

Purification and characterization of an iron-activated alkaline phosphatase produced by *Rhizopus microsporus* var. *microsporus* under submerged fermentation using rye flour

Pedro Henrique de Oliveira Ornela¹, Luis Henrique Souza Guimarães^{2*}

¹Instituto de Química de Araraquara – UNESP, São Paulo, Brazil.

²Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto, USP, São Paulo, Brazil.

ARTICLE INFO

Article history:

Received on: January 17, 2020

Accepted on: May 01, 2020

Available online: July 30, 2020

Key words:

Alkaline phosphatase, enzyme characterization, fungal enzyme, *Rhizopus*

ABSTRACT

Current researches have been carried out to find microorganisms that can produce enzymes for different biotechnological purposes. Among the enzymes, the microbial phosphatases, responsible for hydrolyzing phosphoric acid anhydrides and esters, have been often employed in different sectors such as molecular biology experiments and clinical diagnosis. This work aims to purify and characterize the alkaline phosphatase produced by *Rhizopus microsporus* var. *microsporus* under submerged fermentation. This enzyme was purified 9.9-fold with 13% recovery. The molecular mass for the glycoprotein was 123 kDa estimated with gel filtration and 128 kDa with sodium dodecyl sulfate polyacrylamide gel electrophoresis, indicating that it is a monomeric enzyme. Optimal temperature and pH for the alkaline phosphatase was 45°C and 8.5, respectively, with half-life (t_{50}) of 40 minutes at 50°C. Under alkaline pH, the phosphatase activity was above 50% for 24 hours. FeCl₃ increased the phosphatase activity. Alkaline phosphatase hydrolyzed different substrates, especially *p*-nitrophenylphosphate, with K_m of 0.45 and 0.38 mmol l⁻¹, in presence and absence of FeCl₃, respectively. Thus, alkaline phosphatase from *R. microsporus* var. *microsporus* was characterized, highlighting important characteristics and, thereby, making possible a future application.

1. INTRODUCTION

Phosphorus is an important macronutrient for all living beings. Its assimilation by microorganisms depends on the specific enzymes, as phosphatases (EC 3.1.3), which are able to hydrolyze phosphorus-containing molecules. Organic phosphorus is a common source of phosphorus in soil and for many of these compounds, the phosphorus is linked to a carbon atom through the ester linkage, which can be hydrolyzed by phosphatase action, allowing the mineralization of organic phosphate when levels of free inorganic phosphate in soil are low [1,2]. In general, these enzymes hydrolyze esters and anhydrides of phosphoric acid releasing inorganic phosphate throughout different mechanisms of hydrolysis and substrate specificities [3].

Considering the classic way, phosphatases can be separated into alkaline (EC 3.1.3.1) and acid phosphatases (EC 3.13.2), according to the optimal pH of activity [4]. According to their molecular characteristics, these enzymes are also classified as (1) alkaline phosphatase, (2) high molecular mass acid phosphatase (greater than 90 kDa), (3) low molecular mass acid phosphatase (less than 50 kDa), (4) purple acid phosphatase (metalloenzyme) and (5) V-protein phosphatase [5]. Alkaline phosphatase hydrolyzes different natural and synthetic substrates as adenosine triphosphate (ATP), adenosine diphosphate (ADP), adenosine monophosphate (AMP), *p*-nitrophenyl phosphate, glucose-6-phosphate, glucose-1-phosphate, glyceraldehyde-3-phosphate and pyrophosphate, due to its capacity to act in alkaline pH [6].

Phosphatases are enzymes with multiple functions in living organisms and widely distributed in both prokaryotic and eukaryotic organisms [7]. In this context, filamentous fungi are interesting microorganisms because they contribute with different ecological process as the decomposition of organic molecules and transformation of N and P, among others [1]. For this purpose,

*Corresponding Author

Luis Henrique Souza Guimarães, Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto, USP, São Paulo, Brazil.

E-mail: lhguimaraes@ffclrp.usp.br

fungi are able to produce a myriad of ectoenzymes, including alkaline phosphatases used in the mineralization of organic phosphorus. The phosphatase activities observed in soils are the result of the extracellular enzymes accumulated in soil particles and intracellular enzymes from microorganisms [8]. The alkaline phosphatase is an important enzyme for mineralization process in different environments, especially in alkaline and phosphorus-limited soils [9]. Some alkaline soils as siderite, found in the environment as clay ironstone, are Fe-rich and can form extensive stratified beds [10,11]. Enzymes with the potential to mineralize these types of soils are an interesting focus of study and, according to our knowledge, no fungal alkaline phosphatase activated by iron has been described until this moment.

The investigation on the ability of fungal strains to produce alkaline phosphatases can be performed using submerged fermentation, as demonstrated by Coban and Demirci [12] for *Aspergillus ficuum* and by Suresh and Das [13] for *Aspergillus fumigatus*. As advantages, the composition of the culture medium for SbmF can be precisely defined; the fermentative parameters as temperature and pH are easily controlled, facilitating the scale up of the process; facility of sterilization reducing the contamination; high yield of the product [14,15]. The use of SbmF allows the control of the inorganic phosphate (Pi) concentration in the medium. Pi is an important macronutrient for all organisms and in fungi, the Pi regulates the expression of phosphate responsive genes as *PHO* genes for phosphatases [16]. Different carbon sources have been used for this purpose, including agroindustrial byproducts and residues as sugarcane bagasse, oatmeal and wheat bran [17]. In general, another aspect to be highlighted is the biotechnological potential of the alkaline phosphatases to be used in molecular biology experiments, removing 5'-phosphate groups in the plasmids to block its recircularization, as well as in clinical diagnosis and as biosensor [18].

For an effective understanding of the physiological importance of the phosphatases in the mineralization process, as well as for an effective biotechnological application, the biochemical characteristics of the enzyme should be determined. According to this, the article describes the purification and characterization of an iron-activated alkaline phosphatase produced by the filamentous fungus *Rhizopus microsporus* var. *microsporus*.

2. MATERIALS AND METHODS

2.1. Culture Conditions and Preparation of Crude Extracts

The filamentous fungus *R. microsporus* var. *microsporus* (LH0706) is deposited in the Laboratory of Microbiology of the Faculty of Philosophy, Sciences and Letters of Ribeirão Preto, São Paulo, Brazil. The microorganism was grown in sterile PDA medium (potato dextrose agar) at 30°C for 6–8 days and stored in a refrigerator at 4°C until the use. Submerged fermentation (SbmF) was performed in 125-ml Erlenmeyer flasks containing 25 ml of Khanna medium [19] added with 0.4 mmol l⁻¹ KH₂PO₄ at pH 6.3 and supplemented with 0.5% (m/v) rye flour as additional carbon source. The media were autoclaved at 120°C, 1.5 atm for 25 minutes and inoculated with 10⁶ spores ml⁻¹. Afterward, the cultures were maintained under orbital agitation (100 rpm) at 32°C for 76 hours. After this time, the cultures were harvested

using No.1 Whatman filter paper and a vacuum pump. The free cell filtrate was dialyzed against distilled water for 24 hours at 4°C and used for the determination of the extracellular alkaline phosphatase activity and purification procedure.

2.2. Determination of Alkaline Phosphatase Activity

The alkaline phosphatase activity was determined discontinuously using 2.7 mmol l⁻¹ *p*-nitrophenylphosphate (*p*-NPP) as substrate. The reaction mixture was composed of 100 µl of enzyme sample and 100 µl of *p*-NPP in 100 mmol l⁻¹ Tris-HCl buffer, pH 8.5. The reaction was conducted at 45°C for different periods and it was stopped with 2 ml of a saturated solution of sodium tetraborate. The *p*-nitrophenolate ion was estimated at 405 nm. One unit of enzyme activity (U) was defined as the amount of enzyme required to hydrolyze one micromole of substrate per minute under the assay conditions. All experiments were carried out as triplicates and the values were expressed as the mean ± standard error of the mean.

2.3. Protein Quantification and Carbohydrate Content

Total protein was quantified according to the method described by Bradford [20] using bovine serum albumin (BSA) (20–200 mmol l⁻¹) as standard, and expressed as milligram of protein per ml of sample. The specific enzyme activity was expressed as U/mg of protein.

The carbohydrate quantification was performed according to Dubois et al. [21] using phenol sulfuric acid method with mannose as standard and expressed as percentage of milligram of carbohydrate content per mg of protein.

All experiments were carried out as triplicates and the values were expressed as the mean ± standard error of the mean.

2.4. Purification of Alkaline Phosphatase

The crude filtrate containing alkaline phosphatase was loaded on DEAE-Cellulose chromatographic column (2 × 12 cm) equilibrated with 10 mmol l⁻¹ Tris-HCl buffer, pH 7.5 (buffer A) and eluted by a linear gradient of NaCl (0–500 mmol l⁻¹) in the same buffer. The 3 ml fractions were collected at a flow rate of 0.9 ml minutes⁻¹. Fractions with alkaline phosphatase activity were pooled (25 ml), dialyzed against 5 l distilled water at 4°C for 24 hours, with 3 water exchanges, lyophilized and suspended in the buffer A added with 500 mmol l⁻¹ NaCl, and loaded on Sepharose CL-6B chromatographic column (1 × 70 cm) equilibrated using the same buffer. The 1-ml fractions were eluted using the same buffer at a flow rate of 0.3 ml minutes⁻¹. The fractions with phosphatase activity were pooled (16 ml), dialyzed for 24 hours against 5 l distilled water at 4°C, with 3 water exchanges, and used for electrophoretic analysis and enzyme characterization.

2.5. Denaturing Electrophoresis (SDS-PAGE)

The purified samples were analyzed by polyacrylamide gel electrophoresis in denaturing conditions (12% SDS-PAGE) [22] using the PowerPac™ (Bio-Rad®) power supply adjusted to 100 v and 40 mA. 187 µg of the purified protein sample were applied.

After running for 90 min, the protein band in the gel was stained with Coomassie Blue Silver G-250 solution [23]. The *Precision Plus Protein “Kaleidoscope”* Standards (BIO-RAD®) was used as molecular mass marker with molecular mass ranging from 10 to 250 kDa.

2.6. Determination of the Native Molecular Mass

The native molecular mass for alkaline phosphatase was estimated using the Sepharose CL-6B chromatographic column (1 × 70 cm) as described above. The β -amylase (200 kDa), alcohol dehydrogenase (150 kDa), BSA (66 kDa) and carbonic anhydrase (29 kDa) were used as molecular mass makers.

2.7. Influence of the Temperature and pH on Enzyme Activity

Influence of the temperature on the activity of alkaline phosphatase was evaluated using temperature ranging from 30°C to 70°C, at pH 8.0. The thermal stability, using aqueous solution, was determined at a temperature ranging from 40°C to 60°C, for different periods (5–280 minutes). Aliquots were taken off for each predetermined period and kept in an ice bath until the enzyme assay.

The influence of pH on enzyme activity was analyzed using 100 mmol l⁻¹ citric acid buffer (pH 3.0–4.0), 100 mmol l⁻¹ sodium acetate buffer (pH 4.5–6.5) and 100 mmol l⁻¹ Tris-HCl buffer (pH 7.5–9.0), at 45°C. The stability to pH was assayed using the same buffers at a pH range from 3.0 to 10.0 at 4°C for different periods (5 minutes to 24 hours). The enzymatic samples were added in the ratio of 1:1 (v/v) to the buffers. After incubation, aliquots of 100 μ l were used to determine the residual enzymatic activity.

All experiments were carried out as triplicates and the values were expressed as the mean \pm standard error of the mean.

2.8. Effect of Different Compounds on Alkaline Phosphatase Activity

The effects of chemical compounds on alkaline phosphatase activity were determined by adding 1 mmol l⁻¹ of salts (AgNO₃, BaCl₂, CaCl₂, CoCl₂, CuCl₂, CuSO₄, FeCl₃, Fe₂(SO₄)₃, HgCl₂, KCl, KH₂PO₄, MgCl₂, MgSO₄, MnCl₂, MnSO₄, NaCl, NaF, NH₄Cl, NH₄F, and ZnSO₄), β -mercaptoethanol, ethylenediaminetetraacetic acid (EDTA), SDS and urea, 0.01% and 0.02% (v/v) of Triton X-100 and Tween-20, and 1% and 2% (v/v) of acetone, acetonitrile, ethanol, isopropanol, methanol and n-butanol. The enzymatic reactions were conducted at 45°C and pH 8.5. All experiments were carried out as triplicates and the values were expressed as the mean \pm standard error of the mean.

2.9. Hydrolysis of Different Substrates by Alkaline Phosphatase

The hydrolytic activity of the alkaline phosphatase on the substrates β -glycerophosphate, ATP, phytic acid, D-fructose-6-phosphate, D-glucose-1-phosphate, D-glucose-6-phosphate and *p*-NPP was analyzed according to the Heinonen and Lahti methodology [24]. The inorganic phosphorus released was estimated at 355 nm. One unit of enzyme activity was defined as the amount of enzyme required to produce 1 μ mol of inorganic phosphorus per minute under the assay conditions. All experiments were carried out as

triplicates and the values were expressed as the mean \pm standard error of the mean.

2.10. Determination of Kinetic Parameters

The Michaelis-Menten constant (K_m), maximum velocity (V_{max}) and k_{cat}/K_m were determined for the purified alkaline phosphatase using *p*-NPP (0.1–2.5 mmol l⁻¹) as substrate in both, presence and absence of FeCl₃. The reactions were conducted at 45°C, pH 8.5. The values of K_m , V_{max} and k_{cat} were determined using the SigrafW® software [25]. All experiments were carried out as triplicates and the values were expressed as the mean \pm standard error of the mean.

3. RESULTS

3.1. Enzyme Production and Purification

The Figure 1A depicts the DEAE-Cellulose chromatographic profile for the extracellular alkaline phosphatase. A single peak with enzymatic activity was eluted with 141 mmol l⁻¹ of NaCl demonstrating that alkaline phosphatase interacted with the resin. Fractions with alkaline phosphatase activity were pooled, dialyzed, lyophilized and loaded into Sepharose CL-6B chromatographic column, and a single peak with alkaline phosphatase activity was observed (Figure 1B). After these chromatographic procedures, the alkaline phosphatase was purified 9.9-fold with recovery of 13% (Table 1).

3.1. Molecular Mass Determination and Carbohydrate Content

The native molecular mass of the alkaline phosphatase was 123 \pm 1.8 kDa estimated with gel filtration using Sepharose CL-6B chromatographic column. In addition, the Figure 2 depicts the 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) profile for the purified alkaline phosphatase through DEAE-Cellulose and Sepharose CL-6B chromatographic columns. A single protein band with apparent molecular mass of 128 \pm 0.7 kDa can be observed (lane C).

The carbohydrate content was estimated as 57 \pm 0.6% characterizing the alkaline phosphatase as a glycoprotein.

3.3. Effects of Temperature and pH on Alkaline Phosphatase Activity

The activity of the purified alkaline phosphatase produced by *R. microsporus* var. *microsporus* was the highest at 45°C (Figure 3A). The purified alkaline phosphatase was able to maintain its activity above 80 \pm 0.6% for 210 minutes at 40°C (Figure 3B). Phosphatase activity was reduced to 55 \pm 1.8% in the same period at 45°C. When the phosphatase was maintained at 50°C and 60°C, the half-life (t_{50}) was 40 and 5 minutes, respectively.

According to the Figure 3C, the high alkaline phosphatase activity was obtained at pH 8.5. Considering the enzyme stability to pH (Figure 3D), the enzymatic activity of the alkaline phosphatase was kept close to 90 \pm 0.8% at pH 8.5 throughout the period analyzed. At pH 10.0, the enzymatic activity was reduced with t_{50} of 4 hours.

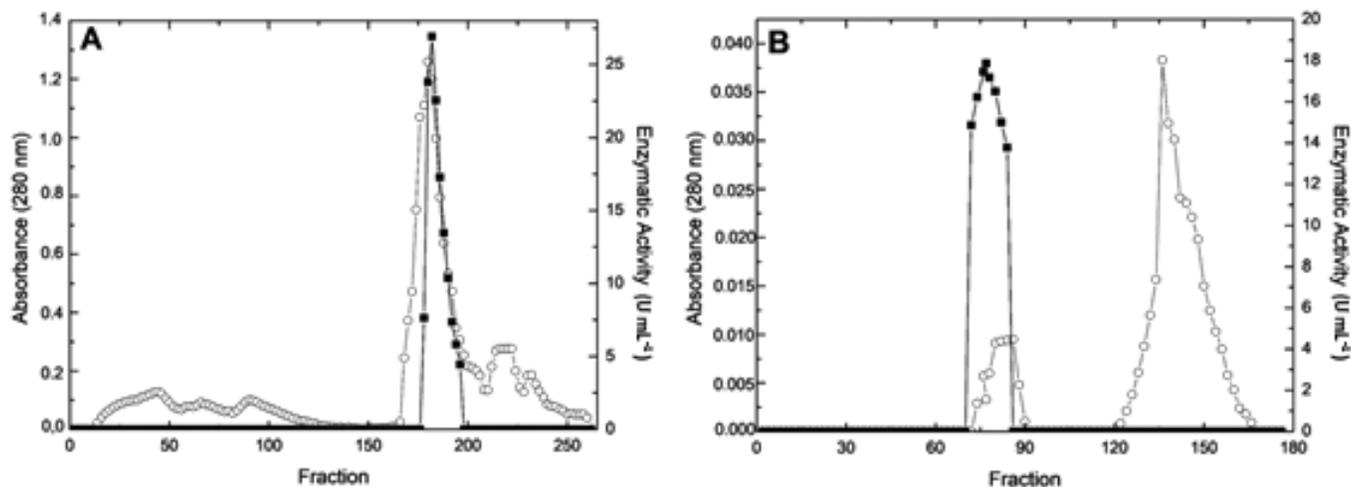


Figure 1: DEAE-Cellulose (A) and Sepharose CL-6B (B) chromatographic profile for the extracellular alkaline *p*-nitrophenylphosphatase activity from *R. microsporus* var. *microsporus*. (■) alkaline *p*-nitrophenylphosphatase activity; (○) Absorbance 280 nm.

Table 1: Purification of extracellular alkaline phosphatase produced by *R. microsporus* var. *microsporus*.

Steps	Vol (ml)	Activity (Total U)	Protein (mg)	SA (U/mg of protein)	Yield (%)	Purification (fold)
Crude extract	90	1980	191.6	10.33	100	1
DEAE-cellulose	25	1160.8	25.74	45.09	58.6	4.3
Sepharose CL-6B	16	257.6	2.51	102.6	13	9.9

The SbmF was performed using Khanna medium with initial pH adjusted to 6.3, with addition of 0.5% (w/v) rye flour as additional carbon source, for 76 hours at 32°C under orbital agitation (100 rpm). SA: specific activity.

3.5. Effect of Different Compounds on Alkaline Phosphatase Activity

The enzymatic activity of alkaline phosphatase was not significantly altered by most of the different salts used at 1 mmol l⁻¹ (Table 2). However, the enzyme activity was inhibited 37%–39% in the presence of AgNO₃, CuCl₂ and CuSO₄, 56 ± 0.5% in the presence of NH₄F, and 79 ± 0% by NaF. KH₂PO₄ also inhibited the phosphatase activity (–54 ± 0.5%). The 1 mmol l⁻¹ FeCl₃ promoted an increase (+12 ± 1.6%) in enzyme activity.

According to this, the influence of different FeCl₃ concentrations on enzyme activity was analyzed (Figure 4). The addition of 0.4 mmol l⁻¹ FeCl₃ stimulated the enzyme activity 1.2-fold if compared to the analysis without its addition.

The alkaline phosphatase activity was kept above 80 ± 1.6% in the presence of EDTA and β-mercaptoethanol. In addition, the activity was significantly inhibited by 1% acetonitrile (–30 ± 1.7%), but kept its activity above 80% for the other solvents. At 2% of solvents, the enzyme activity was reduced to 50%–54% except for the isopropanol (Table 3). The enzyme also kept its activity above 80% in both concentrations of the surfactants Triton X-100, SDS and Tween 20.

3.7. Hydrolysis of Different Substrates by Alkaline Phosphatase and Kinetic Parameters

The alkaline phosphatase from *R. microsporus* var. *microsporus* was able to hydrolyze different substrates (Table 4). The highest

hydrolysis was observed for the synthetic substrate *p*-NPP (6.2 ± 0.8 U ml⁻¹), followed by ATP (5.7 ± 0.9 U ml⁻¹) and glucose-1-phosphate (4.2 ± 0.3 U ml⁻¹). Hydrolysis of the phytic acid was reduced (0.7 ± 0.1 U ml⁻¹). The hydrolysis of the substrate D-glucose-1-phosphate was higher than that observed for D-glucose-6-phosphate and D-fructose-6-phosphate.

The K_m and V_{max} values for the hydrolysis of the *p*-NPP by the alkaline phosphatase were estimated as 0.45 mmol l⁻¹ and 3.95 U/mg of protein, respectively, in the absence of FeCl₃, and 0.38 mmol l⁻¹ and 5.12 U/mg of protein in the presence of this salt. The catalytic efficiency (k_{cat}/K_m) was 1.52-fold higher in the presence of 0.4 mmol l⁻¹ FeCl₃.

4. DISCUSSION

The use of submerged fermentation for the production of phosphatases by filamentous fungi has been reported. For this purpose, different carbon sources have been used, including expensive sources as xylan [26] and *p*-nitrophenylphosphate [13], and agroindustrial byproducts as wheat bran [17]. In spite of the use of agroindustrial byproducts, this is the first time that rye flour is used as carbon source targeting the production of a fungal alkaline phosphatase. Rye flour presents calcium, potassium, sodium and phosphorus as mineral constituents [27] and the presence of phosphorus was demonstrated to be positive for phosphatase production by *R. microsporus* [28]. Phosphorus impacts directly upon the production of phosphatases and for many filamentous fungi, the regulation of genes encoding

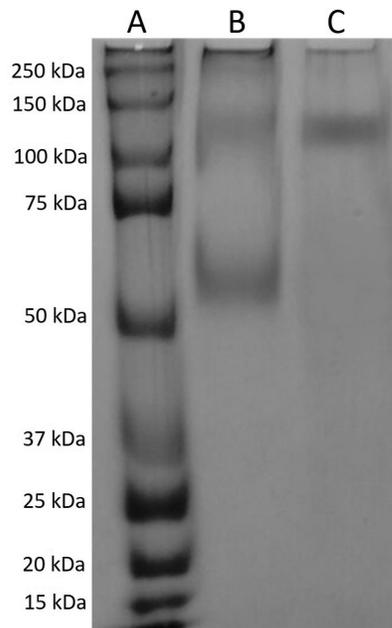


Figure 2: 12% SDS-PAGE profile for extracellular alkaline phosphatase from *R. microsporus* var. *microsporus* purified through chromatographic columns. Proteins were stained by Coomassie Brilliant Blue R-250. Lane A, molecular weight marker BIO-RAD®; lane B, protein bands after DEAE-Cellulose; lane C, alkaline phosphatase band after Sepharose CL-6B (187 µg of protein were applied).

phosphatases is similar to that described for *Saccharomyces cerevisiae* where the *PHO* genes are upregulated. In the presence of ideal intracellular Pi concentration, the *PHO* genes are repressed while these genes are expressed under low Pi concentration [29].

The cultivation of the fungus *R. microsporus* var. *microsporus* under submerged fermentation by using Khanna medium with 0.4 mmol l⁻¹ KH₂PO₃, supplemented with 0.5% (w/v) rye flour, pH 6.3, for 76 hours at 32°C, as determined through preliminary studies (unpublished data), allowed the recovery of the alkaline phosphatase activity (21.7 ± 0.6 U ml⁻¹) secreted into the culture broth. However, the fermentative parameters for maximal alkaline phosphatase production changed according to the microbial source. For instance, Guimarães et al. [26] cultured the fungus *Aspergillus caespitosus* into Segato Rizzatti (SR) medium, pH 6.0, at 40°C for 72 hours, obtaining an activity of 4.5 U ml⁻¹. The fungus *Aspergillus flavus* Indian Type Culture Collection (ITCC) 6720 produced 12 U ml⁻¹ of phosphatase in *Mustard cake* medium with initial pH 6.3, at 32°C for 76 hours [30].

The crude filtrate containing alkaline phosphatase was used for purification procedures and after two chromatographic steps, the factor of purification obtained was higher than the value observed (1.5-fold) for the purification of the alkaline phosphatase produced by *A. fumigatus*, with 46% recovery [13]. On the other hand, the acid phosphatases from *A. caespitosus* and *Aspergillus nidulans* were purified 33.4-fold and 120-fold with recovery of 14.7% and

3.8%, respectively [31,32]. Currently, the chromatography is the most powerful procedure for protein isolation considering the selectivity and recovery. However, it can be influenced by different factors, as the presence of particulate material in the samples, protein concentration and its physical-chemical properties, contaminant properties and characteristics of the chromatography medium [33].

The efficiency of the purification procedure was confirmed with SDS-PAGE analysis that presented a single protein band corresponding to the extracellular alkaline phosphatase. The molecular masses for alkaline phosphatase produced by *R. microsporus* var. *microsporus* estimated by gel filtration and SDS-PAGE were similar, indicating that this enzyme is a monomer, differing from that reported for the alkaline phosphatases from *A. caespitosus* (dimer) [26] and *Neurospora crassa* (tetramer) [34]. The molecular mass of the alkaline phosphatase from *R. microsporus* var. *microsporus* were similar to the values obtained for the enzymes produced by *Scytalidium thermophilum* (132 kDa) [35] and by *Humicola grisea* var. *thermoidea* with molecular mass of 126 kDa [36].

Different fungal alkaline phosphatases have also been reported as glycoprotein, as the enzymes produced by *A. caespitosus* with 56% carbohydrate content [26] and by *N. crassa* with 40% of carbohydrate content [34], as observed for the alkaline phosphatase from *R. microsporus* var. *microsporus*. The glycosylation of proteins is an interesting characteristic that protects the molecule under adverse conditions, as found by extracellular enzymes.

Temperature and pH of the enzyme activity are important parameters to be analyzed aiming not only to characterize the enzyme, but also targeting future applications. Under this view, the optimal temperature observed for the activity of alkaline phosphatase from *R. microsporus* var. *microsporus* resembles those described for the enzyme produced by *A. fumigatus*, which presented better activity between 40 and 45°C [37]. On the other hand, the best alkaline phosphatase activity from *Penicillium citrinum* was obtained at 60°C [38]. The increase in the temperature affects the degree of agitation of the molecule allowing an increase in the enzyme activity, but above a limit value, the protein molecule is denatured and the catalytic activity is depleted. Around 60%–70% of the enzymatic activity was maintained when the alkaline phosphatase from *R. microsporus* var. *microsporus* was incubated at 45°C, an optimal value for the enzyme activity, for long periods.

The optimal value of pH for the alkaline phosphatase activity (8.5) was lower than that reported by Guimarães et al. [35] for the conidial (10.0) and mycelial (9.5) phosphatases from *S. thermophilum*. On the other hand, phosphatase activity remained strictly acidic for *A. fumigatus* and *P. citrinum*, with highest activity at pH 5.5 and 4.8, respectively [37,38]. The pH affects the ionization degree of the molecule increasing or reducing the enzymatic activity. The stability observed at pH 8.5, the best pH value for activity, is attractive, allowing conduction of the reaction under this condition for a long period. Both the stability to temperature and pH can be

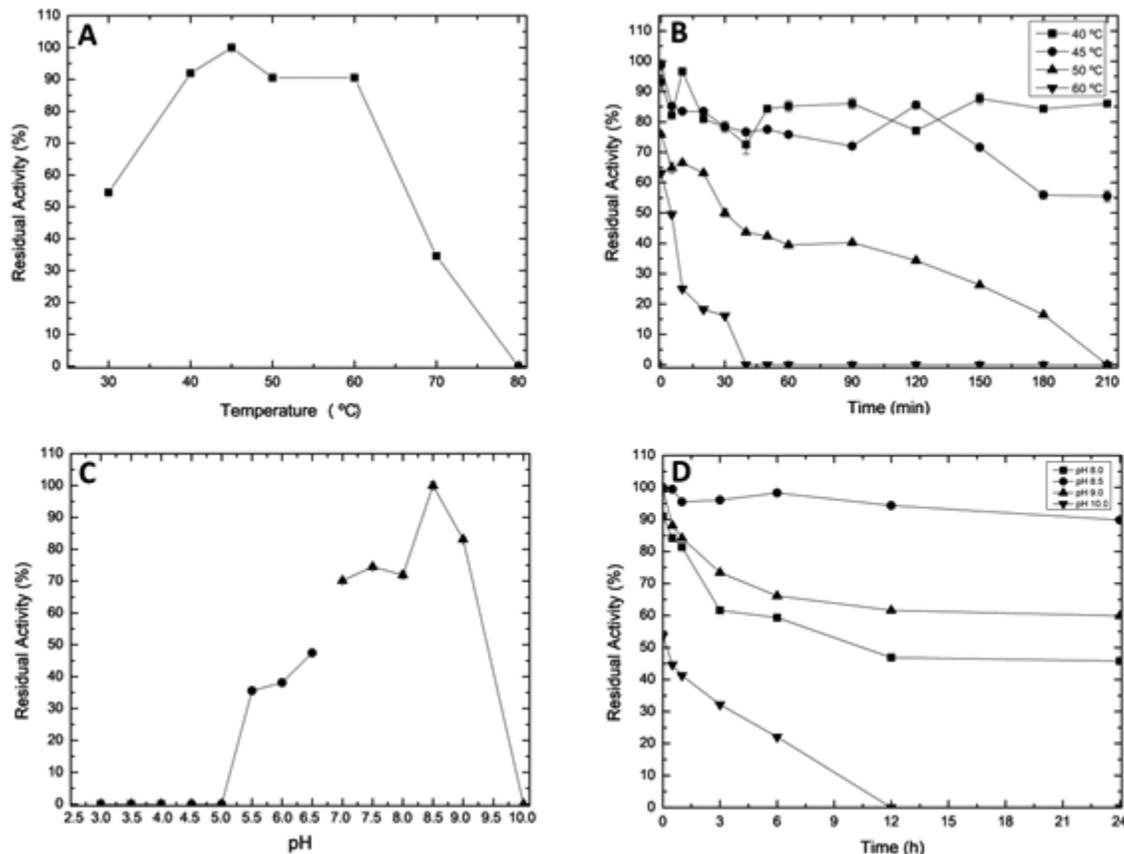


Figure 3: Influence of temperature at pH 8.0 (A) and pH at 45°C (C), thermal (B) and pH stabilities (D) for alkaline phosphatase activity from *R. microsporus* var. *microsporus*. 100% corresponds to 14.38 U ml⁻¹ (A); 16.09 U ml⁻¹ (B); 14.38 U ml⁻¹ (C); 12.06 U ml⁻¹ (D). (■) citric acid buffer pH 3.0–4.0, (●) sodium acetate buffer pH 4.5–6.5 and (▲) Tris-HCl buffer pH 7.5–9.0. The experiments were performed in triplicate and the standard errors varied between 0 and 1.8.

justified by the high degree of glycosylation of the extracellular alkaline phosphatase from *R. microsporus* var. *microsporus*. Enzyme stability is an important parameter to be considered targeting the potential of application. In this sense, the commercial alkaline phosphatase from calf intestine has high specific activity, but its use is limited due to the low thermal stability observed, which hinders the transport, storage and application of this enzyme [39]. The alkaline phosphatase from *R. microsporus* var. *microsporus* can be considered as an alternative to overcome this situation.

The enzymatic activity can also be affected by different chemical compounds. The divalent ions Mg²⁺, Zn²⁺ and Mn²⁺ have been reported as activators of the alkaline phosphatase activity from *H. grisea* var. *thermoidea* and *S. thermophilum* [35,36], differing from the data obtained for the activity from *R. microsporus* var. *microsporus* phosphatase, which was reduced in presence of these ions. Additionally, AgNO₃, CuCl₂, CuSO₄, NH₄F and NaF were the main inhibitors of the enzyme activity. NH₄⁺ and NaF were also reported as inhibitors of alkaline phosphatase activity from *R. microsporus* var. *rhizopodiformis* and *A. fumigatus*, respectively [37,40].

On the other hand, addition of FeCl₃ increased the alkaline phosphatase activity, especially at 0.4 mmol l⁻¹. That is probably because there are enough ions to supply the enzyme demand, turning the substrate hydrolysis more efficient [41]. Enzyme activation by iron can be an advantage for microorganisms living in the environment containing iron. Fe minerals can be found in reducing conditions such as FeS₂ and FeCO₃ in acid soil as pyrite and alkaline soil as siderite, respectively [10]. Microorganisms and Fe oxides coexist in soils, providing possibilities of mutual interaction, where minerals can provide nutrients as Fe, P and S, while microbial community acts on mineral weathering [10]. The mineralization of organic phosphorus in soil containing Fe can be facilitated if the phosphatase is activated by Fe in spite of the low concentration of soluble Fe.

The EDTA is able to form stable complexes with various divalent metal ions, but there was no significant reduction in enzymatic activity of the alkaline phosphatase from *R. microsporus* var. *microsporus*, demonstrating that this enzyme did not depend on a specific divalent ion. The β-mercaptoethanol acts by disrupting enzyme disulfide bonds that may cause a decrease in the catalytic activity of the enzyme. However, the residual activity remained

Table 2: Effect of different compounds on alkaline extracellular phosphatase activity from *R. microsporus* var. *microsporus*.

Compounds (1 mmol l ⁻¹)	Relative activity (%)
Without	100
AgNO ₃	61.8 ± 0.5
BaCl ₂	99.2 ± 3.1
CaCl ₂	84.8 ± 0
CoCl ₂	88.5 ± 0.5
CuCl ₂	63.7 ± 0.5
CuSO ₄	63.7 ± 1
EDTA	95.7 ± 1.6
Fe ₂ SO ₄	96.7 ± 0.5
FeCl ₃	112.2 ± 1.6
HgCl ₂	86.3 ± 0.5
KCl	87 ± 0.5
KH ₂ PO ₄	46.3 ± 0.5
MgCl ₂	86.7 ± 1
MgSO ₄	60 ± 1
MnCl ₂	77.4 ± 0.5
MnSO ₄	87.0 ± 0.5
NaCl	89.6 ± 1
NaF	21.5 ± 0
NH ₄ Cl	88.5 ± 1.6
NH ₄ F	44.8 ± 0.5
ZnSO ₄	88.5 ± 0.5
Urea	82.4 ± 1.0
β-mercaptoethanol	91.2 ± 1.6

The values correspond to the media of three experiments performed at 45°C and pH 8.5. The 100% corresponds to 18.41 U ml⁻¹.

above 90% with this compound indicating that such interactions are not essential for the catalytic activity. The enzyme activity was not affected substantially by Triton X-100 and Tween 20. Organic solvents may increase the solubility of non-polar substrates and decrease water-dependent secondary reactions and surfactants, interfering in polar and nonpolar bonds of the protein [42].

The influence of different sources of phosphorus on the phosphatase activity has been reported, where inorganic phosphate is able either to inhibit the enzyme activity or to repress the enzyme expression [43]. The sensitivity to the repression by phosphate in an extracellular medium may suggest that this is phosphorus-

scavenger enzyme with nutritional role. On other hand, Buainain et al. [36] reported an alkaline phosphatase activity from *H. grisea* var. *thermoidea* unaffected by inorganic phosphate, indicating that the alkaline phosphatases can present different functions in the organisms and in the environments.

Phosphatases are known as enzymes able to act on different substrates. Under this aspect, the best action of the enzyme was observed on the synthetic substrate *p*-NPP. In addition, the hydrolysis of glucose-1-phosphate was higher than that observed for glucose-6-phosphate, indicating a possible preference of the alkaline phosphatase to remove the phosphate groups in the position C-1 of the substrates. The acid phosphatase produced by

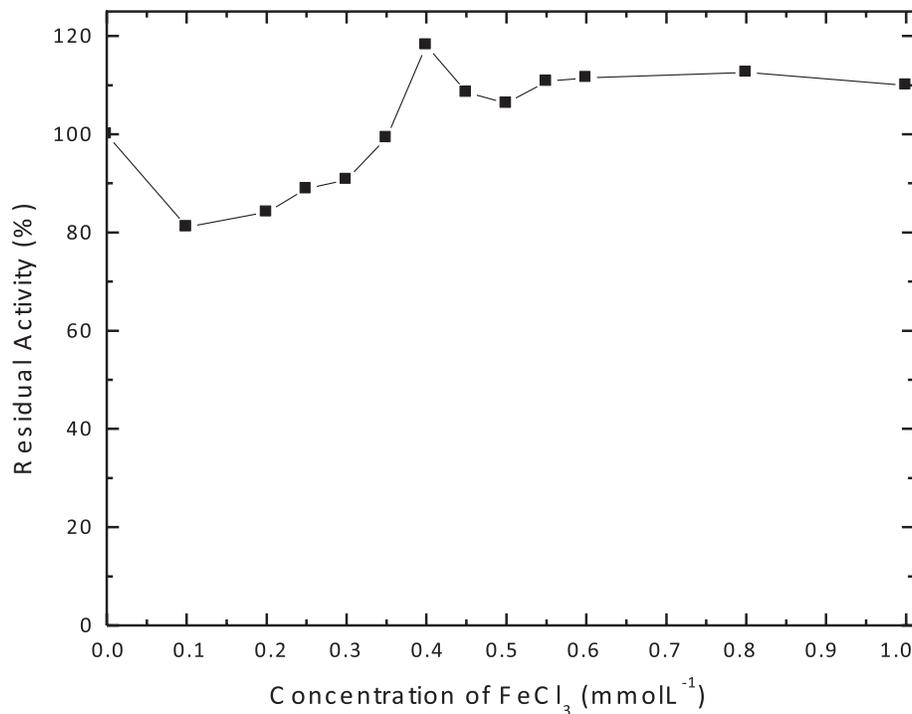


Figure 4: Influence of different concentrations of FeCl₃ on alkaline phosphatase activity produced by *R. microsporus* var. *microsporus*. 100% corresponds to 18.41 U ml⁻¹. The experiments were performed in triplicate and the standard errors varied between 0 and 1.0.

Table 3: Effect of different solvents and surfactants on extracellular alkaline phosphatase activity from *R. microsporus* var. *microsporus*.

Compounds	Relative activity (%)	
	1 % (v/v)	2% (v/v)
Without	100	100
Acetone	87.6 ± 0.6	46.1 ± 0.6
Acetonitrile	70.9 ± 1.7	48.4 ± 0.6
Ethanol	82 ± 1.7	45.3 ± 0.6
Isopropanol	86.8 ± 0.6	72 ± 1.1
Methanol	86.4 ± 1.1	43.7 ± 0.6
n-Butanol	81.2 ± 0.6	40.4 ± 0.6
	0.01% (v/v)	0.02% (v/v)
Triton X-100	90 ± 0.6	81.3 ± 0.6
Tween 20	90.9 ± 0.6	82.2 ± 0.6
	1 mmol l ⁻¹	5 mmol l ⁻¹
SDS	86 ± 1.6	82.4 ± 0.5

The values correspond to the media of three experiments performed at 45°C and pH 8.5. The 100% corresponds to 18.41 U ml⁻¹.

A. caespitosus and the alkaline phosphatases from *H. grisea* var. *thermoidea* and *S. thermophilum* also presented preference for the synthetic substrate *p*-NPP [31,35,36].

According to the kinetic parameters, the alkaline phosphatase was more efficient when FeCl₃ was added to the reaction, with high affinity for the *p*-NPP as substrate. This fact is reinforced by the

k_{cat} value, which was larger in the presence of the salt. Buainain et al. [36] found $K_m = 0.33$ mmol l⁻¹ and $V_{max} = 89$ U/mg of protein for the alkaline phosphatase from *H. grisea* var. *thermoidea*, while Morales et al. [44] reported $K_m = 0.35$ mmol l⁻¹ and $V_{max} = 241$ U/mg of protein for the enzyme from *N. crassa*. Guimarães et al. [35] reported $K_m = 0.10$ and 0.11 mmol l⁻¹, and $V_{max} = 0.79$ and 3.77

Table 4: Hydrolysis of different substrates by alkaline phosphatase from *R. microsporus* var. *microsporus*.

Substrates	Enzyme activity (U ml ⁻¹)
p-NPP	6.2 ± 0.8
Phytic acid	0.7 ± 0.1
ATP	5.7 ± 0.9
Glucose-1-phosphate	4.2 ± 0.3
Glucose-6-phosphate	2.2 ± 0.1
Fructose-6-phosphate	2.3 ± 0.1
β-glycerophosphate	2.0 ± 0.1

U/mg of protein for conidial and mycelial alkaline phosphatases produced by *S. thermophilum*.

5. CONCLUSION

The fungus *R. microsporus* var. *microsporus* was able to produce a monomeric alkaline phosphatase activated by iron, which can be an important advantage for filamentous fungi growing in an iron-rich environment as some alkaline soil types, allowing better phosphorus uptake. The better catalytic efficiency in presence of iron is an evidence for this adaptation. The stability to temperature and pH highlights the potential of the enzyme for future application and indicates possible new studies with applied focus. This aspect is reinforced by the high residual activity observed in presence of solvents, surfactants, β-mercaptoethanol and urea. This first report of an iron-activated alkaline phosphatase is an important contribution for understanding of the phosphatase diversity and future application in the bioremediation of the alkaline soil.

ACKNOWLEDGMENTS

The authors kindly acknowledge the financial support by the research scholarships from Coordenação de Aperfeiçoamento de Pessoal de Nível Superior and from Fundação de Amparo à Pesquisa do Estado de São Paulo (Process nº 2016-11311-5). This manuscript is part of the Master's Degree dissertation from Ornela PHO (2017). We also thank Maurício de Oliveira for the technical assistance.

CONFLICT OF INTEREST

Authors declared that they do not have any conflicts of interest.

REFERENCES

- Treseder KK, Lennon JT. Fungal traits that drive ecosystem dynamics on land. *Microbiol Mol Biol Rev* 2015;79:243–62.
- Ferreira CV, Justo GZ, Souza AC, Queiroz KC, Zambuzzi WF, Aoyama H, et al. Natural compounds as a source of protein tyrosine phosphatase inhibitors: application to the rational design of small-molecule derivatives. *Biochimie* 2006;88:1859–73.
- Wang YP, Houlton BZ, Field CB. A model of biogeochemical cycles of carbon, nitrogen, and phosphorus including symbiotic nitrogen fixation and phosphatase production. *Global Biogeochem Cycles* 2007;21:1–15.
- Qureshi AS, Marium S, Khushk I, Bhutto MA. Production of alkaline phosphatase from newly isolated *Aspergillus fumigatus* EFRL05. *Top Class J Microbiol* 2013;1:67–73.
- Mullaney EJ, Ullah AHJ. The term phytase comprises several different classes of enzymes. *Biochem Biophys Res Commun* 2003;312:179–84.
- Favarin BZ, Andrade MAR, Bolean M, Simão AMS, Ramos AP, Hoylaerts MF, et al. Effect of the presence of cholesterol in the interfacial microenvironment on the modulation of the alkaline phosphatase activity during in vitro mineralization. *Colloids Surf B Biointerfaces* 2017;155:466–76.
- Kapri A, Tewari L. Phosphate solubilization potential and phosphatase activity of rhizospheric *Trichoderma* spp. *Braz J Microbiol* 2010;41:787–95.
- Qin S, Hu C, Oenema O. Differentiating intracellular from extracellular alkaline phosphatase activity in soil by sonication. *PLoS One* 2013;8:e58691.
- Acosta-Martínez V, Acosta-Mercado D, Sotomayor-Ramírez D, Cruz-Rodríguez L. Microbial communities and enzymatic activities under different management in semiarid soils. *Appl Soil Ecol* 2008;38(3):249–60.
- Colombo C, Palumbo G, He JZ, Pinton R, Cesco S. Review on iron availability in soil: interaction of Fe minerals, plants, and microbes. *J Soils Sediments* 2014;14:538–48.
- Roh Y, Zhang CL, Vali H, Lauf RJ, Zhou J, Phelps TJ. Biogeochemical and environmental factors in Fe biomineralization: magnetite and siderite formation. *Clays Clay Miner* 2003;51(1):83–95.
- Coban HB, Demirci A. Screening of phytase producers and optimization of culture conditions for submerged fermentation. *Bioprocess Biosyst Eng* 2014;37:609–16.
- Suresh N, Das A. Purification and characterization of phosphatases from *Aspergillus fumigatus* for applications in industrial uses. *Rasayan J Chem* 2014;7:118–28.
- Singhania RR, Sukumaran RK, Patel AK, Larroche C, Pandey A. Advancement and comparative profiles in the production technologies using solid-state and submerged fermentation for microbial cellulases. *Enzyme Microb Technol* 2010;46(7):541–9.
- Sadh PK, Kumar S, Chawla P, Duhon JS. Fermentation: a boon for production of bioactive compounds by processing of food industries wastes (by-products). *Molecules* 2018;23:2560.
- Dick CF, Dos-Santos ALA, Meyer-Fernandes JR. Inorganic phosphate as an important regulator of phosphatases. *Enzyme Res* 2011;2011:1–7.
- Guimarães LHS, Jorge JA, Terenzi HF, Jamur MC, Oliver C, Polizeli MLTM. Effect of carbon source on alkaline phosphatase production and excretion in *Aspergillus caespitosus*. *J Basic Microbiol* 2003;43:210–7.
- Nalini P, Ellaiah P, Prabhakar T, Girijasankar G. Microbial alkaline phosphatases in bioprocessing. *Int J Curr Microbiol Appl Sci* 2015;4:384–96.
- Khanna P, Sundari SS, Kumar NJ. Production, isolation and partial purification of xylanases from an *Aspergillus* sp. *World J Microbiol Biotechnol* 1995;11:242–3.
- Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 1976;72:248–54.[21]. Dubois M, Gilles KA, Hamilton JK, Rebers PT, Smith F. Colorimetric method for determination of sugars and related substances. *Anal Chem* 1956;28:350–6.
- Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970;227:680–5.
- Candiano G, Bruschi M, Musante L, Santucci L, Ghiggeri GM, Carnemolla B, et al. Blue silver: a very sensitive colloidal Coomassie G-250 staining for proteome analysis. *Electrophoresis* 2004;25:1327–33.
- Heinonen JK, Lahti RJ. A new and convenient colorimetric determination of inorganic orthophosphate and its application to the assay of inorganic pyrophosphatase. *Anal Biochem* 1981;113:313–7.

25. Leone FA, Baranauskas JA, Furriel RPM, Borin IA. SigrafW: an easy-to-use program for fitting enzyme kinetic data. *Biochem Mol Biol Educ* 2005;33:399–403.
26. Guimarães LHS, Terenzi HF, Jorge JA, Leone FA, Polizeli MLTM. Extracellular alkaline phosphatase from the filamentous fungus *Aspergillus caespitosus*: purification and biochemical characterization. *Folia Microbiol* 2003;48:627–32.
27. Brites CM, Igrejas G, León AE. Centeno y triticale. In: León AE, Rosell CM (Eds.). *De tales harinas, tales panes. Granos, harinas y productos de panificación en Iberoamérica*. 1st edition, Hugo Báez Editor, Córdoba, Spain, pp 191–194, 2007.
28. Sato VS, Jorge JA, Guimarães LHS. Characterization of a thermotolerant produced by *R. microsporus* var. *microsporus* biofilm on an inert support using sugarcane bagasse as carbon source. *Appl Biochem Biotechnol* 2016;179:610–24.
29. Gomes-Vieira AL, Widemanb JG, Paes-Vieira L, Gomes SL, Richard TA, Meyer-Fernandes JR. Evolutionary conservation of a core fungal phosphate homeostasis pathway coupled to development in *Blastocladiella emersonii*. *Fungal Genet Biol* 2018;115:20–32.
30. Gaiind S, Singh S. Production, purification and characterization of neutral phytase from thermotolerant *Aspergillus flavus* ITCC 6720. *Int Biodeterior Biodegradation* 2015;99:15–22.
31. Guimarães LHS, Terenzi HF, Jorge JA, Leone FA, Polizeli MLTM. Characterization and properties of acid phosphatases with phytase activity produced by *Aspergillus caespitocsus*. *Biotechnol Appl Biochem* 2004;40:201–7.
32. Nozawa SR, Macheroni Jr W, Stábeli RG, Thedei Jr G, Rossi A. Purification and properties of pi-repressible acid phosphatases from *Aspergillus nidulans*. *Phytochemistry* 1998;49:1517–23.
33. Safarik I, Safarikova M. Magnetic techniques for the isolation and purification of proteins and peptides. *Biomagn Res Technol* 2004;2:7; doi:10.1186/1477-044X-2-7
34. Say JC, Furriel RPM, Ciancaglini P, Jorge JA, Polizeli MLTM, Pizauro JM, et al. Conidial alkaline phosphatase from *Neurospora crassa*. *Phytochemistry* 1996;41:71–5.
35. Guimarães LHS, Terenzi HF, Jorge JA, Polizeli MLTM. Thermostable conidial and mycelial alkaline phosphatases from the thermophilic fungus *Scytalidium thermophilum*. *J Ind Microbiol Biotechnol* 2001;27:265–70.
36. Buainain LB, Kadowaki MK, Polizeli ML, Terenzi HF, Jorge JA. Characterization of a conidial alkaline phosphatase from the thermophilic fungus *Humicola grisea* var. *thermoidea*. *J Basic Microbiol* 1998;38:85–94.
37. Bernard M, Mouyna I, Dubreucq G, Debeaupuis JP, Fontaine T, Vorgais C, et al. Characterization of a cell-wall acid phosphatase (PhoAp) in *Aspergillus fumigatus*. *Microbiology* 2002;148:2819–29.
38. Gawas-Sakhalkar P, Singh SM, Simantini N, Ravindra R. High-temperature optima phosphatases from the cold-tolerant Arctic fungus *Penicillium citrinum*. *Polar Res* 2012;31:11105.
39. Dong G, Zeikus JG. Purification and characterization of alkaline phosphatase from *Thermotoga neopolitana*. *Enzyme Microb Technol* 1997;21:335–40.
40. Barbosa-Júnior A, Guimarães LHS, Terenzi HF, Jorge JA, Leone FA, Polizeli MLTM. Purification and biochemical characterization of thermostable alkaline phosphatases produced by *Rhizopus microsporus* var. *rhizopodiformis*. *Folia Microbiol* 2008;53:509–16.
41. Juma NG, Tabatabai MA. Effects of trace elements on phosphatase activity in soils. *Soil Sci Soc Am J* 1977;41:343–6.
42. Chhokar V, Sangwan M, Beniwal V, Nehra K, Nehra KS. Effect of additives on the activity of tannase from *Aspergillus awamori* MTCC9299. *Appl Biochem Biotechnol* 2010;160:2256–64.
43. Sánchez S, Demain AL. Metabolic regulation of fermentation processes. *Enzyme Microbiol Technol* 2002;31:895–906.
44. Morales AC, Nozawa SR, Thedei Jr G, Maccheroni Jr W, Rossi A. Properties of a constitutive alkaline phosphatase from strain 74A of the mold *Neurospora crassa*. *Braz J Med Biol Res* 2000;33:905–12.

How to cite this article:

Ornela PHO, Guimarães LHS. Purification and characterization of an iron-activated alkaline phosphatase produced by *Rhizopus microsporus* var. *microsporus* under submerged fermentation using rye flour. *J Appl Biol Biotech* 2020; 8(04):016–025. DOI: 10.7324/JABB.2020.80403