J. Microbiol. Biotechnol. (2014), 24(2), 177–187
http://dx.doi.org/10.4014/jmb.1308.08087

jmb

Phytase Production by *Rhizopus microsporus* var. *microsporus* Biofilm: Characterization of Enzymatic Activity After Spray Drying in Presence of Carbohydrates and Nonconventional Adjuvants

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Received: August 28, 2013
Revised: October 24, 2013
Accepted: October 31, 2013

First published online
November 5, 2013

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pISSN 1017-7825, eISSN 1738-8872

Microbial phytases are enzymes with biotechnological interest for the feed industry. In this article, the effect of spray-drying conditions on the stability and activity of extracellular phytase produced by *R. microsporus* var. *microsporus* biofilm is described. The phytase was spray-dried in the presence of starch, corn meal (>150 μm), soy bean meal (SB), corn meal (<150 μm) (CM), and maltodextrin as drying adjuvants. The residual enzyme activity after drying ranged from 10.7% to 60.4%, with SB and CM standing out as stabilizing agents. Water concentration and residual enzyme activity were determined in obtained powders as a function of the drying condition. When exposed to different pH values, the SB and CM products were stable, with residual activity above 50% in the pH range from 4.5 to 8.5 for 60 min. The use of CM as drying adjuvant promoted the best retention of enzymatic activity compared with SB. Spray drying of the *R. microsporus* var. *microsporus* phytase using different drying adjuvants showed interesting results, being quite feasible with regards their biotechnological applications, especially for poultry diets.

Keywords: *Rhizopus microsporus*, spray drying, phytase, enzyme stabilization

Introduction

Phytases (myo-inositol hexakisphosphate phosphohydrolases; EC3.1.3.8 and EC3.1.3.26) catalyze the hydrolysis of phosphomonoester bonds of phytate (salts of myo-inositol 1,2,3,4,5,6-hexakis dihydrogen phosphate), thereby releasing lower forms of myo-inositol phosphates and inorganic phosphate. Phytases are widespread among bacteria, yeast, fungi, and plants, and among animals as well [11, 20]. Regardless of the source, several studies have confirmed the use of phytases in animal nutrition, environment protection, and human health [27, 39].

The demand for enzymes with desirable properties for application may be accomplished by screening different microbial strains isolated from different environments, or may also be performed through modification using methods of protein engineering and molecular evolution [25]. Many bacteria and fungi have been reported as phytase producers under different fermentative process as, for example, the phytase produced by the fungus *Rhizopus oryzae* under solid-state fermentation [29] and by *Thermoascus aurantiacus* in submerged fermentation [22]. However, the possibility of using new ways of cultivation to maximize the production of enzymes, as biofilm fermentation, is attractive. The use of biofilms and their potential to release enzymes with biotechnological potential has been mentioned [10].

One of the main priorities in the marketing of enzymes is to maintain the molecule stability in aqueous solution, as

well as the development of formulations that are stable during packaging, transportation, and long-term storage [19]. Spray-drying is a technology that has been considered as a good alternative for this purpose. It is the most common method for the drying of liquid compositions. The commutation of a solution suspension or emulsion into a powder in a one-step process can be considered as the main advantage of this methodology [35]. The high drying efficiency and the reduced cost compared with the freeze-drying should be also considered [26]. Despite the high drying temperature, the cooling effect caused by the solvent evaporation leads the dried product to relatively low exposure temperature. Therefore, enzymes can be dried without appreciable loss of activity [18]. This method is widely used in dairy and pharmaceutical industries for the dehydration of various substances, such as milk, whey, antibiotics, and vitamins [19].

The addition of stabilizing agents is a common method used to protect the native protein when submitted to drying [2]. The carbohydrates, polymers, and polyols (*e.g.*, mannitol, sucrose, trehalose, lactose, arabic gum, cyclodextrins, and maltodextrin) are widely used. Some enzymes have been successfully spray-dried and characterized as, for example, the lipase of *Cercospora kikuchii* [5] and the β-fructofuranosidase from *Fusarium graminearum* [8]. However, to our knowledge, the properties of fungal spray-dried phytase have not been reported yet. According to this, the present paper describes the characterization of spray-dried extracellular phytase produced by *R. microsporus* var. *microsporus* biofilm in the presence of several carbohydrates and nonconventional drying adjuvants.

Materials and Methods

Microorganism and Culture Condition

The filamentous fungus *Rhizopus microsporus* var. *microsporus* was isolated from Brazilian soil, identified by 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 30ºC for 7 days, and then stored at 4ºC.

The *R. microsporus* var. *microsporus* biofilms were developed on polyethylene inert supports (2.0 × 2.0 cm) previously washed with detergent and distilled water, and then sterilized under UV radiation. The sterilized supports were immersed in an aqueous spore suspension (10^6 spores/ml) at 30°C under agitation (50 rpm) for 2 h for spore adhesion to the support. The supports were then 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 Vogel medium [38] with different agro-industrial products (soya mince, rye flower, sugarcane bagasse, wheat bran, and orange peel) and sugars (fructose, galactose, glucose, maltose, and sucrose) as carbon sources, pH 6.0, and previously autoclaved at 121ºC, 1.5 atm for 30 min. The cultures were maintained at 30°C under agitation (50 rpm) for 48 h. The media values obtained were compared using the Tukey test with the p value fixed at 0.05.

Determination of Phytase Activity and Protein Quantification

After cultivation, the biofilm was removed and the cell-free extracellular extract containing phytase was obtained, dialyzed overnight at 4°C against distilled water, and used for the enzyme activity assay. The phytase activity was assayed according to Gulati *et al*. [9] with modifications. The reaction mixture was 50 µl of enzyme sample incubated with 50 µl of 1% (w/v) phytic acid (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% TCA (trichloroacetic acid) and 300 µl of distilled water was added in each test tube. Then 0.9 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 taken at 820 nm. Controls without addition of enzyme were included to estimate the non-enzymatic hydrolysis of the substrate. One unit of enzyme activity was defined and expressed as the amount of enzyme that releases 1 µmol of phytate per minute under the assay conditions. The spray-dried phytase activity was determined in the same conditions, but using the powder diluted in 0.5 M sodium acetate buffer at pH 4.0, taking into account the residual solids present in the soluble extract.

Protein content was quantified according to Bradford [1] using bovine serum albumin (BSA) as standard.

Scanning Electron Microscopy (SEM)

The *R. microsporus* var. *microsporus* biofilm on polyethylene as an inert support obtained from the cultivation using sugarcane bagasse as carbon source was dehydrated with increasing concentrations of ethanol in water (10–90%, by volume) and finally with absolute ethanol, sprayed with colloidal gold, and submitted to SEM analysis using a Zeiss equipment EVO50 (Carl Zeiss, Oberkochen, Germany) with IXRF Systems 500 digital processing.

Drying and Operation Conditions

Drying was conducted in a bench-top spray dryer (model SD-05; Lab-Plant, Huddersfield, UK), with a concurrent flow regime. The drying chamber had a diameter of 215 mm and a height of 500 mm. The main components of the system were a feed system for the drying gas, consisting of a blower and an air filter; a temperature control system for the drying gas; and a product collection system (cyclone). The crude extract was placed into the

spray dryer through a feed system, consisting of a peristaltic pump, a two-fluid atomizer (inlet orifice diameter of 1.0 mm), and the air compressor. The feed flow rate of atomizing was 17.0 L/min at a pressure 1.5 kgf/cm^2. The flow rate of the drying air was maintained constant at 60 m^3/h.

The drying operation started with the injection of drying air into the SD-05 spray dryer. The air was heated at 100°C, and then the crude extract was fed at a preset flow rate (4 g/min) together with the atomizing air at a pressure of 141 kPa and flow rate of 17.0 L/min. Different proportions (2, 5, and 10% (w/v)) of the adjuvants starch, corn meal (>150 µm), soybean, corn meal (<150 µm) and maltodextrin were added to the enzymatic extract before spray drying. Measurements of the outlet gas temperature (T_{gs}) were taken at regular intervals to detect the moment when the dryer attained a steady state (15 min). Once steady state was attained, samples of the dried product were collected and used for physical and chemical characterization.

Measurement of Powder Properties

The powder properties were characterized through the determination of phytase activity, powder moisture content, water activity, and particle size. The determination of the residual moisture content of the samples was performed in a Karl Fischer titrator (Titrino Plus model 870; Metrohm). The water activity was measured using a water activity meter in Aqua Lab 4TEV (Decagon Devices, Inc., Pullman, WA, USA). For the determination of particle size distribution, dried sample was sprinkled on a glass slide and images were obtained with the aid of an optical microscope coupled with a digital camera (Olympus model BX60MIV). Photographs were analyzed using the Image Pro Plus 7.0 software, and the average diameter of particles (which refers to the average length of the diameter measured at intervals of two degrees and passing through the center of particles) was determined. The powder production yield or product recovery of the spray-drying was also evaluated as a measure of dryer performance. The product recovery was determinate by mass balance, defined as the ratio between the total mass of the solids introduced into the dryer and the total mass collected by the cyclone. The powder on the internal wall of the cyclone was not considered, and assumed as powder losses.

Biochemical Characterization of the Powders

The powders containing phytase that had the best residual activity and product recovery were characterized. Reactions to determine the optimum pH and temperature of phytase activity after drying were conducted between 30°C and 80°C, and pH between 2.5 and 10.5, using 0.5 M citrate buffer (2.5–3.5), sodium acetate buffer (pH 3.5 to 6.0), Tris-HCl buffer (pH 6.0–8.5), and Ampol buffer (pH 8.5–10.5). The stability to pH and temperature was determined using the pH range from 2.5 to 10.5 for 30 to 60 min and temperature range between 30°C and 80°C for certain periods (5–180 min). For this goal, the powders obtained with soy bean and corn meal (<150 µm) were diluted in 0.5 M sodium acetate buffer at pH 4.0 (10.15 g/100 ml of buffer) as described previously and maintained at different pH and temperature conditions. It was used the proportion of 1:1 (v/v) between the enzymatic sample and the incubation buffer. During the incubation, the tubes were mixed manually. The assayed samples were placed on ice to decrease the temperature as soon as possible and the phytase activity was determined as described previously. The enzymatic activity of the spray-dried extract measured after the completion of the drying (time 0) was used as a control.

Results and Discussion

Production of Phytase by *R. microsporus* var. *microsporus* Biofilm

The *R. microsporus* var. *microsporus* biofilm produced good levels of extracellular phytase using saccharides, considering the specific activity, especially with sucrose (121.7 U/mg of protein) and glucose (95.4 U/mg of protein) as carbon sources. In the absence of carbon source, however, the enzyme was also produced. These results indicate that the phytase production by this fungus is constitutive. On the other hand, the best production using agro-industrial products was obtained using sugarcane bagasse (44.8 U/mg of protein) and rye flour (16.4 U/mg of protein) as carbon source (Table 1). Despite the best production using saccharides, the biofilm mass obtained was lower than the biomass obtained in the presence of agro-industrial products

Table 1. Carbon sources used for phytase production by *R. microsporus* var. *microsporus* biofilms.

Carbon source	Extracellular phytase activity		Dryed biomass
	U/ml	U/mg of protein	g
None	42.87 ± 0.47^{a}	114.75 ± 0.23^{a}	0.06
Crushed soy	23.38 ± 0.33^{b}	6.48 ± 0.20^{b}	1.13
Fructose	29.12 ± 0.14^{c}	43.13 ± 0.09^{c}	0.13
Galactose	30.92 ± 0.70^{c}	88.7 ± 0.43^{d}	0.34
Glucose	30.09 ± 0.33^{c}	95.37 ± 0.20^{a,d,g}	0.28
Maltose	28.9 ± 0.20^{c,d}	67.4 ± 0.13^{e}	0.47
Oat meal	27.71 ± 0.52^{d}	14.76 ± 0.32^{f}	1.53
Rye flour	30.21 ± 0.28^{c}	16.43 ± 0.17^{f}	1.62
Sucrose	32.91 ± 0.89^{c,e}	121.69 ± 0.55^{g}	0.51
Sugarcane bagasse	35.81 ± 0.11^{e}	44.8 ± 0.99^{c}	1.29
Wheat bran	26.67 ± 0.28^{d}	13.4 ± 0.17^{f}	2.81

Cultures were performed using Vogel medium, pH 6.0, at 30°C for 48 h under orbital agitation (50 rpm). The media values were compared using the Tukey test ($p < 0.05$) and the same lower case letters in the columns show that there is no statistically significant difference. The biomass was determined after biofilm drying at 40°C using a stove.

as carbon sources. In addition, the development of biofilm in the absence of carbon source was extremely reduced (0.06 g). In this situation, the specific activity was directly influenced by the biofilm biomass since a reduced growth also reduced protein secretion and, consequently, gave a higher specific activity. Considering this aspect as well as that the enzyme production using sugarcane bagasse was 2.7 times higher than that obtained for rye flour, the former was selected as the carbon source for phytase production. In addition, the specific activity obtained when saccharides were used as carbon sources showed higher values, due to the low levels of protein. Various authors have used sugarcane bagasse as carbon source for the production of enzymes [23, 30]. According to Pandey *et al*. [27], the energy required and the physical support for the fungus growth as well as the production of the desired metabolite are primarily provided by the carbon source. Thus, probably the sugarcane bagasse has additional nutrients that promote better development of the fungus and, consequently, an increase in the enzyme production. There are reports in the literature on the use of other types of waste as carbon source to produce enzymes, such as the work of Spier *et al*. [34] that shows the production of phytase using citrus pulp. Wheat flour is well reported for the production of various enzymes with industrial application, such as cellulases, xylanases, and phytases from *Aspergillus japonicus*. Roopesh *et al*. [31] optimized production of phytase by *Mucor racemosus* in wheat bran. However, for the production of phytase by *R. microsporus* var. *microsporus*, this carbon source was less pronounced. Being an agro-industrial residue, sugarcane bagasse has low cost, providing more viable conditions for large-scale production. The maximum production of extracellular phytase by *R. microsporus* var. *microsporus* biofilms was obtained in 48 h. The maximum phytase activity from *Saccharomyces cerevisiae* CY was also obtained with 48 h of cultivation with 10% galactose as carbon source [13]. Other different periods for phytase production by microorganisms have been reported as, for example, 14 h for *E. coli* phytase [14] and 120 h for *Bacillus* sp. KHU-10 [4].

The enzyme production is also influenced by the way in which the fungus grows. Fig. 1 shows the morphology of the *R. microsporus* var. *microsporus* biofilm after 48 h of growth in medium containing sugarcane bagasse as carbon

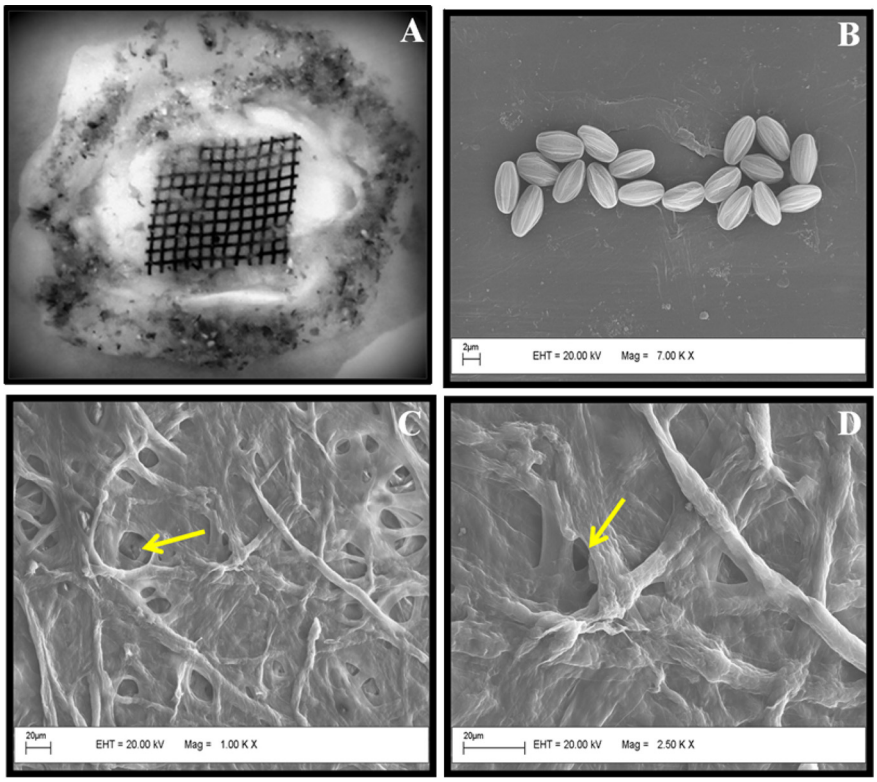

Fig. 1. Biofilm (**A**) and scanning electron micrograph of *R. microsporus* var. *microsporus* spores (**B**) and biofilm obtained with 48 h of growth (**C** and **D**).
Channels are indicated by arrows.

source. The development of the fungal biofilm depends on sequential steps where the spore adhesion in the inert support, using adhesive molecules, is the first one. Then, the germinative tube is provided and, consequently, the hyphae develop. According to the SEM micrograph (Fig. 1B), the spores from *R. microsporus* var. *microsporus* have an ellipsoid morphology with rugous surface that can facilitate its interaction with the inert support. The biofilm can be characterized by several intertwined hyphae, which form an ordered array, with the formation of various channels that allow efficient exchange of nutrients between the microbial cells and the environment (Figs. 1C and 1D). Morphological patterns similar to *R. microsporus* var. *microsporus* biofilms were observed in other studies with biofilms, as demonstrated for the biofilm of *A. niger* in polyester fabric [36, 37].

Phytase Activity of Spray-Dried Product

The maintenance of the enzyme structure and its properties, as well as the improvement in its stability, is an important challenge for biotechnological application of this type of molecule. To protect the enzyme during the drying process, the extracellular crude extract containing phytase from *R. microsporus* var. *miscrosporus* was added with different protective adjuvants in different proportions. Some carbohydrates that are recognized as agents that protect the enzymatic activity at high temperatures were used as adjuvants, as well as some alternative adjuvants like corn bran and soybean meal poultry feed used in animal feed [32]. Table 2 presents the effects of the different adjuvants and their respective concentrations, as well as the outlet drying gas temperature (T_{gs}) and feed flow rate of enzyme composition (W_{real}) on product recovery (R_{EC}), drying efficiency (X), and phytase activity (R_{PA}).

During the drying process, the outlet air temperature was maintained between 68°C and 72°C. Similar results were observed in studies by other authors [5, 6, 8], using similar drying conditions. According to results obtained by Namaldi *et al*. [21], the loss of the enzyme activity becomes more pronounced when the inlet temperature of the drying air is increased. In spite of the drastic conditions of spray drying, the enzyme activities can be maintained if their native structure is preserved by the use of protective adjuvants. This makes the spray-drying a convenient method for

Table 2. Effects of type and concentration of drying adjuvants on process yield, phytase activity, residual phytase activity (R_{PA}), and outlet drying gas temperature (T_{gs}).

Experimental conditions		Responses				
Adjuvants	Concentration (%)	T_{gs} (°C)	W_{real} (g/min)	R_{EC} (%)	X (%)	R_{PA} (%)
Starch	10	68	4.3	63.1	41.2	44.8
	5	69	4.1	60.2	39.9	40.8
	2	68	4.1	53.6	41.2	10.7
Corn meal (>150 μm)	10	69	4.4	20.6	38.9	40.5
	5	69	4.1	22.1	39.8	30.5
	2	69	4.0	24	36.1	25.7
Soybean meal	10	71	4.1	32.6	37.6	60.4
	5	69	4.1	30.6	37.7	59.2
	2	69	4.2	22.4	36.5	59.6
Corn meal (<150 μm)	10	71	4.1	55.7	39.4	49.3
	5	71	4.2	57.5	38.1	59.5
	2	70	4.0	41.2	37	59,6
Maltodextrin	10	72	4.1	46.2	39.1	58
	5	72	3	5.6	37.8	57.9
	2	71	4	6.9	37.8	56.6
Aqueous solution phytase						100

T_{gs}: outlet drying gas temperature; **W_{real}**: feed flow rate of enzyme composition; **R_{EC}**: product recovery; **X:** drying efficiency; **R_{PA}**: residual phytase activity (residual phytase activity was determined by comparing the activities after being spray-dried and the enzyme in aqueous solutions before drying). The 100% corresponds to 36.8 U/ml.

enzyme drying. The α-amylase (Fungamyl 800L, Novozymes A/S) obtained from *Aspergillus oryzae* dissolved in a solution of maltodextrin and submitted to the spray-drying showed a variation in outlet and inlet air temperatures from 50°C to 107°C and 160°C to 220°C, respectively [33]. For serine alkaline proteases spray-dried, the inlet air temperature was from 70°C to 130°C, and the outlet air temperature ranged from 56°C to 90°C [21]. In the study of drying of probiotics for *Bifidobacterium lactis* BB12, the inlet and outlet temperatures were 80°C and 48°C, respectively [16].

The best product recovery, after the spray-drying, was found using starch (60–63.1%) and corn meal <150 μm (55.7–57.1%) as adjuvants (Table 2). On the other hand, low yields of the drying process (5.6% and 6.9%) were obtained using maltodextrin. This may be related to the physicochemical properties of the resulting mixture of enzymatic extract with maltodextrin. The low yield in some cases might be due to the inefficient powder collection system of the spray dryer, which leads to a carryover of fine particles along the exhaust air. In studies conducted by Costa-Silva *et al*. [5], the powder deposition in the chamber during the spray-drying was also cited as a possible factor responsible for reduced yields.

The highest residual phytase activity was found for the powders containing soybean at all concentrations (average 59.7%), as well as when used corn meal was (<150 μm) as adjuvant (59%) at 2% and 5% (w/v). The powder containing maltodextrin also presented good residual phytase activity, but the recovery was reduced, as previously presented. For α-amylase from *Aspergillus oryzae,* the residual activity obtained for the enzyme dried in presence of maltodextrin varied from 51.9% to 91.8% [33]. The extract containing *F. graminearum* β-fructofuranosidase was better when spray-dried with the modified starch (10% (w/v)) and trehalose (2% (w/v)), with 63.6% and 56.9% of recovery, respectively [8].

According to Stahl *et al*. [35], the reduction of the feed flow rate can reduce the particle size. If the same amount of liquid is sprayed into smaller particles, the total surface area of droplets is increased, resulting in an extensive contact with high temperature. It can lead to a reduction in the enzyme activity. In addition, other factors have been recognized as important for the reduction of the enzymatic activity during spray-drying. The proteins are presumed to be exposed to high shear forces during the atomization process, and contact with the air flow of high temperature, and thus suffer from heat stress resulting in irreversible structural changes and thermal denaturation, among others [41].

To reduce the degree of activity, loss of an enzyme during drying, adjuvants as, for example, the carbohydrates (as used for spray-drying of *R. microsporus* var. *microsporus* phytase) can be used. Two mechanisms may explain, at least in part, the role of carbohydrates in the protection of the enzyme. In the first case, the water is replaced by carbohydrates to preserve the native structure of the protein. In aqueous medium, water and protein establish hydrogen bonds, as can be also observed for carbohydrates, which form hydrogen bonds with proteins in the solid state. A second hypothesis is the formation of a crystalline matrix, which hinders the change of protein conformation, thus preserving its activity [19].

Physical Properties of the Spray-Dried Powders

The average particle diameters of spray-dried extract using corn meal (28.45 μm) were significantly higher than that observed for the powders obtained with maltodextrin (17.86 μm) and with starch (13.25 μm) (Table 3). The micro- and macroscopic aspects were clearly observed in powders

Table 3. Physical properties of the powders containing phytase in the presence of different adjuvants.

Powders	Responses		
	A_w	X_p (%)	dp (μm)
ST 2%	NA	10.7 ± 0.67	13.83 ± 8.33
ST 5%	NA	9.78 ± 0.75	13.54 ± 5.37
ST 10%	NA	7.62 ± 0.65	12.39 ± 5.05
CM1 2%	NA	10.24 ± 0.4	NA
CM1 5%	NA	8.90 ± 0.38	NA
CM1 10%	NA	7.72 ± 0.63	NA
SB 2%	NA	9.73 ± 0.3	NA
SB 5%	NA	8.85 ± 0.62	NA
SB 10%	0.2 ± 0.01	7.66 ± 0.36	NA
CM2 2%	NA	9.3 ± 0.14	23.13 ± 22.8
CM2 5%	NA	7.78 ± 0.46	32.87 ± 25.8
CM2 10%	0.24 ± 0.01	6.96 ± 0.69	29.37 ± 23.8
MT 2%	NA	10.6 ± 0.77	15.45 ± 17.05
MT 5%	NA	8.46 ± 0.22	18.24 ± 30.72
MT 10%	0.23 ± 0.01	6.54 ± 0.49	19.90 ± 37.31

ST: starch; CM1: corn meal (>150 μm); SB: soybean; CM2: corn meal (<150 μm); MT: maltodextrin; A_w: water activity; X_p: product moisture content; dp: particle diameter; NA: not available.

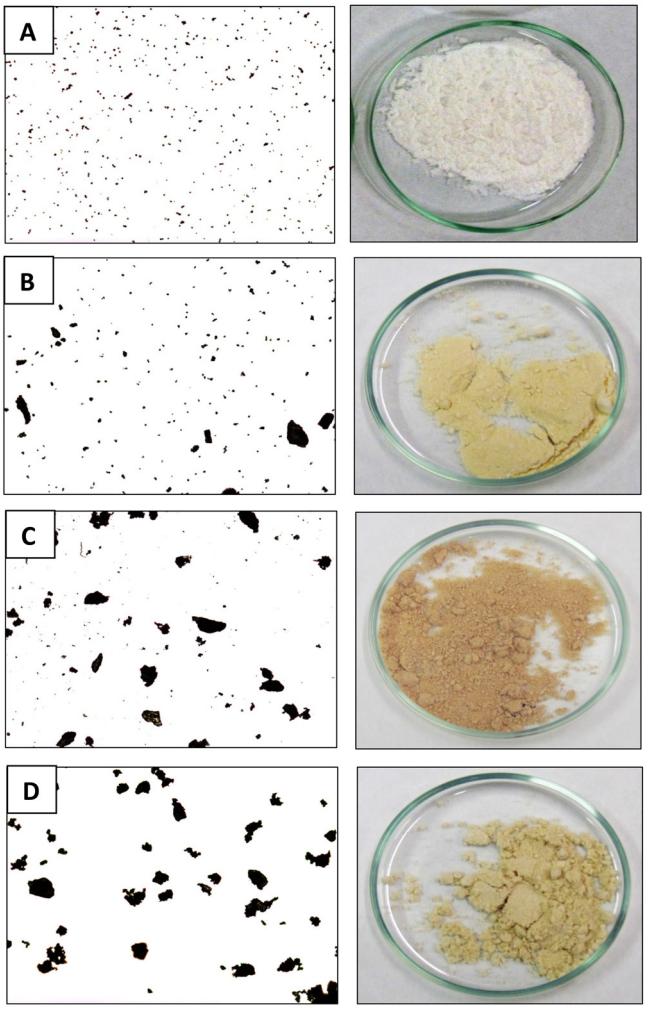

Fig. 2. Spray-dried phytase powder using different adjuvants: starch 2% (**A**), corn meal (<150 µm) 2% (**B**), soybean meal 2% (**C**), and corn meal 2% (>150 µm) (**D**).
Images were obtained in an optical microscope coupled with a camera (Olympus model BX60MIV). Magnification = 40x.

containing the enzyme (Fig. 2). The soybean and corn meal (>150 µm) are visually amorphous, when compared with the starch and corn meal (<150 µm). It was not possible to determine the average diameter of the corn meal (>150 µm) and soybean meal powders because of the particles aggregation. This may be due to a high moisture content, since water is a determinant factor for both the integrity of the solid matrix and the protein-adjuvant interaction [17]. It was mentioned that the dry powder containing trypsin using lactose, dextrin, and cyclodextrin as adjuvants was almost all amorphous, whereas the powder obtained with sucrose as adjuvant showed different degrees of crystallinity and that with mannitol was highly crystalline [19].

The water activity (A_w) and the moisture content of the product are important aspects that should be considered for the stability of some processed foods and for inhibition of the microbial growth. The dried extracts that had the best phytase activity and product recovery had minimum values of water activity, which is desired to guarantee product stability (Table 3). The growth of bacteria that influences the deterioration of the product ceases at A_w below 0.9. Most yeast and filamentous fungi will not grow at A_w below 0.85 and 0.70, respectively. These values are higher than those found in the spray-dried powder containing phytase described in this paper. The moisture content ranged from 6.54 to 10.5, depending on the concentration and type of adjuvant added to the feed composition.

Biochemical Characterization of Spray-Dried Products

The determination of the effects of temperature and pH on enzymatic activity is very important to define the process parameters for enzyme utilization, as well as its stability during the process. According to this, the optimum temperature and pH of extracellular phytase for the spray-dried powders using soybean and corn meal (<150 µm) as adjuvants were determined, as well as for the free enzyme. Two peaks of phytase activity, one at pH 4.5 and another at pH 8.5, for both soybean meal and corn meal (150 µm) spray-dried powders were obtained (Figs. 3A and 3C). The phytase activity under alkaline pH is an uncommon and interesting characteristic for fungal phytases, which showed activity at a range of pH 4.5 to 6.0 as demonstrated for free *R. microsporus* var. *microsporus* phytase with two peaks of activity at 4.5 and 6.5 (data not shown). However, alkaline phytases have been reported for bacteria such as *Bacillus* [9]. In addition, significant phytase activity (>70%) was also observed for the pH range from 5.0 to 8.0. It is possible that the *R. microsporus* var. *microsporus* produces two phytases: one is acidic and the other is a new alkaline enzyme. Another explanation would be the production of a bifunctional enzyme that is able to act in both conditions. Fungal alkaline phytase has not been documented until this moment. Production of two different enzymes has been reported as observed for the mesophilic fungus *Aspergillus ficuum* NRRL 3135 that produced two different phytases designated as phyA and phyB, one with optimum pH of 5.5 and the another with optimum pH of 2.0, respectively [12]. As an example of a bifunctional enzyme, *Rhizopus oryzae* phytase produced under solid-state fermentation that acts at pH 1.5 and 5.5 can be cited [29].

The optimal temperature for phytase activity after drying

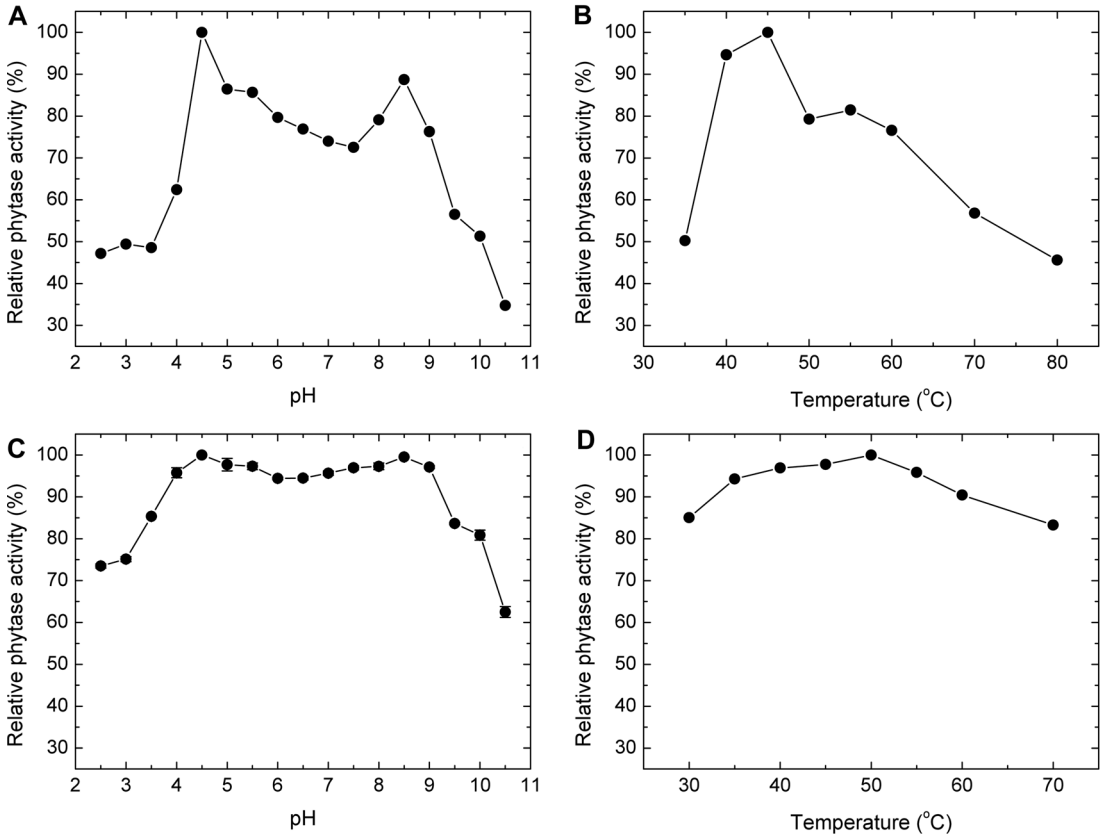

Fig. 3. Influence of pH (**A** and **C**) and temperature (**B** and **D**) on the activity of the spray-dried phytase from *R. microsporus* var. *microsporus* in the presence of soybean (**A** and **B**) and corn meal (<150 μm) (**C** and **D**) as adjuvants.

was 45°C using soybean (Fig. 3B) and 50°C using corn meal (<150 μm) (Fig. 3D) as adjuvants, both higher than that found for the free enzyme (40°C). Several studies have investigated the best temperature of reaction for microbial phytases, with optimal activity reported between 40°C and 60°C, as obtained for the enzymes from *Aspergillus fumigatus* (37°C) [28] and *Aspergillus ficuum* NRRL 3135 (55°C) [12]. Then, the spray dryer technology can be used to also improve some enzymatic properties if compared with the free enzyme, because of the protective action of the adjuvants.

The soybean meal and corn meal powders containing phytase were exposed to different pH values for 30 and 60 min. The phytase activity in soybean powder was maintained higher than 60% for 60 min at a pH range from 4.5 to 8.5 (Fig. 4A). For the powder with corn meal (<150 μm) as adjuvant, the phytase activity was maintained above 90% at pH value from 2.5 to 10.0 (Fig. 4C). There are no significant differences in the stability profile for 30 and 60 min considering both adjuvants. For the free enzyme, the stability was reduced at extreme pH values with a half life (t_{50}) of 120 min at pH 2.0, 3.0, and 10.5 (data not shown), a profile similar to that found in the presence of soybean as adjuvant. The differences observed between the stabilities obtained in the presence of both soybean meal and corn meal can be explained by the interactions of these adjuvants with the enzyme molecule. The corn meal adjuvant was able to protect the enzyme structure better than soybean meal, especially at extreme pH. The reduced dimension of corn meal (<150 μm) adjuvant allows the higher surface of contact to interact with the enzyme molecule if compared with each other. The phytase from *Schizophyllum commune* maintained 100% of its activity at pH 6.0 after 72 h [40]. According to Rani and Ghosh [29], the optimum temperature and pH for phytase activity from *Rhizopus oryzae* was 55°C, and 1.5 and 5.5, respectively. Despite the few studies on the stability of dried phytase as well as data on enzyme storage at room temperature or at higher temperatures, the results obtained here for spray-

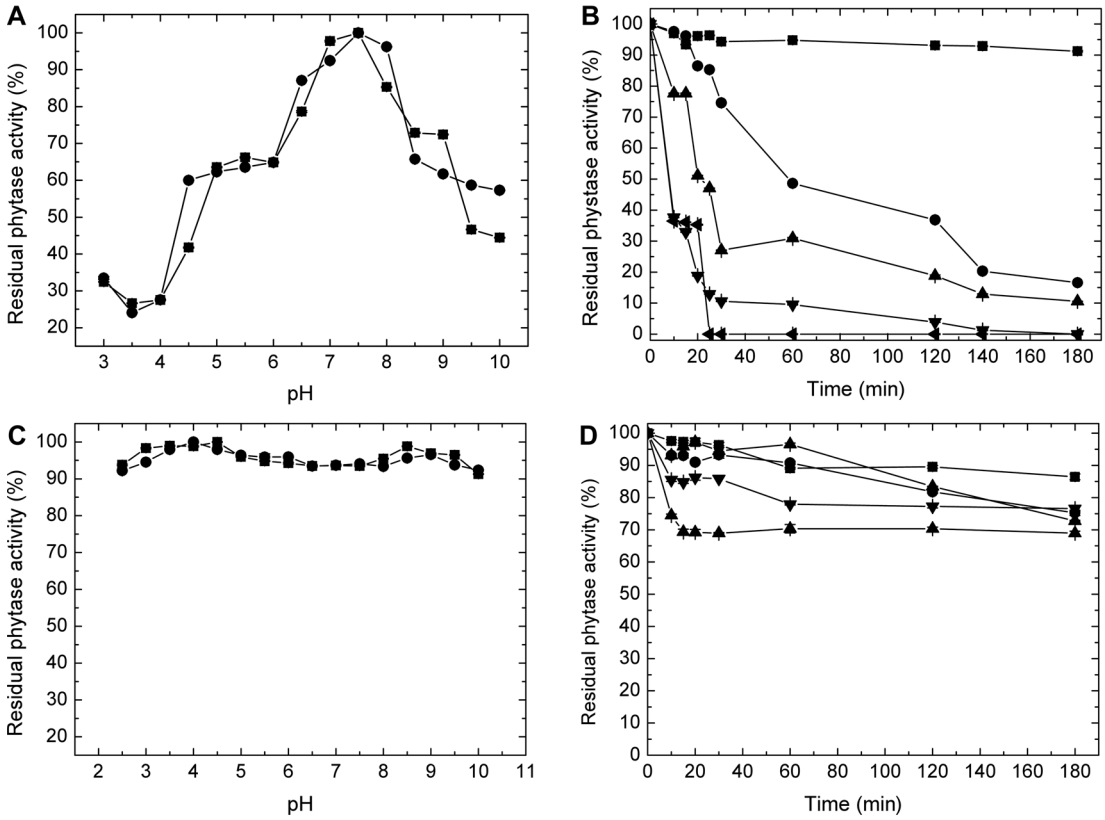

Fig. 4. pH stability (**A** and **C**) for 30 min (■) and 60 min (●), and thermal stability (**B** and **D**) at (■) 30°C, (●) 37°C, (▲) 40°C, (▼) 50°C, and (◀) 60°C, and at (■) 37°C, (●) 40°C, (▲) 50°C, (▼) 60°C, and (◀) 70°C for spray-dried phytase in the presence of soybean meal (**A** and **B**) and corn meal (<150 μm) (**C** and **D**), respectively.

dried *R. microporus* var. *microsporus* phytase are promising for future application. For correct action, these enzymes should be resistant both to the action of the stomach pH and the temperature of food processing, as well as capable to support the storage condition [24].

The phytase activity based on soybean meal as spray-drying adjuvant was fully stable at 30–37°C for 30 min. (Fig. 4B). On the other hand, the enzyme activity was reduced when the dried extract was maintained at 50°C and 60°C, with a half-life (t_{50}) of 8 min (Fig. 4B). The use of corn meal (<150 μm) as adjuvant allowed good thermal stability, with 75% of phytase activity maintained for 180 min from 37°C to 60°C, and 70% of activity at 70°C (Fig. 4D). The *A. niger* ATCC 9142 phytase retained 22% residual activity after 3 min at 80°C [3]. The spray-dried phytase from *R. microsporus* var. *microsporus* was found to be more stable than the other reported phytases [7, 15]. When compared with the pH and temperature stability of the crude extract, the spray-dried enzyme in the presence of corn meal (<150 μM) had its relative activity improved in 5%. The interaction between the adjuvant corn meal and the protein provided greater protection than that observed for the use of soybean meal as adjuvant. The addition of some stabilizing agents would prevent the direct contact of the protein with high temperatures and or stabilize the conformational structure of the protein [5].

In conclusion, the production of *R. microsporus* var. *microsporus* phytase for use in a biofilm is an alternative and viable process for future applications. In addition, the enzyme production was dependent on the carbon source used. The extracellular enzyme obtained under this condition was successfully spray-dried and its stability properties were improved compared with the soluble enzyme, allowing the use of this enzyme for biotechnological applications, especially in diets for poultry. In addition, this is the first time that an alkaline activity has been mentioned for a fungal phytase, deserving future investigation and characterization of these interesting properties.

Acknowledgments

We thank Fundação de Amparo a Pesquisa do Estado de São Paulo (FAPESP) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) for financial support, and Maurício de Oliveira and M.Sc. Marcelo Luís Lombardi Martinez for the technical assistance.

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