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Pichia fermentans B4-1 phytase with the potential for enhancing soil phosphorous bioavailability

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Abstract

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A yeast strain *Pichia fermentans* B4-1, newly isolated from traditional fermented Bulgarian boza was chosen as a perspective producer of an extracellular phytase to degrade organic phytate and improve soil mineral conditions. The crude enzyme preparation could be applied at temperatures up to 50 °C and pH 5.0. It preserved 100% of its activity after incubation at 45°C for 90 min. In addition to sodium phytate, the *P. fermentans* B4-1 phytase can hydrolyze a broad range of substrates, such as ATP, glucose 6-phosphate, fructose 1,6-biphosphate, and glycerol 2-phosphate. Partial inhibition of phytase activity was detected (59–73% residual activity) in the presence of mono- and divalent cations like K^+, Na^+, Zn^{2+}, Ca^{2+}, Cu^{2+}, and Mn^{2+} at 1 M concentration.

Keywords: yeast; phytase; phosphorus; PSM; soil fertility

Introduction

Plants can absorb only soluble inorganic phosphate in the form of HPO_4^{2-} and $H_2PO_4^-$ ions. Between 30 and 65 % of the phosphorus in soil is contained in the organic biomass, but plants cannot use it until it is released into the soil through the specific action of phosphate-hydrolysing enzymes (Poirier et al., 2022). Phytases, or myoinositol (1,2,3,4,5,6)-hexakisphosphate phosphohydrolases, are enzymes responsible for the hydrolysis of phosphate-containing organic compounds. They catalyze the gradual removal of phosphorus from myo-inositol hexakisphosphate molecules or phytate. This process involves the separation of orthophosphate groups from the inositol ring of phytic acid, resulting in the generation of free inorganic phosphorus and a series of lower phosphorus esters (from inositol pentaphosphate to inositol monophosphate) as intermediates (Lei & Porres, 2007). Moreover, metal cations bound to the negatively charged phosphate groups in the phytate molecule are also released and become available for plant absorption (Rizwanuddin et al., 2023). Although phytates are abundant in soil, they are relatively unavailable to plants, because they lack phytase activity in their root systems. Adding phytase enzymes or phytase-synthesizing microorganisms to the soil would solve this problem and even improve agricultural productivity (Singh & Satyanarayana, 2011). In this aspect, research on the production of phytases and the characterization of their properties is an important issue related to soil sustainability, nutrition, and quality of life.

Microbial phytases have become a promising prospect for commercial use in agriculture due to their crucial role in the phosphorus nutrient cycle in the soil (Kaur, 2020; Mohite et al., 2022). Their application is considered a precise method for improving plant growth and productivity worldwide. Among the described phytate-degrading microorganisms, yeasts have been outlined as good candidates for extracellular phytase production because of their short

fermentation and easy post-fermentation extracellular enzyme processing and recovery. Although several yeast phytate-degrading enzymes have already been studied (Pandey et al., 2001; Vohra and Satyanarayana, 2004; Ragon et al., 2009; Nuobariene et al., 2011; Das & Ghosh, 2013; Pires et al., 2019), the discovery of new phytases offering better properties than those described so far is of growing commercial importance.

Following these current tendencies, the present study describes certain physicochemical properties of the extracellular phytase produced by the *Pichia fermentans* B4-1 yeast strain, originally isolated from a Bulgarian traditional fermented beverage (boza).

Material and Methods

Strains, media, and growth conditions

The yeast strain producing extracellular and intracellular phytase was isolated from a Bulgarian boza and taxonomically identified as *Pichia fermentans*. Phytase production was achieved after cultivation of the strain (48 h at 28°C) in liquid PSM medium (Palla et al., 2017), with some modifications (10 g/L glucose, 2 g/L yeast extract, 4 g/L Na-phytate, 2 g/L $CaCl_2$, 5g/L $NaNO_3$, 0.5g/L KCl, 0.5 g/L $MgSO_4 \times 7H_2O$, 0.01g/L $FeSO_4 \times 7H_2O$, 0.01g/L $MnSO_4 \times H_2O$; pH – 5.0). Sodium phytate (Sigma) was sterilized separately through a membrane filter and added to the medium before inoculation.

Crude enzyme preparation

After growing the yeast cells for 48 h in the modified liquid PSM medium they were removed by centrifugation at 5000 rpm for 15 min. Resulted supernatants were used as a source of an extracellular phytase. It was further concentrated and partially purified by ultrafiltration using a Millipore PM 30 membrane (Millipore, Bedford, MA, USA). The obtained concentrate was used in further assays for the characterization of the phytase's properties.

Phytase activity assay

The phytase activity was assayed according to the method of Tran et al. (2010). It is based on the spectrophotometric measurement of phosphomolybdate at 415 nm, obtained from the interaction of phosphorus released in the enzymatic reaction with ammonium heptamolybdate. A solution of 4.5 mM Na-phytate in an appropriate buffer was used as a substrate in the enzyme reaction. The reaction mixture was incubated for 30 minutes at different temperatures. The reaction was terminated by the addition of the color-developing reagent – 10% ammonium heptamolybdate, 0.24% ammonium vanadate, and 65% HNO_3 (1.5:1.5:1). The absorption of the samples was measured against a control, prepared in parallel with the samples without incubation.

Lowry`s method was used for quantitative determination of protein using bovine serum albumin as a standard (Lowry et al., 1951).

Characterization of the properties of extracellular phytase from P. fermentans B4-1

pH and temperature optima of crude phytase enzyme

The pH optimum of the enzyme was determined in 0.25 M sodium acetate buffer at 45 °C with pH ranging from 4.0 to 6.5 with step 0.5. The temperature optimum was defined in the 15 °C to 60 °C range at pH 5.0.

Thermostability

Thermostability of the phytase concentrate was determined in the absence of the substrate sodium phytate by pretreatment of the enzyme solution at 45, 50, 55, and 60 °C for a certain time and subsequent determination of the residual enzyme activity using the method of Tran et al., 2010.

Effect of metal ions and inhibitors on enzyme activity

The enzyme concentrate was preincubated for 30 minutes at 45 °C in the presence of different ions: $ZnSO_4.7H_2O$; KCl; NaCl; $CaCl_2$; $MnSO_4$; $MgSO_4$ and $CuSO_4.5H_2O$ at a final concentration of 1 M, and the residual activity was then determined at 45 °C.

The influence of ethylenediaminetetraacetic acid (EDTA), urea, dithiothreitol (DTT), and sodium dodecyl sulfate (SDS) at a final concentration of 0.1 % in the reaction mixture was studied according to the same scheme. Residual phytase activity was determined at 45 °C against a control without the tested chemicals.

Substrate specificity of the enzyme

The substrate specificity of the phytase enzyme was determined in the presence of the following phosphate-containing substrates at a final concentration of 4.5 mM: glucose 6-phosphate, ATP-Na_2, glycerol 2-phosphate-Na_2, fructose 1,6-diphosphate. The enzyme reaction was carried out at 45 °C, pH 5.0.

Data analysis

The analyses were performed in triplicate and the data used represent the mean values with Standard error of the mean (± SEM) of the three independent experiments. The statistical analysis was performed using Microsoft Office 365 Excel 2020 software.

Results and Discussion

Extracellular phytase concentrate preparation

Extracellular phytase from the centrifuged culture broth of *Pichia fermentans* B4-1 strain was concentrated by ultrafiltration. The measured total extracellular phytase activity before the ultrafiltration procedure was 0.69 U/ml. A 2.3-fold purification of the enzyme was achieved and the specific phytase activity in the resulting concentrate was doubled – 4.5 U/mg.

Temperature optimum and thermostability

P. fermentans B4-1 extracellular phytase was active in the range of 15 to 60 °C with temperature optimum at 45 °C. Its activity at 50 °C was about 60 % of those measures at 45 °C (Fig. 1), while at 35–37 °C, about 85–90 % of the enzyme activity was retained. According to other authors the fungal phytases have maximal activity between 37 °C and 65 °C (Yin et al., 2007).

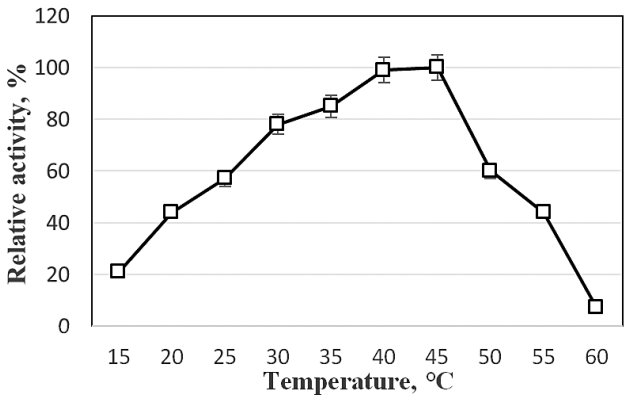

Fig. 1. Temperature optimum of *Pichia fermentans* B4-1 crude phytase. The relative activity was determined as a percentage of the maximal specific activity – 4.5 U/mg

The effect of temperature on the stability of the studied phytase was determined after pretreatment of the crude enzyme preparation using two different schemes. Initially, the thermostability of phytase concentrate was evaluated at 45 °C for 30, 45, 60, and 90 min. Further, the effect of 60 min incubation at higher temperatures (50, 55, and 60 °C) on the enzyme activity was studied (Fig. 2). It was revealed that the enzyme retained 100% of its activity after being heated at 45 °C for 30 minutes. By extending the incubation time to 90 min, a slight decrease in the phytase activity was registered (only 3%). Moreover, similarly to the extracellular phytases from *Pichia kudriavzevii* OG32 and *Candida tropicalis* BOM21 (Ogunremi et al. 2020) the *P. fermentans* B4-1 enzyme retains up to 90% of its initial activity after exposure to 50 °C for 60 min. However, at higher temperatures (55 °C – 60 °C) the residual activity sharply decreased (remaining only 5% of the initial one).

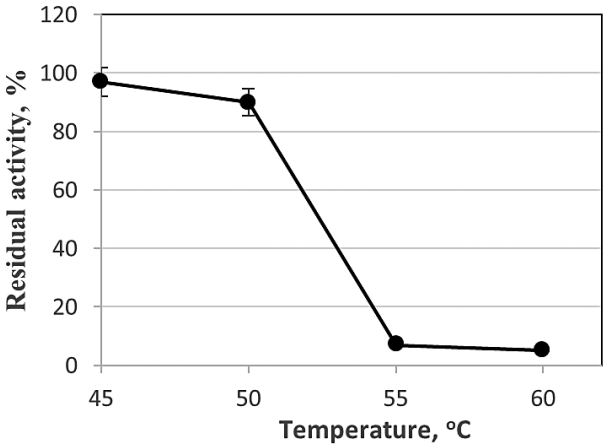

Fig. 2. Thermostability of *Pichia fermentans* B4-1 crude phytase preparation

pH optimum

pH profile for the activity of the phytase from *P. fermentans* B4-1 resembled to some extent that of most yeast phytases, which vary in the range 4–7 (Nakamura et., 2000; Bohn et al., 2008, Ogunremi et al. 2020). The enzyme was active at pH values from 4.0 to 6.5 with the maximum phytase activity reported at pH 5 at a temperature of 45 °C (Fig. 3). The observed residual activity at pH 4 and pH 6.5 was 67%, indicating a certain level of flexibility of the enzyme. As for the possible use of this phytase in crop production, there is a range of crops such as rice, maize, and papuda (Fageria & Zimmermann, 1998) that grow at a more acidic soil pH, suggesting the suitability of the enzyme for phytate degradation according to different agricultural demands.

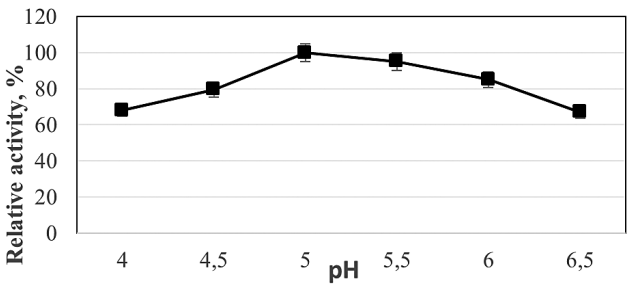

Fig. 3. pH optimum of *Pichia fermentans* B4-1 crude phytase preparation

Effect of metal ions and other compounds on phytase activity

When the effect of seven metal ions at a concentration 1 M (K^+, Na^+, Ca^{2+}, Cu^{2+}, Mn^{2+}, Mg^{2+}, Zn^{2+}) on the phytase activity was studied, an inhibitory influence for all of the tested metals was observed with 25–40% decrease in activity compared to the untreated enzyme (Fig. 4). The weakest influence on phytase action is exerted by potassium (73.6% residual activity) and calcium (72% residual activity) ions. Minimal residual catalytic activity was reported for the copper ions (59%), followed by that of the sodium (61%) and zinc ions (62.5%). The literature is rich in conflicting data on the action of metals on phytases obtained from different species and even strains of microorganisms. While Yao et al. (2014) reported an increase in phytase activity by Mg^{2+}, In et al. (2008) reported a negative effect. The case with Cu^{2+} is similar (Demir et al., 2017; Yu & Chen, 2013). On the other hand, calcium, sodium, and manganese ions appear to have a positive effect (Yao et al., 2014; Li et al., 2008), while zinc ions exhibit inhibitory properties (Li et al., 2008). Similar results of no stimulatory effect of metal ions, even at concentrations lower than 1 mM, on enzyme activity were reported for phytase from *Pichia anomala* (Vohra & Satyanarayana, 2002). The observed inhibitory effect may be the result of the formation of slightly soluble complexes of metal ions with phytic acid, which may reduce its active concentration in the reaction mixture (Vohra & Satyanarayana, 2002). While EDTA does not affect the phytase action, described by Li et al. (2008) or may have a mild stimulatory effect (Haefner et al., 2005), a partial inhibition (73% residual activity) of the phytase from *P. fermentans* B4-1 was detected when this chelating agent was added to the crude enzyme solution (Fig. 5). As EDTA is well-known chelator of divalent cations, the result obtained suggested that the addition of this compound to the media probably causes a depletion of such ions and correspondingly negatively affects the activity or stability of the investigated phytase. In support of these results are the investigations of other authors, which showed that calcium ions have a positive effect on enzyme activities of some microbial phytases (Yao et al., 2014; Li et al., 2008). The performed study revealed that the reducing agent dithiothreitol (DTT) had an even more pronounced inhibitory effect on the studied enzyme (66% residual activity), contradicting the data, reported by Quan et al. (2002) for a positive influence of the compound on phytase activity. The disulfide bonds appear to be a crucial factor for the catalytic activity, considering the negative effect of DTT. The most detrimental to phytase was sodium dodecyl sulfate (SDS), in the presence of which only 20% of the initial phytase activity remained. This suggested that protein conformation and function strongly depend on non-covalent interactions. A similar result of very low residual activity in the presence of SDS was reported for extracellular phytase from *Pichia anomala* (Vohra & Satyanarayana, 2002). However, this enzyme was not inhibited by EDTA and DTT, in contrast to the investigated phytase from the B4-1 strain.

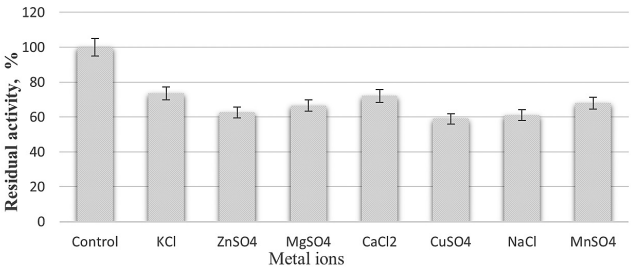

Fig. 4. Influence of some metal ions on the crude phytase activity. The residual activity was calculated as a percentage of the maximal specific activity – 4.5 U/mg

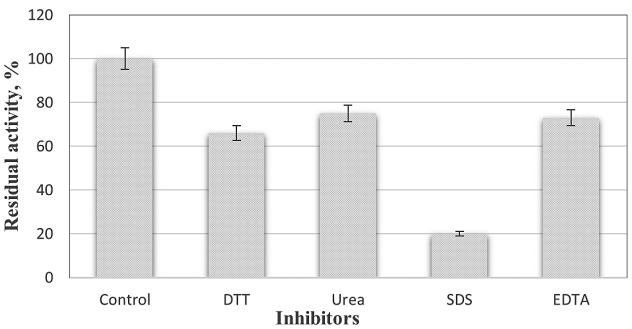

Fig. 5. Influence of some inhibitors on the crude phytase activity. The residual activity was calculated as a percentage of the maximal specific activity – 4.5 U/mg

Substrate specificity

The substrate specificity of the *P. fermentans* B4-1 phytase was determined in the presence of five phosphate-containing compounds: Na-phytate, glucose 6-phosphate, ATP-Na_2, glycerol 2-phosphate-Na_2 and fructose 1,6-biphosphate (Table 1). Similar to other yeast phytases (Vohra & Satyanarayana, 2002), those from *P. fermentans* B4-1 showed a broad substrate specificity and catalyzed the hydrolysis of all tested compounds. Maximal specific activity was registered when sodium phytate was used as a substrate. A lower phosphorus formation rate was determined after hydrolysis of the other four substrates, and the specific phytase activity varies between 69–81% of that when sodium phytate was used as a substrate.

Table 1. Specific phytase activity towards different phosphate-containing substrates.

Substrate	Specific phytase activity [U/mg]
Glucose 6-phosphate	3.65
ATP-Na_2	3.52
Glycerol 2-phosphate-Na_2	3.14
Fructose 1,6-biphosphate	3.26
Na-phytate	4.5

Conclusion

Enhancing the quality of life requires the development of new approaches to ecological and sustainable agriculture. The highly active and stable extracellular phytase produced by the yeast strain *P. fermentans* B4-1, shows promising characteristics for an efficient phytate hydrolysis, which determines its possible application in improving the soil bioavailability of phosphorus and other minerals, stimulating the nutrition and growth of various crops.

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