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INFLUENCE OF CULTURE CONDITIONS ON THE BIOSYNTHESIS OF *SCHWANNIOMYCES CASTELLII* PHYTASE

Christel Lambrechts, Hélène Boze, L. Segueilha, G. Moulin* and P. Galzy.
Chaire de Microbiologie Industrielle et de Génétique des Micro-organismes ENSA-INRA,
34060 Montpellier, France

SUMMARY

The influence of several factors on the biosynthesis of *Schwanniomyces castellii* phytase was studied in continuous culture. The level of phytase production increased with pH and dilution rate. It decreased when the phytic acid or phosphate content increased.

INTRODUCTION

Phytic acid salt or phytate, myo-inositol hexakisphosphate, occurs as a storage phosphorus source in all nutrition substances derived from plants (Graf, 1986). Phytic acid is considered to be an anti-nutritional factor since it chelates minerals such as calcium, zinc, magnesium, iron and may also react with proteins, thereby decreasing the bioavailability of proteins and nutritionally important minerals (Cheryan, 1980; Morris, 1986).

The enzymes produced by micro-organisms and which catalyse conversion of phytate to inositol and inorganic phosphorus are known as phytases. Phytase-producing micro-organisms include bacteria such as *Bacillus subtilis* (Powar and Jagannathan, 1982), fungi such as *Aspergillus ficuum* (Ullah and Gibson, 1987) and yeasts such as *Saccharomyces cerevisiae* (Nayini and Markakis, 1984).

A previous study (Lambrechts *et al.*, 1992) had shown that the yeast *Schwanniomyces castellii* had high phytase production potential in comparison with four other phytase-producing yeasts. In order to optimise the production of phytase by *Schwanniomyces castellii*, we have studied the effects of the following factors on the enzyme biosynthesis: pH, dilution rate, dissolved oxygen, concentration of inorganic phosphate and of phytate.

MATERIALS AND METHODS

Organism

The strain used is listed at the Centraal bureau voor schimmelculture (Delft) as *Schwanniomyces 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 litre: $(NH_4)_2SO_4$, 3 g; $MgSO_4.7H_2O$, 0.5 g; KCl, 0.5 g; $CaCl_2$, 0.1 g; $MnSO_4.H_2O$, 7.5 mg. Galactose 10 $g.l^{-1}$ was used as carbon substrate to prevent any repression by glucose. Different concentrations of sodium phytate (Sigma) were added to this medium. Galactose, sodium phytate and mineral salts were autoclaved separately at 120°C for 30 minutes. Vitamins, trace elements and $FeCl_3$ were sterilised by filtration through an HA 0.45 µm Millipore membrane and added at the same concentrations as in G medium described by Galzy (1964).

Continuous cultures were performed in a Biolafitte fermenter with a useful capacity of 3.6 l. The pH was adjusted by the addition of NaOH (2N) or H_2SO_4 (2N), and the temperature maintained at 30°C. The biostat was aerated at 4 v.v.m. and agitation maintained at 500 rpm. Dissolved oxygen was measured by a polarographic probe.

Analytical methods

Dry weight was determined after desiccation at 108°C.

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

The culture supernatant phytic phosphate content 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.

Galactose was determined in the culture supernatant using the method described by Kurz and Wallenfels (1974).

Enzyme preparation and assay

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 on both fractions at 37°C using Ullah and Gibson's (1987) method. One phytase activity unit (U) was defined as the amount of enzyme that liberates 1 µmol of phosphate per minute. Activity measured in the ultrafiltered culture supernatant was about 20% (± 4%) of the total activity under all the culture conditions used.

RESULTS

Influence of pH (Fig. 1)

The study was performed using continuous culture at dilution rate (D) of 0.2 h^{-1} with a pO_2 of over 80% of the saturation in the presence of 2 $g.l^{-1}$ sodium phytate, i.e. 1.69 mM phytic acid (10 mM of phytic phosphate). At equilibrium, with pH of 3.5 to 5.5, biomass varied from 5.8

to 6.8 $g.l^{-1}$, specific phytase activity increased slightly (0.6 to 1.4 $U.g^{-1}$), as did volumetric activity (3.7 to 8.5 $U.l^{-1}$). At pH 5.5, 41% of the initial phytic phosphate was hydrolysed and the residual phosphate content was 0.3 mM.

When the pH was over 6, there was a distinct increase in specific phytase activity (S.A. = 8 $U.g^{-1}$ at pH 7) and volumetric activity (V.A.= 40 $U.l^{-1}$ at pH 7). This can be explained either by a direct and positive effect of pH on the biosynthesis or by an indirect effect: the removal of repression by phosphate resulting from a decrease in the residual phosphate content (less than 0.1 mM).

The amount of phytic phosphate hydrolysed decreased when the pH was over 6; at pH 7, only 19% of the initial phytic phosphate was hydrolysed. Segueilha *et al.* (1992) showed that at these pH values phytase functioning was less than 5% of that observed at pH 4.4. In addition, the culture medium contained cations likely to form non-hydrolysable complexes with phytic acid at these pH levels.

Nevertheless, sufficient phosphate was available for the strain at pH 6.5 and D = 0.2 h^{-1} to conserve a biomass yield ($Y_{X/S}$, gram of biomass produced per gram of galactose consumed) close to 0.5.

Influence of the dilution rate (Fig. 2)

The study was performed at pH 6.5 under the conditions described above.

Increasing the dilution rate (D) caused a decrease in biomass at equilibrium (X) from X = 5.9 $g.l^{-1}$ (D = 0.10 h^{-1}) to X = 0.7 $g.l^{-1}$ (D = 0.27 h^{-1}). Yield $Y_{X/S}$ also decreased ($Y_{X/S}$ = 0.59 at D = 0.10 h^{-1} and $Y_{X/S}$ = 0.19 at D = 0.27 h^{-1}).

Volumetric phytase activity varied little until D = 0.25 h^{-1}; this automatically caused an increase in specific activity from 1.6 $U.g^{-1}$ to D = 0.10 at 4.9 $U.g^{-1}$ to D = 0.25 h^{-1}.

Whatever the dilution rate, the quantity of phytic phosphate hydrolysed remained between 2 and 2.9 mM as the increased phytase content compensated the shorter time of contact between the enzyme and its substrate. The residual phosphate content also remained lower than 0.1 mM. Under these conditions, it is possible that a low phosphate content might be a limiting factor for growth with a dilution rate greater than 0.2 h^{-1} and induce diversion of the metabolism to fermentation.

Influence of the dissolved oxygen content

At D = 0.2 h^{-1}, pH 6.5 and 1.69 mM phytic acid, the partial oxygen pressure (pO_2) had little effect on yeast growth and phytase biosynthesis. Yield ($Y_{X/S}$ = 0.50) was stable for pO_2 between 80% and 40%. The metabolism became oxido-fermentative at less than 40% and yield decreased ($Y_{X/S}$ = 0.39 when pO_2 = 10%). Specific phytase activity increased from 3.6 $U.g^{-1}$ (pO_2 = 80%) to 5.1 $U.g^{-1}$ (pO_2 = 10%). Oxygen limitation seemed to slightly enhance phytase synthesis. The amount of phytic phosphate hydrolysed varied by only 20% with pO_2 (1.7 to 2.1 mM).

Influence of the phosphate content (Fig. 3)

The sodium phytate content was reduced to 0.6 $g.l^{-1}$ during the study (i.e. 0.51 mM phytic acid and 3.06 mM phytic phosphate) to prevent possible inhibition of phytase synthesis by phytic acid. Increasing amounts of phosphate in KH_2PO_4 form (0 to 2.5 mM) were added.

Yield ($Y_{X/S}$) decreased from 0.47 without phosphate to 0.31 with 2.5 mM phosphate.

Phytase synthesis was strongly repressed from 1.0 mM phosphate onwards. Specific activity was divided by 4.2 and subsequently stabilised at a very low level (0.3-0.7 $U.g^{-1}$).

A curious contrasting feature was that the amount of phytic phosphate hydrolysed varied little (40 to 50% of the phytic phosphate applied) whatever the phosphate concentration. It was little affected by an increase to 1 mM in phosphate content whereas the amount of enzyme in the medium was divided by 4 (volumetric activity fell from 9.8 to 2.4 $U.l^{-1}$). This phenomenon could result from the fact that the rate of hydrolysis is limited rather by the sodium phytate content and by the time of reaction than by the amount of phytase present in the fermenter. It could be also explained by the improvement of phytase functioning. Since no positive effect of phosphate on phytase activity has been observed *in vitro* (Segueilha *et al.*, 1992), this improvement could be accounted for by better solubility of phytic acid. Indeed, added phosphate may have played the role of cation chelator in competition with phytic acid and thus limit the formation of phytate-cation complexes.

Influence of the phytate content (Fig. 4)

This was investigated by using 0.6 to 5.0 $g.l^{-1}$ sodium phytate in the medium (corresponding to 0.51 and 4.23 mM phytic acid respectively).

Yield increased distinctly (+ 23%) up to 2.54 mM phytic acid concentration. It then remained close to the optimum level (0.56 - 0.58).

Phytase synthesis decreased as the phytic acid content increased. Specific activity was divided by 2.3 when phytic acid increased from 0.51 mM to 4.23 mM.

Phytic acid hydrolysis increased with the amount added up to 2.54 mM (4.5 mM of phytic phosphate released) and then decreased strongly with 3.38 mM of phytic acid (1.6 mM of phytic phosphate released, i.e. 7% of the initial amount used).

DISCUSSION

In continuous culture, biosynthesis of *Schwanniomyces castellii* varies by a factor of 26 (0.3 to 8.0 U g^{-1}) according to growth conditions. It increases with the pH (above pH 6) and the dilution rate (until D = 0.25 h^{-1}). In contrast, synthesis is repressed when the phytic acid or phosphate content increases.

The results concerning functioning of the enzyme show that the variation in the amount of phytic acid hydrolysed (factor of 4) was not correlated with the variation of the amount of phytase present in the culture (factor of 20). This shows that enzyme functioning is closely dependent on medium composition (cations, phosphate and phytic acid contents) and pH. These

factors may have opposing effects on synthesis and phytase activity. In particular, they indirectly influence the effectiveness of enzymatic hydrolysis by enhancing or hindering the formation of phytic acid-cation complexes.

Whatever the culture conditions, less than 50% of the phytic phosphate in the medium is hydrolysed. Study of *in vitro* hydrolysis of phytic acid by *Schwanniomyces castellii* (Segueilha *et al.*, 1992) shows that the first half of phytic phosphate (hydrolysis of hexa- to tetraphosphate inositols) is released in half the time required for the second half. It is therefore difficult to obtain hydrolysis of phytic phosphate greater than 50% in continuous culture in which phytase operation time is limited to fermenter renewal time (1/D).

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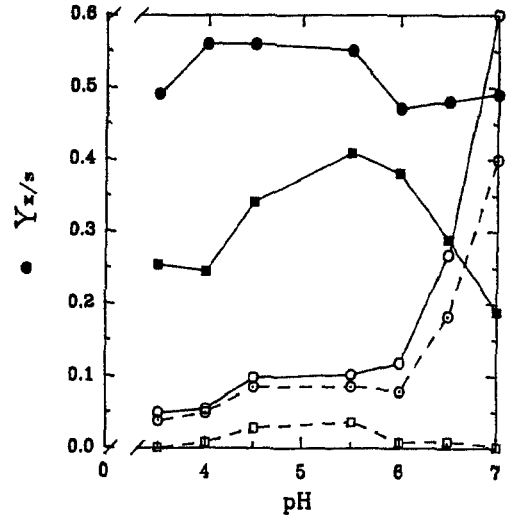
Fig. 1:



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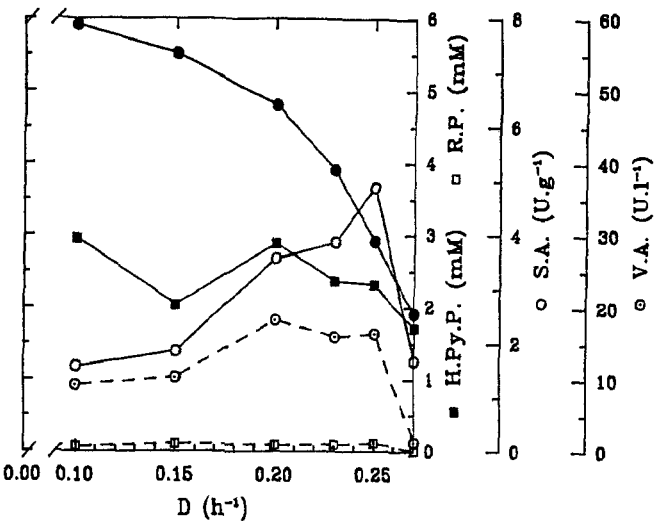

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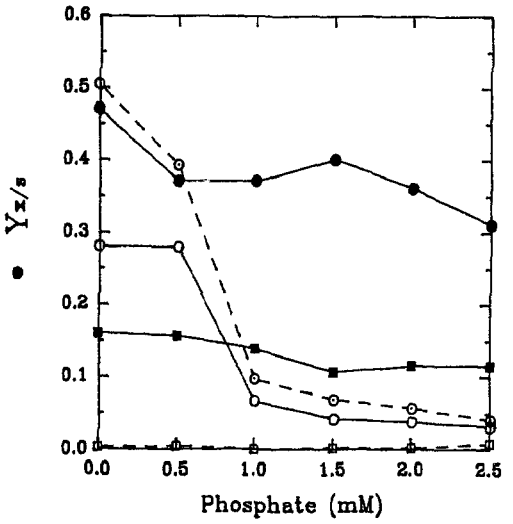

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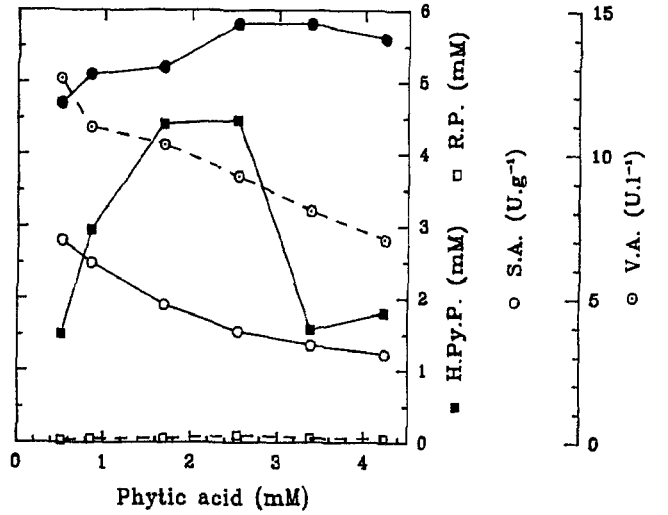

Figures: Effects of different factors on Yield ($Y_{X/S}$), Specific Phytase Activity (S.A., enzymatic unit per gram dry weight), Voluminal Phytase Activity (V.A., enzymatic unit per litre), Hydrolysed Phytic Phosphate (H.Py.P., mM) and Residual Phosphate (R.P., mM) during continuous culture of *Schwanniomyces castellii* CBS 2863 in presence of MSA medium, galactose 10 g.l^{-1} and phytic acid. Dissolved oxygen was maintained over 80%.

Fig. 1: Influence of pH. Dilution rate was 0.20 h^{-1} and phytic acid concentration was 1,69 mM.

Fig. 2: Influence of dilution rate (D). pH was 6.5 and phytic acid concentration was 1.69 mM.

Fig. 3: Influence of phosphate addition. D was 0.20 h^{-1}, pH was 6.5 and phytic acid concentration was 0.51 mM.

Fig. 4: Influence of phytic acid concentration. D was 0.20 h^{-1} and pH was 6.5.