^{-1} mL^{-1}, respectively, were very similar to that observed previously. The final phosphate contents (0.69 mM with wheat bran and 0.96 mM with cotton flour) corresponded to complete hydrolysis of phytic acid, as with sodium phytate.

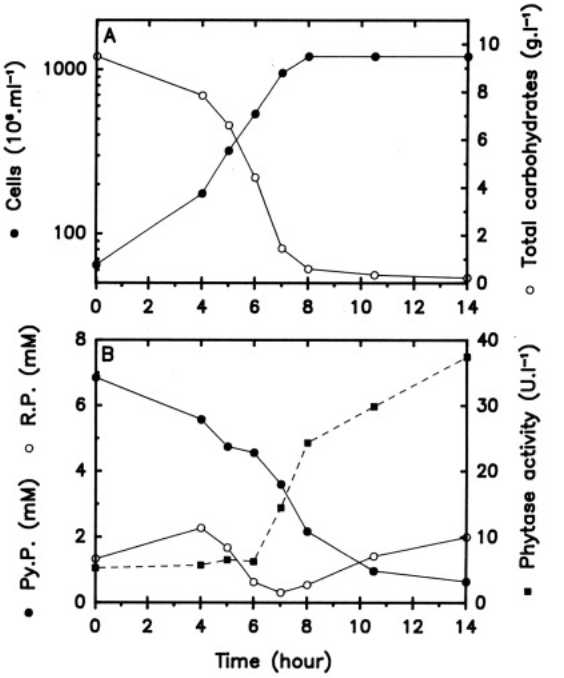

Figure 2. Evolution of various parameters during culture of *S. castellii* with soft wheat bran (25 g L^{-1}), MSA medium and starch (10 g L^{-1}), pH 4.0, T = 30 °C, initial phytic acid content 1.18 mM: (A) cell count and total soluble carbohydrates; (B) phytic phosphate (Py.P.) and residual phosphate (R.P.) contents, total phytase activity.

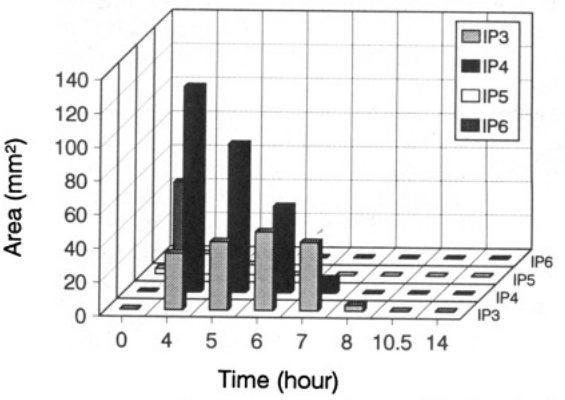

Figure 3. Evolution, during culture of *S. castellii* with soft wheat bran, of the HPLC peak areas for the various inositol phosphates in culture supernatants.

The phytase from *S. castellii* thus hydrolyzed the phytic acid in both substances in the same way, in terms of speed and overall yield, as when added in the form of pure sodium phytate solution.

Batch Culture of *S. castellii* in the Presence of 25 g L^{-1} Wheat Bran (Figures 2 and 3). The generation time of *S. castellii* on MSA medium with added starch (10 g L^{-1}) and wheat bran (25 g L^{-1}) was 1.5 h. Growth terminated after 8 h of culture (1.2×10^9 cells mL^{-1}) when 94% of the carbohydrates had been used (Figure 2A).

At time 0, the medium contained 1.18 mM phytic acid (IP6), 68% of which was in the supernatant. This corresponded to 6.9 mM of phytic phosphate assayed enzymatically (Figure 2B). Phytic acid was hydrolyzed from the start of culture: the phytic phosphate content had decreased by 33% after 6 h. Hydrolysis was performed mainly by phytase (approximately 6 units L^{-1}) coming from preculture. Synthesis of *S. castellii* phytase appeared to be repressed by the phosphate in the medium: the residual phosphate content (R.P.) was greater than 1 mM during the first 5 h of culture (Figure 2B).

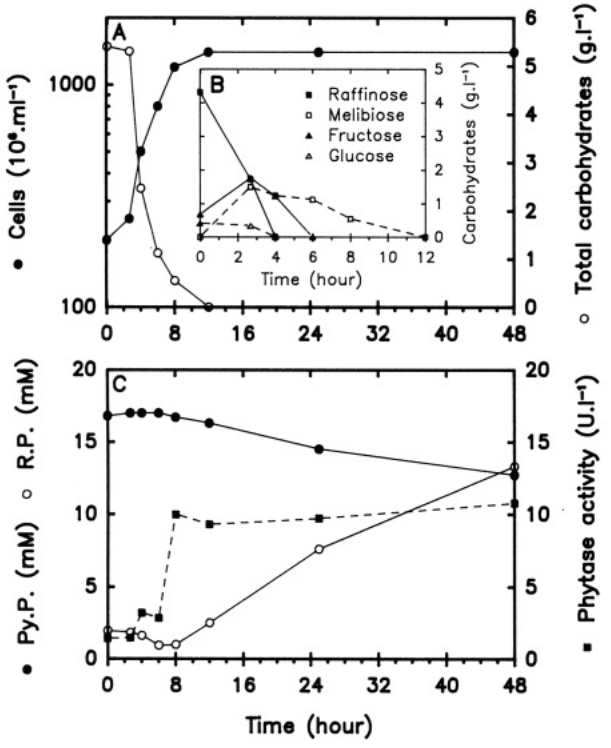

Figure 4. Evolution of various parameters during culture of *S. castellii* with glandless cotton flour (50 g L^{-1}) and MSA medium, pH 4.0, T = 30 °C, initial phytic acid content 3.00 mM: (A) cell count and total soluble carbohydrates; (B) raffinose, melibiose, fructose, and glucose contents; (C) phytic phosphate (Py.P.) and residual phosphate (R.P.) contents, total phytase activity.

The residual phosphate content fell from 2.3 to 0.3 mM between the fourth and seventh hour of culture. The phytase content of the medium increased from 6.3 (hour 6) to 24.3 units L^{-1} (hour 8). Hydrolysis of phytic phosphate increased correlatively: 35% of the initial amount was hydrolyzed between culture hours 6 and 8. At the end of culture, 91% of the initial phytic phosphate content (6.3 mM) was hydrolyzed and the medium contained 2 mM of residual phosphate and 37 units L^{-1} of phytase.

Chromatographic analysis (Figure 3) showed that the phytic acid (IP6) in the supernatant was fully hydrolyzed at culture hour 5. It was converted into IP5, IP4, and IP3, which were in turn hydrolyzed after 6, 8, and 10.5 h of culture, respectively. No inositol phosphate was detected in the supernatant at the end of culture and only 0.06 mM of IP6 was measured in the pellet. Ninety-five percent of the phytic acid added was therefore hydrolyzed.

Batch Culture of *S. castellii* with 50 g L^{-1} Cotton Flour (Figures 4 and 5). As raffinose, the most abundant carbohydrate in cotton flour, can be assimilated by *S. castellii*, culture was performed on MSA medium with 50 g L^{-1} cotton flour and without addition of carbon substrate.

At time 0, the culture medium contained 4.3 g L^{-1} raffinose, 0.4 g L^{-1} glucose, and 0.7 g L^{-1} fructose (Figure 4B), i.e., a total of 5.4 g L^{-1} soluble carbohydrates (Figure 4A). After 2.6 h of culture, 60% of raffinose had been hydrolyzed into melibiose and fructose, while the total carbohydrate content remained stable (5.3 g L^{-1}). Growth then proceeded by uptake of fructose and then melibiose with a generation time of about 2 h (Figure 4A). It was completed in hour 12 (1.4×10^9 cells mL^{-1}) when all of the meliobiose had been used up.

At the start of culture, the medium contained 3.00 mM phytic acid (IP6), 1.33 mM (44%) of which was in the

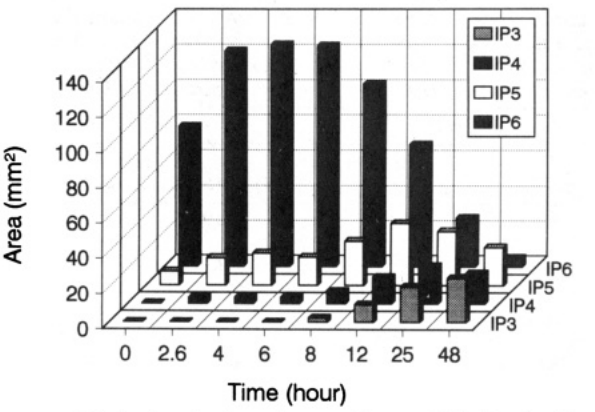

Figure 5. Evolution, during culture of *S. castellii* with glandless cotton flour, of the HPLC peak areas for the various inositol phosphates in culture supernatants.

supernatant. Supernatant phytic acid content increased after 2.6 h (Figure 5) because of the dissolution of a further part of the cotton flour phytic acid. The level stabilized at about 2.0 mM. IP6 was hydrolyzed slowly only after culture hour 6. At the end of culture, the total phytic acid content of the medium was 0.12 mM (0.08 mM of which was in the supernatant), i.e., a reduction of 96% in relation to the initial value. However, the inositol phosphates formed from phytic acid were little hydrolyzed (Figure 5); the total phytic phosphate content only decreased by 25% (4.4 mM) during culture (Figure 4C).

The phytase content of the medium remained lower than 3.2 units L^{-1} until hour 6 of culture (Figure 4C). Synthesis of the enzyme occurred mainly between the sixth (2.8 units L^{-1}) and eighth (10 units L^{-1}) hour of culture when the residual phosphate content of the medium was minimum (1 mM). The phytase content then remained constant.

DISCUSSION

S. castellii developed on both the substrates tested with no other phosphate addition. Growth was not correlated with the phytic phoshate hydrolysis rate. Indeed, whereas the greater proportion of the latter (4.7 mM, i.e., 68%) had been released at the end of growth (hour 8) with wheat bran, only 4.1% (0.7 mM) of the phytic phosphate provided had been hydrolyzed at the end of growth (hour 12) with cotton flour. In the latter case, it would seem that growth occurred mainly because of uptake of free phosphates (2 mM) coming from preculture and cotton flour.

Phytase synthesis was significant only at the end of growth in both cultures, when the medium phosphate content was minimum. This content was over 1 mM at the start of culture, causing strong repression of synthesis, as observed during continuous culture by Lambrechts *et al.* (1993). Total synthesis during culture with wheat bran (31.7 units L^{-1}, i.e., 26 units/10^{12} cells) was distinctly greater than that observed with cotton flour (9.4 units L^{-1}, i.e., 6.7 units/10^{12} cells). Since the increase in the phytic acid content also represses phytase synthesis (Lambrechts *et al.*, 1993), the weakness of synthesis with cotton flour can be accounted for by the larger amount of phytic acid present during culture with this substrate (3.00 mM compared to 1.18 mM with wheat bran).

The rate of hydrolysis of phytic phosphate (in micromoles per hour) was calculated between each measurement point to compare phytase functioning levels in the two culture types. The rate was then divided by the average of the phytase contents (in units per liter) measured at each of the two points. The results (in micromoles per

Table I. Rate of Hydrolysis of Phytic Phosphate (in Micromoles per Hour) Related to the Quantity of Phytase (in Enzyme Units) in the Medium during Cultures of *S. castellii* in the Presence of Wheat Bran and Cotton Flour

wheat bran		cotton flour	
time (h)	rate of hydrolysis (μmol h^{-1} $unit^{-1}$)	time (h)	rate of hydrolysis (μmol h^{-1} $unit^{-1}$)
0		0	
	58		0
4		2.6	
	136		0
5		4	
	30		0
6		6	
	93		23
7		8	
	74		10
8		12	
	18		14
10.5		25	
	3		8
14		48	

hour per unit) were used to assess the phytase functioning level independently of the quantity of enzyme in the medium (Table I). We calculated the weighted average of the values obtained during the active phases of hydrolysis of phytic phosphate: from time 0 to time 10.5 h during culture with wheat bran (58 μmol h^{-1} $unit^{-1}$) and from hour 6 to hour 48 during culture with cotton flour (11 μmol h^{-1} $unit^{-1}$). The fact that the average rate measured with wheat bran is 5.3 times as high as that obtained with cotton flour shows that there is a limit *in vivo* to the functioning of phytase in the presence of the latter substrate. This phenomenon was not observed *in vitro* during the study of hydrolysis using purified phytase extract. It therefore seems that a combination of cotton flour and MSA medium is unfavorable for action of the enzyme. The presence in MSA medium of cations that easily form complexes with cotton flour phytates may reduce substrate availability for phytase. Thus, only 25% (4.3 mM) of phytic phosphate was released with cotton flour in comparison with 91% (6.3 mM) in the case of wheat bran.

However, almost all (90–95%) of the phytic acid was hydrolyzed in both cultures. Batch culture of *S. castellii* is therefore a good way of reducing the phytate content of wheat bran and glandless cotton flour while producing biomass. This process could be used to produce animal food free of phytic acid and rich in protein. In the case of wheat bran, a fermented bran with high fiber content, the starch being consumed by yeast, could be easily separated from biomass by decantation. Furthermore, *S. castellii* could be used to make bioavailable the phytate phosphorus of many livestock foods so that the phosphate supply could be reduced.

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