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Characterization of a Thermotolerant Phytase Produced by *Rhizopus microsporus* var. *microsporus* Biofilm on an Inert Support Using Sugarcane Bagasse as Carbon Source

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Abstract The *Rhizopus microsporus* var. *microsporus* biofilm was able to produce increased levels of an extracellular thermotolerant phytase using polyethylene and viscose as an inert support in both modified NBRIP medium and modified Khanna medium containing sugarcane bagasse as the carbon source. The enzyme production was strictly regulated by the phosphorus content with optimal production at 0.5 mM of sodium phytate and KH_2PO_4. The extracellular phytase, RMPhy1, was purified 4.18-fold with 4.78 % recovery using DEAE-cellulose and CM-cellulose. A single protein band with a molecular mass of 35.4 kDa was obtained when the samples were subjected to 10 % SDS-PAGE. The optimum temperature for activity was 55 °C and the optimum pH was 4.5. *R. microsporus* var. *microsporus* phytase exhibited high stability at 30 and 40 °C with a half-life of 115 min at 60 °C. The enzyme activity increased in the presence of Ca^{2+} and was inhibited by Zn^{2+}, arsenate, and sodium phosphate. Phytase demonstrated high substrate specificity for sodium phytate with $K_m = 0.72$ mM and $V_{max} = 94.55$ U/mg of protein and for *p*-NPP with $K_m = 0.04$ mM and $V_{max} = 106.38$ U/mg of protein. The enzyme also hydrolyzed ATP, AMPc, glucose 6-phosphate, glucose 1-phosphate, and UDPG. This is the first report on phytase characterization delivered with biofilm technology. The properties of the enzyme account for its high potential for use in biotechnology and the possibility of application in different industrial sectors as feed in the future.

Keywords Enzyme characterization · Fungal biofilm · Phytase · *Rhizopus microsporus*

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Introduction

Phosphate is a vital nutrient required in large amounts in order to maintain optimum levels of key cell molecules necessary for growth, division, and cellular metabolism, such as ATP, nucleic acids, and phospholipids; phosphorus is also a pivotal mediator in the regulation of many metabolic processes [1, 2]. The main form in which phosphorus is stored in plants is in the phytate form, for example, in cereal and legumes, which are the major components of commercial animal feeds. However, phytate cannot be fully hydrolyzed by the digestive tract of monogastric animals. In addition, phytate is an important antinutritional agent able to complex with cations such as Ca^{2+}, Zn^{2+}, Mn^{2+}, Fe^{2+}, Cu^{2+}, and Mg^{2+} as well as with proteins, thus reducing the bioavailability of these nutrients [3].

Phytases (myo-inositol hexakisphosphate phosphohydrolases) are enzymes that compose a special class of phosphatases. Phytases catalyze the hydrolysis of the phosphomonoester bonds of phytate (salts of myo-inositol 1,2,3,4,5,6-hexakis dihydrogen phosphate), releasing lower forms of myo-inositol and inorganic phosphates. On the basis of the site at which phytate dephosphorization begins, phytases can be grouped into three classes: 3-phytases (EC 3.1.3.8), 6-phytases (EC 3.1.3.26), and 5-phytases (EC 3.1.3.72). The fungal phytases are included in the 3-phytases class, in which the dephosphorization starts at the third phosphate group. Because of their catalytic mechanism, the fungal phytases belong to the histidine acid phosphatase class [3].

Phytases show great potential for application in different sectors such as in animal nutrition, human nutrition, aquaculture, and pharmacology. These enzymes are considered as a green feed additive that can be used to neutralize the antinutritional effects of phytate, thereby increasing the bioavailability of phosphorus and other minerals. They also contribute to the reduction of environmental pollution by phosphorus [4, 5]. Some isomers of myo-inositol phosphates have interesting pharmacological properties such as anti-inflammatory, antiangiogenic, and antitumor effects. The numbers and position of the phosphate residues in the myo-inositol ring are determinative for the effect of each myo-inositol isomer [6].

Microorganisms are the main source of phytases with biotechnological characteristics, and the enzymes used in the feed industry are mesophilic or thermophilic. Kinetic characterization of phytases from different sources indicated that the microbial enzymes are about 100,000 times more effective than those obtained from other sources [7]. In this context, filamentous fungi can be highlighted as important producers of phytases with interesting biotechnological characteristics such as thermal stability. Some fungal phytases have been characterized as those obtained from *Rhodotorula mucilaginosa* [4], *Aspergillus niger* [5], *Paecilomyces variotii* [8], *Aspergillus caespitosus* [9], *Rhizopus oligosporus* [10], and *Aspergillus flavus* [11].

Phytases can be produced using both submerged fermentation (SmF) and solid-state fermentation (SSF) [12]. However, new alternatives for the production of microbial enzymes have been used such as via the use of fungal biofilms, which is an innovative way of cultivating filamentous fungi. Fermentation conducted with fungal biofilm accounts for a homogenous system of production under the liquid environment, similar to SmF, but with the efficiency of SSF [13]. Biofilms can be developed on polymeric matrices in a way that allows high structural organization of the organisms that are important in the production process [14]. Sato et al. [15] reported the production of phytases using the *Rhizopus microsporus* var. *microsporus* biofilm. The crude extract containing phytase was spray-dried and some properties of the dried product obtained were evaluated. However, the purification and characterization of *R. microsporus* var. *microsporus* phytases were not reported. Enzyme

characterization is an important factor for determining its potential in commercial application. Therefore, in this study, we aimed to describe the production of an extracellular thermotolerant phytase by *R. microsporus* var. *microsporus* biofilm as well as its purification and biochemical characterization.

Material and Methods

Microorganism and Culture Condition

The filamentous fungus *R. microsporus* var. *microsporus* was isolated from Brazilian soil; identified at the Laboratory of Microbiology from the Federal University of Pernambuco, Brazil; and deposited in the Laboratory of Microbiology of the Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto from the University of São Paulo, Brazil. The microorganism was maintained on slants of PDA medium (Acumedia, Lansing, MI, USA) at 30 °C for 7 days and then stored at 4 °C.

The *R. microsporus* var. *microsporus* biofilms were developed initially on polyethylene inert supports (2.0 × 2.0 cm), previously washed with detergent and distilled water, and then sterilized under UV radiation. For spore adhesion, the sterilized supports were immersed in aqueous spore suspension (10^6 spores/mL) at 30 °C under agitation (40–80 rpm) for 2 h. Then, the supports were rinsed twice with distilled water for 30 min under agitation (50 rpm) in order to remove the unadhered spores. The supports with adhered spores were transferred to 250 mL Erlenmeyer flasks containing 50 mL of different culture media: modified Khanna [16], Czapeck [17], Vogel [18], Segato-Rizzatti (SR) [19], and modified National Botanical Research Institute's phosphate growth medium (NBRIP) [20]. Different agro-industrial by-products (2.0 % *w*/*v*) (soya mince, rye flower, sugarcane bagasse, wheat bran, and orange peel) and saccharides (2.0 % *w*/*v*) (fructose, galactose, glucose, maltose, and sucrose) were used as carbon sources, and the initial pH was adjusted to 6.0. The media were previously autoclaved at 121 °C, 1.5 atm for 30 min. The cultures were maintained at 30 °C under agitation (50 rpm) for 48 h. The values from the media obtained were compared using Tukey's test with p value fixed at 0.05.

Influence of Different Inert Supports on Biofilm Formation

Different inert supports (TNT—100 % polypropylene, polyurethane foam, nylon, viscose, polyester, ethylene vinyl acetate (EVA), and polyethylene) were used for *R. microsporus* var. *microsporus* biofilm formation. For this purpose, the cells were cultured on modified NBRIP medium using sugarcane bagasse as carbon source (20 g/L) under agitation (50 rpm) at 30 °C for 48 h.

Influence of KH_2PO_4 and Sodium Phytate Upon Phytase Production

Phytase production by *R. microsporus* var. *microsporus* biofilm was analyzed using modified NBRIP medium and modified Khanna medium added with different concentrations of sodium phytate (Sigma®) (0–20 mM) with sugarcane bagasse (2 % *w*/*v*) as carbon source, initial pH 6.0. Additionally, the influence of different KH_2PO_4 concentrations (0.1 to 20 mM) added to modified Khanna medium upon enzyme production was also analyzed.

Determination of *p*-Nitrophenylphosphatase Activity

The *p*-nitrophenylphosphatase activity was determined discontinuously using 1 mM *p*-nitrophenyl phosphate (*p*-NPP) as substrate in 100 mM sodium acetate buffer pH 5.0 at 45 °C. The reaction was stopped by adding 2.0 mL saturated solution of sodium tetraborate at different time intervals. The *p*-nitrophenol released was determined using a spectrophotometer at 420 nm. One unit of enzyme activity (U) was defined as the amount of enzyme required to release 1 μmol of *p*-nitrophenol per min under the assay conditions. The specific activity was expressed as units per milligram of protein.

Determination of Phytase Activity

The phytase activity was assayed according to the method described by Gulati et al. [21] with some modifications. The reaction mixture was composed of 50 μL of the enzyme sample and 50 μL of 1 % (*w*/*v*) sodium phytate (dodecasodium salt; Sigma) in 0.2 M sodium acetate buffer pH 4.5. After 30 min, the reaction was stopped by the addition of 100 μL of 15 % trichloroacetic acid (TCA) at room temperature, and 300 μL of distilled water was added to each test tube. Thereafter, 900 μL of the chromogenic reagent (0.76 M sulfuric acid, 10 % ascorbic acid, and 2.5 % ammonium molybdate; 3:1:0.5 (*v*/*v*/*v*)) was added and the tubes were incubated at 50 °C for 20 min. The absorbance was read at 820 nm. Controls without the addition of the enzyme were included for the estimation of nonenzymatic hydrolysis of the substrate. One unit of enzyme activity was defined as the amount of enzyme that releases 1 μmol of inorganic phosphate per min under the assay conditions. The specific activity was expressed as units per milligram of protein.

Protein Quantification

The protein content was quantified according to Bradford [22] using bovine serum albumin (BSA) as the standard and expressed as milligram of protein.

Purification of Phytase Produced by *R. microsporus* var. *microsporus* Biofilms

After incubation as described above, the biofilm was removed from the medium containing sugarcane bagasse as the carbon source using a sterile tweezer, and the free cell liquid containing the extracellular enzyme was dialyzed overnight at 4 °C against distilled water and loaded in DEAE-cellulose chromatographic column (1 × 12 cm) equilibrated with 50 mM sodium acetate buffer, pH 5.0. Fractions of 3 mL were collected at a flow rate of 1 mL per min using a linear gradient of NaCl (0–1 M). Fractions with *p*-nitrophenylphosphatase activity were monitored and two enzyme peaks with phytase activity were obtained. Peak I (RMPhy1), which did not interact with DEAE-cellulose resin, was loaded in the chromatographic column (1 × 8 cm) of CM-cellulose equilibrated with 50 mM sodium acetate buffer, pH 5.0. Fractions of 3 mL were collected at a flow rate of 0.5 mL per min using a linear gradient of NaCl (0–1 M) in the same buffer. Fractions with phytase activity were pooled, dialyzed overnight at 4 °C, and concentrated using a 30-kDa cutoff Amicon membrane (Millipore) at 8000*g* for 15 min at 4 °C. The concentrated fraction was used for the electrophoresis procedure and enzyme characterization.

Electrophoresis

The purified sample was applied to polyacrylamide gel electrophoresis under denaturing conditions (SDS-PAGE 10 %) [23], using a power source adjusted to 120 V and 40 mA. After running, the gels obtained were stained using Coomassie Brilliant Blue-R250. For SDS-PAGE, the Precision Plus Protein Standards Kaleidoscope (Bio-Rad®) was used as molecular weight markers (10–250 kDa).

Characterization of RMPhy1 Phytase

The effect of temperature (30–80 °C) and pH (100 mM sodium acetate buffer pH 3.5 to 6.0; 100 mM Ampol buffer pH 6.0 to 7.0; 100 mM Tris-HCl buffer pH 7.0 to 10.0) on phytase activity was determined.

The thermal stability analysis was performed by maintaining the enzymatic samples at different temperatures (30–70 °C) for 120 min. At different time periods, samples were withdrawn and maintained in an ice bath for subsequent measurement of enzyme activity. The pH stability was determined in the pH range of 3.5 to 10.0 using the same buffer solutions cited above for 30 and 60 min at room temperature (29 °C).

Effect of Different Compounds on Enzyme Activity

The effects of 1 and 2 mM of KCl, $MgCl_2$, $ZnSO_4$, $CuCl_2$, $CoCl_2$, and $CaCl_2$; 0.2 and 0.4 mM of $FeSO_4$; 5 and 10 mM of EDTA; 0.25 and 1 mM of sodium arsenate; and 1 and 5 mM of potassium phosphate on enzyme activity were analyzed. The reactions were carried out in a 100 mM acetate buffer at pH 4.5 containing 1 % sodium phytate as substrate. Residual activity was calculated by considering the value obtained during the absence of compounds as 100 %.

Hydrolysis of Substrates and Estimation of Kinetic Parameters

The ability of the enzyme to hydrolyze sodium phytate (1 mM), *p*-NPP (2 mM), ATP (2 mM), AMPc (0.5 mM), uridine diphosphate glucose (UDPG) (2.5 mM), and glucose 6-phosphate and glucose 1-phosphate (both at 2 mM) was analyzed under the same conditions described for sodium phytate. The kinetic parameters V_{max}, K_m, and V_{max}/K_m were estimated using sodium phytate as the substrate (0.01 to 4 mM) and *p*-NPP (0.01 to 5 mM), as described above. The calculation and graphical representation were fit using the OriginPro 8.0 software according to Lineweaver-Burk [24].

Results and Discussion

Formation of *R. microsporus* var. *microsporus* Biofilm

The level of agitation during the process of biofilm formation, and consequently, in enzyme production, is an important aspect that should be considered. Initially, the *R. microsporus* var. *microsporus* biofilms were developed in Khanna medium using sugarcane bagasse as the carbon source, as previously reported [15], and 0.5 mM of KH_2PO_4 was added. As seen in Table 1, the use of agitation from 40 to 60 rpm allowed the formation of a biofilm without free

cells in the culture medium, indicating greater resistance of the *R. microsporus* var. *microsporus* biofilm structure. The biomass of the biofilm during each condition was similar. However, when 70- and 80-rpm stirrings were used, the biomass of the biofilm that adhered to the support decreased (0.90 and 0.35 g, respectively), and free biomass was observed in the culture medium, indicating a minor resistance of biofilms under these conditions of agitation. High level of agitation can promote de-adhesion of the spores of the inert support leading to the free mycelium formation.

Therefore, fungal morphology and, consequently, the enzyme production can be affected by the culture conditions; under SmF, the fungal growth can vary between the pelleted and filamentous forms. The orientation of growth in both SmF and biofilm fermentation (BF) systems is different; pellets increase in density rather than depth under SmF, while the opposite is observed for BF [13]. However, specific aspects of the relation between enzyme production and fungal morphology under BF need to be investigated in the future.

Effect of Different Concentrations of KH_2PO_4 on Phytase Production

In order to improve the production of extracellular phytase by *R. microsporus* var. *microsporus* biofilms, the effect of different concentrations of KH_2PO_4 (0–5 mM) added to modified Khanna medium (without yeast extract) was analyzed. Maximum phytase production (Fig. 1) was observed at 0.5 mM KH_2PO_4 (61.6 U/mg of protein) and maintained without significant differences at high KH_2PO_4 concentrations. On the other hand, the specific activity of the enzyme decreased above 0.5 mM KH_2PO_4. These results indicate that phytase production by *R. microsporus* var. *microsporus* biofilm is stimulated by the phosphorus source in the medium, suggesting that there is a link between the use of exogenous phosphate, mobilization of reserves, and enzymatic production. Similar results were obtained for phytases and other microbial phosphatases [25, 26]. The regulation of enzymatic synthesis by Pi is coordinated by the PHO pathway that has been reported for different organisms, including fungi. In *Saccharomyces cerevisiae*, high Pi levels are responsible for the activation of a cyclin-dependent kinase complex that phosphorylates the positive regulator Pho4 (encoded by the *PHO4* gene), which remains in the cytoplasm. However, at low Pi levels, the unphosphorylated Pho4 travels to the nucleus and activates the *PHO* gene transcription allowing the secretion of the acid phosphatases and other proteins related to the Pi uptake [27].

Production of Phytase by *R. microsporus* var. *microsporus* Biofilm Using Different Culture Media

Table 2 shows the production of phytase by *R. microsporus* var. *microsporus* biofilms as the function of different culture media added with 0.5 mM KH_2PO_4. The maximum phytase

Table 1 Influence of agitation in the formation of *R. microsporus* var. *microsporus* biofilms

Agitation (rpm)	Biofilm biomass (g)	Free biomass (g)
40	2.15	–
50	2.48	–
60	2.10	–
70	0.90	0.39
80	0.35	0.16

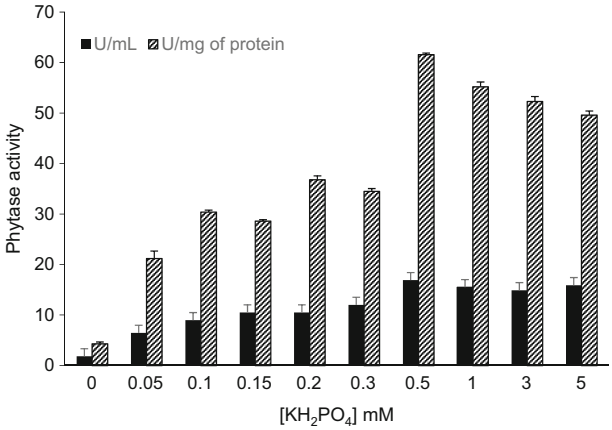

Fig. 1 Phytase production by *R. microsporus* var. *microsporus* biofilms at different concentrations of KH_2PO_4

production was detected in modified NBRIP medium (without the addition of glucose) (69.9 U/mg protein) followed by modified Khanna medium (without yeast extract) (46.3 U/mg protein). Production using modified NBRIP medium was 20-fold higher than that observed using SR medium. NBRIP medium has been used in the search and selection of phosphate-solubilizing microorganisms [28]. In addition, the result obtained for modified NBRIP medium was 1.5-fold higher than that observed for the modified Khanna medium used previously.

Influence of Different Inert Supports Upon Biofilm Formation and Phytase Production

Considering the high enzymatic production obtained when using modified NBRIP medium, the biofilm formation using different inert supports was analyzed (Table 3). The supports were selected on the basis of availability, low cost, homogeneous distribution of particle size, and surface roughness (this provides a larger surface area for adhesion of microorganisms). All supports were colonized by *R. microsporus* var. *microsporus* with good spore adhesion and biofilm formation. Couto et al. [29] stated that filamentous fungi have a great affinity for inorganic and organic surfaces; however, the adhesion mechanisms are not yet understood well. Different materials have been reported for use as fungal adhesion materials such as synthetic foams (polyurethane), nylon sponge, geotextile webs of polyamide, and polyester cloth [14, 29, 30]. Thus, as seen in Table 2, a higher level of phytase production was observed when viscose (169.61 U/mg) and polyethylene (163.0 U/mg) were used as supports, and the specific enzyme activities were also highest (3-folds higher) for these compared to the use of

Table 2 Phytase production by *R. microsporus* var. *microsporus* biofilms in different culture media

Medium	U/mg protein
Modified Khanna	46.3
Czapeck	8.79
Vogel	11.5
M5	15.7
SR	3.41
Modified NBRIP	69.9

The cultures were maintained under agitation (50 rpm) at 30 °C for 48 h

Table 3 Phytase production by *R. microsporus* var. *microsporus* biofilms obtained on different supports using modified NBRIP medium

Supports	U/mg protein
TNT	138.5 ± 0.20
Polyurethane foam	71.6 ± 0.67
Nylon	79.5 ± 0.80
Viscose	169.61 ± 0.70
Polyester	128.0 ± 0.80
Polyethylene	163.0 ± 0.20
EVA	53.3 ± 0.67

The cultures containing sugarcane bagasse were maintained at 30 °C for 48 h at 50 rpm

EVA as a support. The choice of inert support for fungal adhesion and biofilm formation is essential in designing an effective system for particular purposes.

Effect of Different Concentrations of Sodium Phytate on Phytase Production

The addition of 0.5 mM sodium phytate in both modified Khanna medium and modified NBRIP medium increased phytase production 2.6-fold and 1.4-fold, respectively, as compared to the absence of phytate condition (Fig. 2). At this concentration, enzymatic production using modified NBRIP medium was 4-fold higher than that obtained when using modified Khanna medium. However, at high phytate concentration (20 mM), the production was reduced for both media used. These results indicate that the expression of *R. microsporus* var. *microsporus* phytase can be regulated by the phytate concentration in the culture medium.

Purification and Molecular Mass Determination

The chromatographic profile by loading crude filtrate obtained in modified NBRIP medium onto DEAE-cellulose is presented in Fig. 3a. Two enzymatic peaks (I and II) were obtained. Peak I (RMPhy1) does not interact with the resin, while peak II (RMPhy2) was eluted with 0.25 M NaCl using a linear gradient (0–1 M). Both pooled peaks presented phytase activity. After dialysis, the RMPhy1 pool was loaded onto the CM-cellulose chromatographic column and eluted with 0.5 M NaCl (Fig. 3b). Different approaches have been used for the purification of microbial phytases such as precipitation using acetone followed by Macro-prep high Q-column and ultrafiltration used for *A. flavus* ITCC 6720 phytase [11] and ammonium sulfate precipitation, ion exchange chromatography, and gel filtration chromatography for *Bacillus licheniformis* PFBL-03 phytase [31].

The phytase RMPhy1 was purified 4.18-fold with a 4.78 % yield (Table 4). This yield was better as compared to the values obtained for the purified phytase from *A. flavus* ITCC 6720 [11] and two purified isoforms of intracellular phytase from *R. oligosporus* after five steps of purification with a recovery of 1.3–1.6 % [10].

A single protein band of 35.4 ± 2.6 kDa was obtained under SDS-PAGE for RMPhy1 (Fig. 4, lane D). This molecular mass value was close to that found for *Cladosporium* sp. FP-1 phytase (32.6 kDa) [32] and for *A. niger* 307 phytase (39 kDa) [33]. According to Yao et al. [34], phytases are proteins with highly variable molecular mass, ranging from 40 to 500 kDa. The phytases from *A. niger* commercialized by La Roche and BASF have molecular masses of 89.14 and 65.73 kDa, respectively [35]. The phytases produced by *R. oligosporus* showed a molecular mass of 45 kDa [10]. The efficiency of each purification step, i.e., DEAE-cellulose

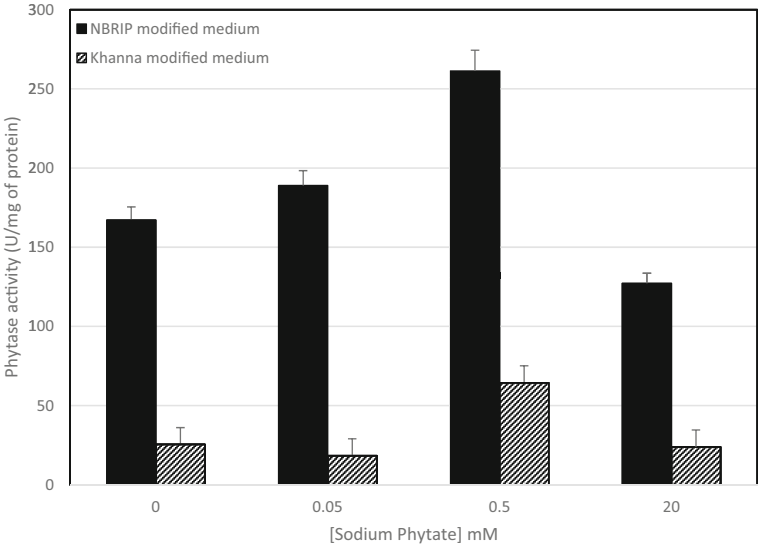

Fig. 2 Phytase production by *R. microsporus* var. *microsporus* biofilms at different concentrations of sodium phytate added to the modified NBRIP and modified Khanna media

(Fig. 4, lane B), CM-cellulose (Fig. 4, lane C), and after filtration through a 30-kDa cutoff membrane (Fig. 4, lane D), also can be observed, with significant reduction of the amount of contaminant proteins.

Influence of pH and Temperature Upon RMPhy1 Phytase Activity

The influence of temperature and pH on the activity of the enzyme is an important characteristic that needs to be considered before its possible biotechnological application. Figure 5 shows that the optimum activity for phytase produced by *R. microsporus* var. *microsporus* biofilm was achieved at pH 4.5 and 55 °C. Most phytases present optimum enzyme activity at a pH range from 4.0 to 6.0 for that produced by *A. niger* (pH 5.0–5.5), *A. fumigatus* (pH 4.0–6.0), and *Peniophora lycii* (pH 4.0–5.0), among others [3]. RMPhy1 phytase was able to act

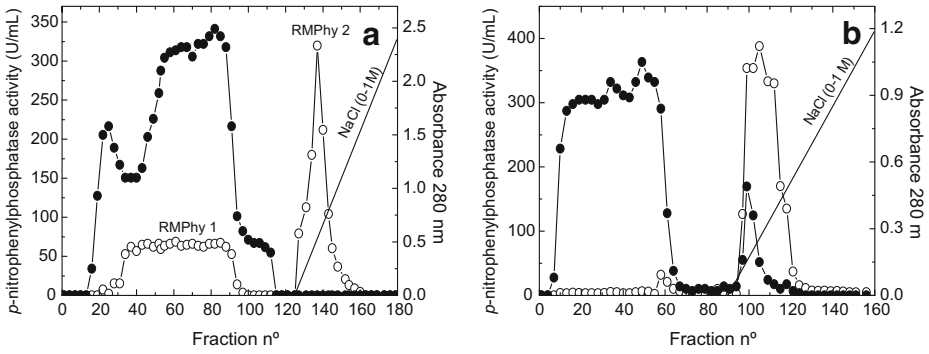

Fig. 3 Chromatographic profiles of DEAE-cellulose (**a**) and CM-cellulose (**b**) for the phytases produced by *R. microsporus* var. *microsporus* biofilms in modified NBRIP medium using sugarcane bagasse as carbon source. Symbols: *filled circle*, absorbance 280 nm; *empty circle*, *p*-nitrophenylphosphatase activity

Table 4 Purification of the phytase RMPhy1 produced by *R. microsporus* var. *microsporus* biofilms

Step	Volume (mL)	Activity (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Crude extract	180	9546.0	17.5	545.5	100	1
DEAE-cellulose	95	2098.5	8.3	252.8	22.0	0.5
CM-cellulose	25	457.0	0.2	2285.0	4.78	4.18

under alkaline conditions (pH 8–10.5) maintaining 65–75 % of its activity. This characteristic is very interesting because there is no report on a fungal phytase acting at an alkaline pH for the genus *Rhizopus*. Until now, only a phytase produced by *A. niger* ATCC 9142 was described as acting under alkaline pH conditions [36]. Studies have also investigated the optimum temperature for phytase activity in the range from 40 to 70 °C [34]. Despite maximal activity observed at 55 °C, the RMPhy1 phytase maintained 85 and 73 % of its activity at 60 and 65 °C, respectively. The activity from *Cladosporium* sp. FP-1 phytase was optimum at 40 °C, while the phytases from *Aspergillus oryzae* [37] and *B. licheniformis* PFBL-03 [31] showed optimum activity at 55 °C.

The RMPhy1 phytase produced by *R. microsporus* var. *microsporus* biofilms was stable at 30 and 40 °C for 120 min, maintaining 80–95 % of its activity (Fig. 5d). At 60 °C, the *R. microsporus* var. *microsporus* phytase showed a half-life (t_{50}) of 115 min, which was better than that observed for the *Escherichia coli* phytase that maintained only 24 % of its activity at 60 °C for 1 h [38] and better than that observed for *Rh. mucilaginosa* phytase with a half-life of

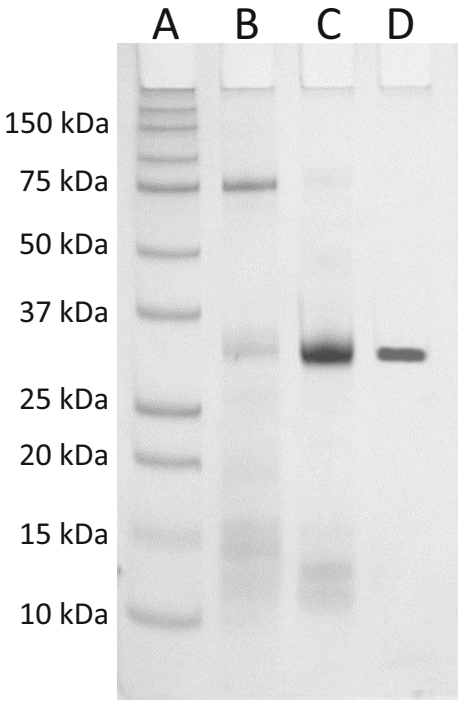

Fig. 4 Ten percent SDS-PAGE for purified RMPhy1 phytase produced by *R. microsporus* var. *microsporus* biofilms stained with Coomassie Brilliant Blue-R250. *Lane A* molecular weight markers, *B* peak I (RMPhy1) obtained from DEAE-cellulose, *C* phytase containing fraction after CM-cellulose, *D* phytase containing fraction after filtration through a 30-kDa membrane (Amicon, Millipore). For details, see "Material and Methods" section

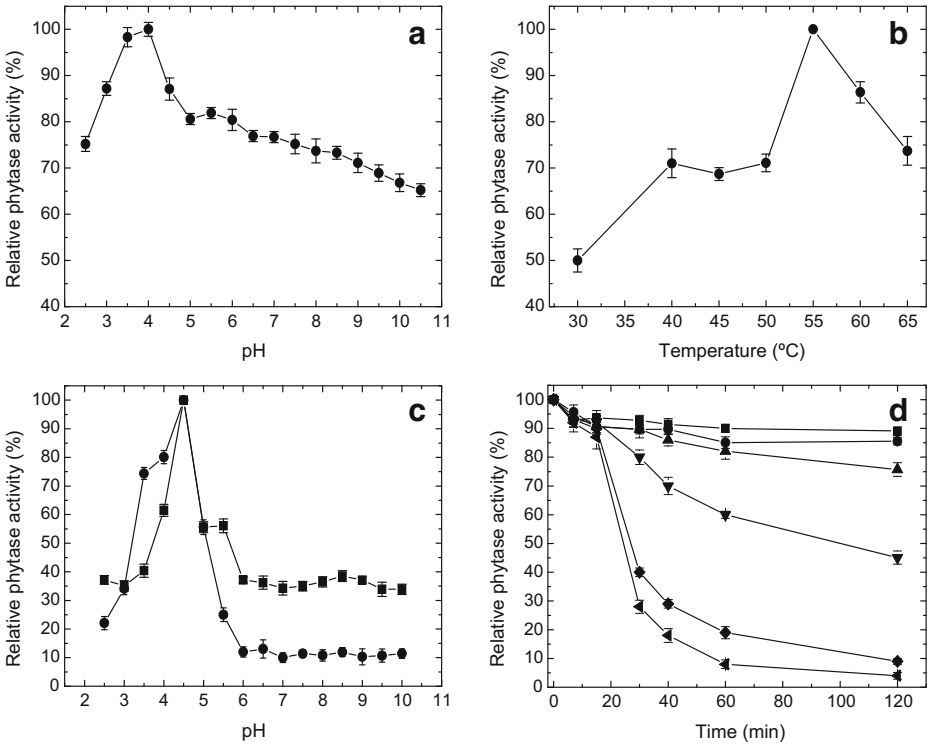

Fig. 5 Determination of optimum of pH (**a**), temperature (**b**), and pH stability (**c**) for 30 (*filled square*) and 60 min (*filled circle*), and thermal stability (**d**) at 30 °C (*filled square*), 40 °C (*filled circle*), 50 °C (*filled triangle*), 60 °C (*filled inverted triangle*), 65 °C (*filled diamond*), and 70 °C (*filled left-pointing arrowhead*) for RMPhy1 phytase produced by *R. microsporus* var. *microsporus* biofilms

10 min at 60 °C [4]. At 65 and 70 °C, the half-life was 28–31 min, higher than that observed for the phytase produced by *Rh. mucilaginosa* [4]. Thermostability is an important characteristic for preserving the nutrition of the animal feed because the enzyme needs to resist the temperature of pelletization [3].

The *R. microsporus* var. *microsporus* RMPhy1 phytase maintained 75–100 % of its activity in the pH range from 3.5 to 4.5 for both 30 and 60 min of incubation (Fig. 5c). The phytase activity from *Bacillus nealsonii* ZJ0702 decreased dramatically at a pH of 4.0 with incubation for 30 min [39], while the *A. niger* NCIM 563 phytase showed broad pH stability from 2.0 to 9.0 [40].

Effect of Different Compounds Upon RMPhy1 Phytase Activity

Table 5 displays the influence of different compounds, such as ions and chelating agents, on RMPhy1 phytase activity. *R. microsporus* var. *microsporus* phytase activity was significantly affected primarily by Ca^{2+} (+24-42 %); this was also observed for the enzymes produced by *A. flavus* ITCC 6720 [11] and *A. niger* [41]. On the other hand, phytase RMPhy1 activity reduced in the presence of K^+, Zn^{2+}, Cu^{2+}, and Co^{2+}. This inhibitory effect could be due to the formation of poorly soluble complexes of the metal ions with phytate, which can reduce the concentration of phytate available for hydrolysis in the assay [42]. Inhibition of enzyme activity by Cu^{2+} and Zn^{2+} was also observed for *A. niger* phytase [41], while the enzyme

produced by *Schizophyllum commune* was activated in the presence of $CuSO_4$ and $ZnSO_4$ [43]. Ions can interact with charged amino acids on the protein surface or in its active site, promoting conformational modifications that can positively or negatively affect the enzyme activity.

R. microsporus var. *microsporus* phytase RMPhy1 was not affected by ethylenediaminetetraacetic acid (EDTA), suggesting that it is not a metalloenzyme. EDTA, a chelating compound, can form a complex with various important metal ions, negatively affecting the catalytic activity of enzymes, as reported for the enzyme produced by *Mucor indicus* [44]. On the other hand, EDTA can also stimulate phytase activity, as reported for the enzyme produced by *A. fumigatus* [7].

Phosphate and arsenate can be considered as potent inhibitors of *R. microsporus* var. *microsporus* phytase RMPhy1 activity. Arsenate is a structural analog of phosphate and it is assumed that its inhibiting action is because of its linking to the catalytic site of the enzyme. Pi is a product obtained via the action of phytase and it is a specific inhibitor of enzyme activity [45]. Pi can act through a competitive inhibition model. Inhibition of the phytase RMPhy1 activity by sodium phosphate suggests that the concentration of inorganic phosphate regulates not only enzyme synthesis and induction but also enzymatic activity.

Hydrolysis of Substrates and Kinetic Parameters

Phytase RMPhy1 was able to hydrolyze not only sodium phytate and *p*-NPP but also all other substrates used (Table 6). The enzymatic activity in the presence of sodium phytate was higher than that obtained for the use of ATP, AMPc, UDPG, glucose 6-phosphate, and glucose 1-phosphate. On the other hand, the enzyme activity using *p*-NPP as substrate was 33 % higher

Table 5 Effect of different compounds on RMPhy1 phytase activity from *R. microsporus* var. *microsporus* biofilm

Compounds	Relative phytase activity (%)	
	1 mM	2 mM
Control	100	100
KCl	60.20 ± 0.33	49.69 ± 0.12
$MgCl_2$	102.82 ± 0.44	108.46 ± 0.13
$ZnSO_4$	60.12 ± 0.89	15.38 ± 0.35
$CuCl_2$	95.24 ± 0.66	88.46 ± 0.33
$CoCl_2$	98.32 ± 0.28	54.60 ± 0.88
$CaCl_2$	124.4 ± 0.44	142.92 ± 0.42
	0.2 mM	0.4 mM
$FeSO_4$	99.45 ± 0.66	109.80 ± 0.62
	5 mM	10 mM
EDTA	98.34 ± 0.48	96.92 ± 0.88
	0.25 mM	1 mM
Sodium arsenate	0.93	0.84
	1 mM	5 mM
Potassium phosphate	0.03	0.00

Phytase activity 100 %; 161.69 U/mg protein

Table 6 Hydrolysis of different substrates using the purified enzyme produced by *R. microsporus* var. *microsporus*

Substrates	Specific activity (U/mg of protein)	Relative activity (%)
Sodium phytate (1 mM)	78.27	100
p-NPP (2 mM)	104.82	133.92
ATP (2 mM)	37.47	47.87
AMPc (0.5 mM)	55.63	71.07
UDPG (2.5 mM)	13.10	16.74
Glucose 6-phosphate (2 mM)	57.70	73.72
Glucose 1-phosphate (2 mM)	18.73	23.93

Enzymatic sample obtained after filtration through the 30-kDa membrane

than that observed for sodium phytate. Additionally, it is possible to observe that the enzyme has preference to hydrolyze the phosphate located in carbon 6 in the molecule of glucose. Phytase produced by *M. indicus* hydrolyzed sodium phytate, *p*-NPP, ATP, and AMP as well [44]. The kinetic parameters K_m and V_{max} were estimated as 0.72 mM and 94.55 U/mg of protein for the hydrolysis of sodium phytate and as 0.04 mM and 106.38 U/mg of protein for *p*-NPP by phytase RMPhy1 produced by *R. microsporus* var. *microsporus* biofilms. This shows higher affinity demonstrated by the synthetic substrate than that by the natural one. The value obtained for the relation V_{max}/K_m was 131.32 and 2659.5 U/mg of protein mM^{-1} for sodium phytate and *p*-NPP, respectively. The K_m value obtained using sodium phytate was lower than that reported for *A. niger* NCIM 563 phytase PhyI (2.01 mM) [40], for *A. niger* (K_m 0.92 mM) [33], and for *R. oligosporus* phytase RO1 (1.6 mM) [10], indicating that the enzyme from *R. microsporus* var. *microsporus* has more affinity toward this substrate than these enzymes. On the other hand, the K_m value was higher than that observed for phytase RO2 (0.13 mM) from *R. oligosporus* [10] and the phytases produced by *S. commune* (0.16 mM) [43] and *Rh. mucilaginosa* JMUY14 (0.24 mM) [4].

Conclusion

Biofilm fermentation can be used effectively to produce high levels of phytase via *R. microsporus* var. *microsporus* with a high potential for use in biotechnology, as demonstrated by its high thermal and acidic pH tolerance; its ability to hydrolyze different natural substrates, especially sodium phytate; and the effect of ions on reducing its activity. The effect of temperature on enzyme activity can also be considered an interesting characteristic for its biotechnological application. In addition, we demonstrated that phytase production and activity are regulated by phosphate. This is the first description of the characterization of fungal phytase produced using biofilm technology. Phytase produced by *R. microsporus* var. *microsporus* shows good potential for future industrial applications, and its use in animal feed may be emphasized.

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