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Phytate dephosphorylation by free and immobilized cells of *Saccharomyces cerevisiae*

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SUMMARY

Saccharomyces cerevisiae in the form of baker's yeast, cells cultivated on a yeast extract–peptone–glucose medium, as well as cells immobilized in 18% (w/v) polyacrylamide gel showed the ability to hydrolyze 1.727 mM sodium phytate solution at 45 °C, pH 4.6, in a stirred tank reactor. Seventy percent yield of dephosphorylation was observed after 2 h using a baker's yeast concentration of 5.8 g dry matter per 100 ml. Hydrolytic activity at 1.8–2.0 μM Pi min^{-1} was observed between 1st and 3rd h of the reaction in cells cultured 24 or 48 h. No inhibition by the substrate was found at sodium phytate concentrations of 0.587–1.727 mM. After 1.5 h of hydrolysis a single, well distinguished peak of *myo*-inositol-triphosphate was the main product found. By means of immobilization the stability of the biocatalyst was enhanced 3.3-fold and reached its half-life at 64 ninety-minute runs.

INTRODUCTION

Phytates are known to be a principal storage form of phosphorus in plant seeds, and because of their strong chelating properties, as an antinutritional factor decreasing bioavailability of minerals (Ca^{2+}, Mg^{2+}, Fe^{3+} and Zn^{2+}) from the gastrointestinal tract of monogastric animals and humans [15].

There are several reasons why methods to enzymatically hydrolyze (in vitro or in vivo) plant phytate need to be developed: firstly, to deprive certain food and feed components of their antinutritional properties [3,8]; secondly, to supply additional available phosphorus for monogastric animals [20,29,39], and at the same time, to decrease the amount of phosphorus in animal manures [13] and thirdly, in order to shorten the steeping time during corn wet milling [2].

Phytase catalyzes the hydrolysis of *myo*-inositol-hexakis dihydrogenphosphate—IP_6 (phytic acid), and its salts—phytates, to inositol and inorganic phosphate in a stepwise manner yielding different *myo*-inositol phosphates (IP_x) as intermediate products. Negative effects of phytic acid (IP_6) salts on nutrient availability and also on protein digestion are known to be largely attributed to the presence of inositol hexa- and pentaphosphates [14,26]. The role of lower inositol phosphates is not fully understood, but *myo*-inositol-triphosphate (IP_3) has been claimed as a stabilizer against oxidation caused by free radicals and against heavy metals intoxication [31].

Several preparations of phytate-degrading enzymes have been tested for these applications up to now. Special attention has been attracted by preparations derived from molds, and especially those from *Rhizopus oligosporus* [33], *Aspergillus ficuum* [8], *Aspergillus oryzae* [34], and *Aspergillus niger* [35–39]. *Saccharomyces cerevisiae* have been known for a long time to play a role in decomposing phytate in yeast-leavened bread making [24,25]. Due to the presence of phytase in wheat usually used in this process it was difficult to separate the effects of yeast from that of a wheat phytase. Nayini and Markakis [19] purified a phytase from baker's yeast providing direct evidence of the yeast's dephosphorylating abilities. Recently, a thermostable phytase has been found in *Schwaniomyces castellii* [27].

The objective of the present study was to explore dephosphorylating abilities of the whole yeast cells in a free and immobilized form, as well as to examine the elution profiles of inositol phosphates formed in the course of the reaction.

MATERIAL AND METHODS

Chemicals

Dimethylaminopropionitryle, Dowex 1 × 8 Cl^-, and sodium phytate were purchased from Sigma Chemical Co., St Louis, MO, USA. Acrylamide and *N,N'*-methylenebis-acrylamide were from Reanal (Hungary). Peptone and yeast extract were the products of Warsaw Vaccines Factory (Poland). Commercial wort was from Krakow Brewery (Poland). All other chemicals used were of analytical grade.

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Strains

The following strains and forms of the yeast *Saccharomyces cerevisiae* were investigated: commercial baker's yeast from the Lublin Yeast and Distillers Company (Poland), DLC Active Dried Yeast (Distillers Company, Yeast Ltd, Sutton, UK). The yeast strains *Saccharomyces cerevisiae* 'MF', and *Saccharomyces cerevisiae* 'DF' were from the culture collection of the Department of Food Biotechnology, University of Agriculture, Krakow, Poland.

Yeast cultivation

The strains 'MF' or 'DF' of *Saccharomyces cerevisiae* were cultivated on yeast extract (3 g L^{-1})–peptone (10 g L^{-1}) –glucose (50 g L^{-1}) medium (YEPG). After dissolving the components, pH of the medium was regulated to 6.8, then 100 ml of the medium was poured into 300-ml Erlenmeyer flasks, and after autoclaving, the cultivation proceeded from 24 to 144 h at 30 °C, in a shaken water bath using 200 rpm. In a single experiment a commercial brewery wort diluted with tap water to 8 g dry matter per 100 ml was used as a medium.

Dephosphorylation of sodium phytate by free or immobilized Saccharomyces cerevisiae *cells*

A weighted sample of yeast (standardized according to dry mass) was incubated with 50 ml of 1.727 mM sodium phytate solution at 45 °C, pH 4.6 with constant stirring. Samples of 1.5 or 2 ml were withdrawn at specified time intervals, centrifuged and the liberated phosphate was determined in the supernatant by the Heinonen and Lahti [11] method.

Separation of inositol phosphates (IP_x)

The *myo*-inositol phsophates formed during hydrolysis were separated by ion-exchange chromatography on a 'Dowex' (1 × 8 (Cl^-) 200–400 mesh) 15 × 70 mm column. The hydrolysate was put on the column, and inositol phosphates were eluted for 4 h with a linear gradient of 0–1 M HCl using 'ISCO' equipment for liquid chromatography. The flow rate was 2 ml min^{-1}, and fractions of 5 ml were collected.

Determination of the elution profile of IP_x

The elution profile of *myo*-inositol phosphates was determined colorimetrically with sulfosalicyclic acid as described previously [36]. Three milliliters of 2.5 M acetate buffer (pH 4.0) and 1 ml of sulfosalicyclic acid reagent (0.3 g L^{-1} of $FeCl_3 \cdot 6H_2O$ and 3 g L^{-1} of sulfosalicylic acid in double distilled water) were added to a 1-ml sample of the eluate and the absorbance was read at 500 nm against a blank containing water instead of eluate.

Identification of inositol phosphates

Fractions of separated peaks were combined, dephosphorylated by autoclaving as described by Hara et al. [9]. The phosphorus content in dephosphorylated fractions was measured by the method mentioned above, while the inositol content was determined by the method of Agranoff et al. [1].

Immobilization of the Saccharomyces cerevisiae *cells in polyacrylamide gel*

Cells of *Saccharomyces cerevisiae* were grown on YEPG medium for 48 h, then washed out with physiological saline solution and finally with 0.1 M acetate buffer pH 4.6. Four grams of wet cells were mixed with 16 ml of a monomer solution containing 8.8 g of acrylamide and 0.6 g of *N,N'*-methylenebisacrylamide dissolved in 50 ml of 0.05 M Tris-HCl buffer pH 7.0. 0.4 ml of 25% ammonium persulfate solution was added and the suspension was poured dropwise into 80 ml of soybean oil, stirred and kept under the temperature of ca. 10 °C. When droplets of appropriate size (diameter of ca. 1 mm) were formed, 0.4 ml of 50% dimethylaminopropionitryle was added and the biocatalyst collected by centrifugation (2 min, 100 *g*), washed with acetate buffer and transferred aseptically into 100 ml of YEPG medium, and cultivated for 24 h under conditions similar to those described for free cells cultivation. The resulting biocatalyst was stored at 4 °C after appropriate washing.

RESULTS AND DISCUSSION

Recently the screening for yeast strains with phytase activity was reported by Lambrechts et al. [16] and *Saccharomyces cerevisiae* were found among yeasts with moderate dephosphorylating activity. Those data however might be misleading since an autoclaved solution of sodium phytate was used as a medium component throughout that work, while autoclaving is known as a means to partly hydrolyze the phytic acid molecule [23,37].

In this study intact cells of *Saccharomyces cerevisiae* (commercial baker's yeast) showed the ability to release inorganic phosphate when incubated in a sodium phytate solution at 45 °C and pH 4.6. Using a biocatalyst concentration of 5.8 g d.s. per 100 ml, 70% yield of dephosphorylation was observed after 2 h of the reaction. After that time the hydrolysis ratio remained constant (Fig. 1). The initial reaction velocity was found to be proportional to the biocatalyst concentration in the substrate solution within the range studied (from 0.8 to 5.8 g dry matter per 100 ml).

Effect of substrate concentration

Phytases are usually inhibited by a high substrate concentration. Above a critical concentration of sodium phytate, a decrease in the reaction rate is observed. Complete or partial inhibition of different phytases by sodium phytate has been reported [3,7,30,32]. Nayini and Markakis [19] reported similar phenomenon with a phytase isolated from baker's yeast and found 2 mM as the critical concentration of the substrate. In this study no inhibition by the substrate was found at sodium phytate concentration from 0.587 to 1.727 mM (Fig. 2).

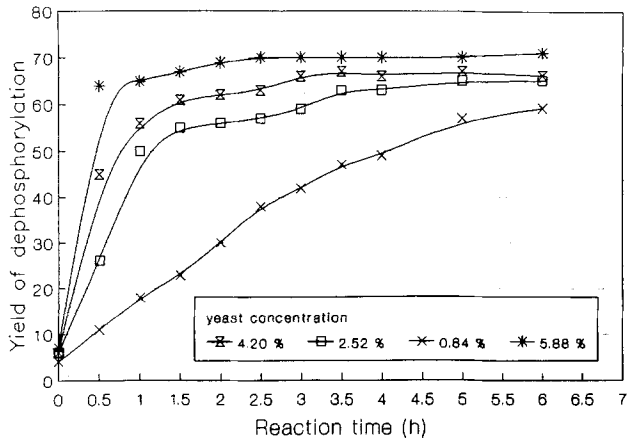

Fig. 1. Progression curves of 1.727 mM sodium phytate hydrolyzed by different concentration of baker's yeast at 45 °C and pH 4.6.

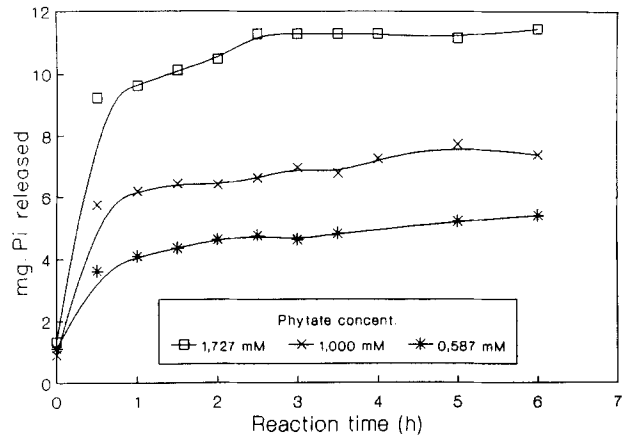

Fig. 2. Inorganic phosphorus released by 5.88% (w/v) suspension of baker's yeast from different concentrations of sodium phytate.

Effect of yeast form on dephosphorylation rate

Saccharomyces cerevisiae cells cultivated on a medium containing yeast extract, peptone and glucose (YEPG) revealed a slightly higher initial reaction velocity and final yield of dephosphorylation (after 5 h reaction) in comparison to commercial baker's or dried yeasts (Fig. 3). The yeast *Saccharomyces cerevisiae* 'MF' cultivated on a wort solution was much less active, while the strain 'DF' grown on YEPG medium was the most active among the yeast tested.

Effect of culture age

The amount of phytase synthesized by molds is influenced by the time of cultivation. Howson and Davis [12] observed the maximum enzyme biosynthesis between the eighth and eleventh day of *Aspergillus ficuum* cultivation. In order to find out how the time of cultivation may influence the hydrolyzing abilities of the yeast cells, 24 h, 48 h, and 144 h cultivation times were applied. The hydrolytic activity of the yeast *Saccharomyces cerevisiae* 'DF' was similar after 24 and 48 h of cultivation on the YEPG medium but much lower

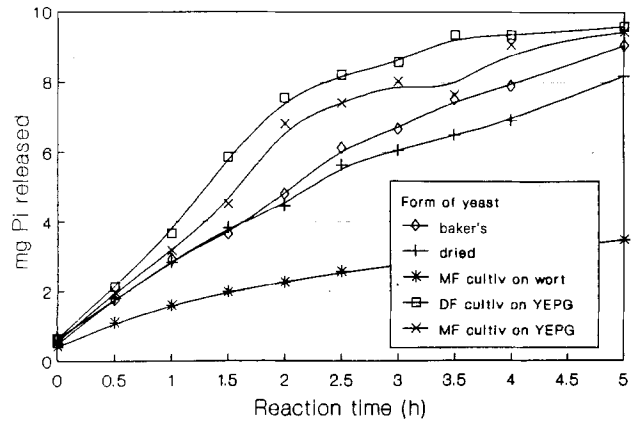

Fig. 3. Inorganic phosphorus released by 0.84% (w/v) suspension of different forms of *Saccharomyces cerevisiae* from 1.727 mM sodium phytate solution.

in cells from 144 h culture. The highest hydrolytic activity 1.8–2.0 μM Pi min^{-1} was observed between 1 and 3 h of the reaction (Fig. 4).

The mode of action

Several investigators suggest that a direct conversion of phytic acid (IP_6) to IP_4 takes place during enzymatic hydrolysis [6,18,36–38], and reports by Phillippy et al. [23] and Hayakawa et al. [10] indicated the presence of IP_5 isomers in a reaction mixture containing a phytase of plant or mold origin.

The IP_6 hydrolysis by the yeast cells was accompanied by the accumulation of a *myo*-inositol-triphosphate (IP_3) isomer, which was observed on the chromatogram as a single, well-distinguished peak after 1.5 h of hydrolysis (Fig. 5). The dephosphorylation pattern of IP_6 by the yeast cells differs substantially from those previously reported for plant and mold phytases and seems to be a unique feature of a yeast phytase. Similar conclusions can be drawn from the

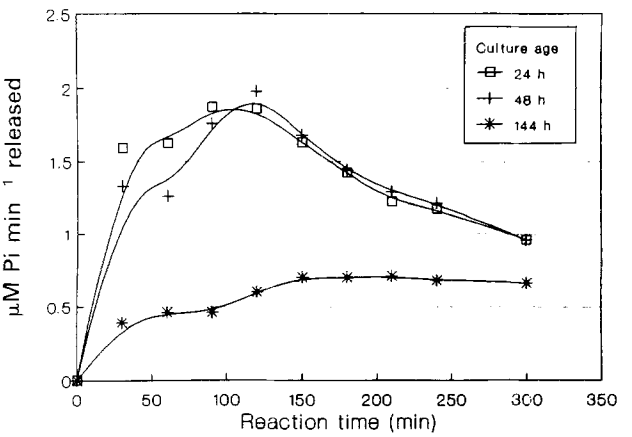

Fig. 4. The influence of the culture age of *Saccharomyces cerevisiae* 'DF' grown on YEPG medium on their dephosphorylating activity.

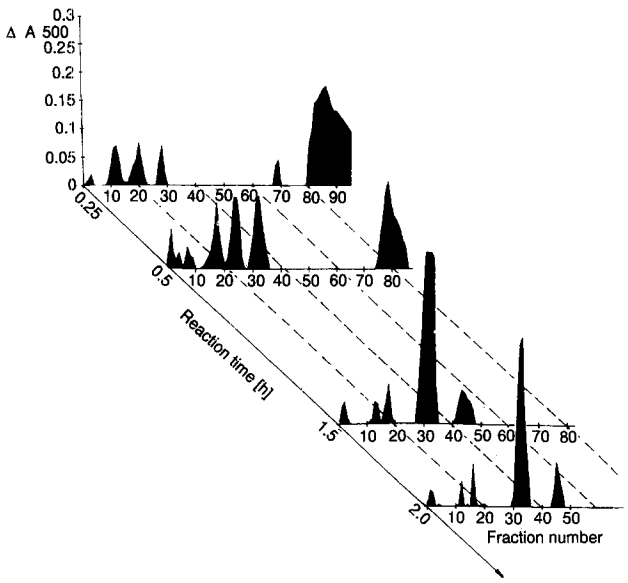

Fig. 5. Elution profiles of inositol phosphates formed in the course of hydrolysis of 1.727 mM sodium phytate for 2 h at 45 °C and pH 4.6 by 0.84% (w/v) suspension of *Saccharomyces cerevisiae* 'DF'.

data reported recently for a phytase from *Schwaniomyces castellii* [27]. There is a potentiality therefore, to use the yeast in a free or immobilized form as a biocatalyst for the production of *myo*-inositol-triphosphate (IP_3).

Effect of immobilization on biocatalyst stability

Immobilization of yeast cells in a polyacrylamide gel was reported as a means to produce chemically and mechanically stable biocatalysts for different applications, under relatively mild conditions [5,17,21,28]. In this study immobilization of *Saccharomyces cerevisiae* 'DF' cells resulted in a substantial loss (90%) of initial dephosphorylating activity. In gel-immobilized cells of *Saccharomyces cerevisiae* a similar phenomenon was reported for catalase [28], as well as for invertase and acid phosphatase activities [22]. After 24 h recultivation of immobilized cells in the EDPG medium, the activity was recovered as a result of yeast growth inside the gel matrix. Stability of the catalyst obtained was then compared to the stability of baker's yeast, as well as to cultivated free cells of *Saccharomyces cerevisiae* 'DF'. Each form of biocatalyst (0.84% w/v) was incubated with 1.727 mM sodium phytate solution, several times in a 50-ml stirred tank reactor for 90 min at 45 °C until the residual activity dropped below 50% of its initial value. Before any decrease was observed the yeast cells showed a significant increase in dephosphorylating activity. When the immobilized yeast cells were suspended in 50 ml of 1.727 mM of sodium phytate solution at 45 °C, pH 4.6, the activity increased significantly and grew during subsequent applications with new portions of the substrate to reach 200% of its initial value in the tenth run. The activity of free yeast cells also increased under these conditions and amounted to 136% in

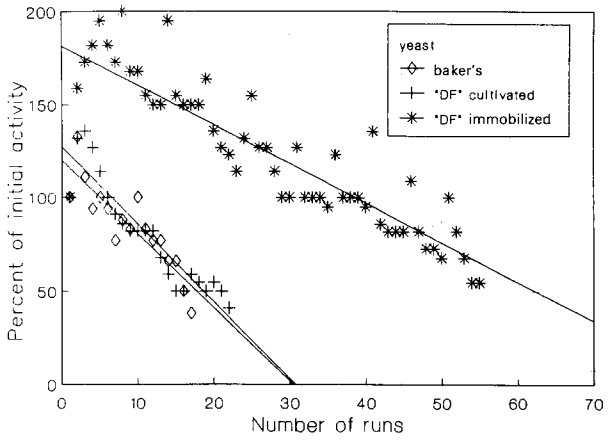

Fig. 6. The comparison of stability (half-life time determination) of free cells (baker's yeast, and *Saccharomyces cerevisiae* 'DF' cultivated on YEPG medium) and *Saccharomyces cerevisiae* 'DF' immobilized in 16% polyacrylamide gel.

the third application (Fig. 6). Chibata [4] described similar phenomenon with aspartase activity in *Escherichia coli* cells immobilized in a polyacrylamide gel. It was explained by an increase in the membrane permeability for the substrate or the product, or due to autolysis of the cells in the gel lattice. In the present study with stirred tank reactor, the stability of dephosphorylating activity in *Saccharomyces cerevisiae* cells entrapped in polyacrylamide gel was found to be 3.3-fold higher than the stability in free cells (Fig. 6).

In conclusion, it can be stated that the whole cells of *Saccharomyces cerevisiae* can be an inexpensive and readily available biocatalyst to perform conversion of phytic acid into *myo*-inositol-triphosphate, a compound that may have many different potential applications in the food technology. The stability of the biocatalyst can be improved by immobilization of the cells in a polyacrylamide gel.

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