Full Length Research Paper

Optimization of phytase production by *Aspergillus japonicus* Saito URM 5633 using cassava bast as substrate in solid state fermentation

Keila A. Moreira^{1*}, Polyanna N. Herculano^{1,2}, Marília de H. C. Maciel³, Tatiana S. Porto¹, Michele R. Spier⁴, Cristina Maria Souza-Motta³, Ana L. F. Porto² and Carlos R. Soccol⁴

¹Academic Unit of Garanhuns, Federal Rural University of Pernambuco, CEP 55292-270, Garanhuns, PE, Brazil.

²Department of Morphology and Animal Physiology, Federal Rural University of Pernambuco, CEP 52171-900, Recife, PE, Brazil.

³Department of Mycology, Federal University of Pernambuco, Cidade Universitária, CEP 50670-420, Recife, PE, Brazil.

⁴Bioprocess Engineering and Biotechnology Division, Chemical Engineering Department, Federal University of Paraná, P.O. Box 19011, CEP 81531-970, Curitiba, PR, Brazil.

Accepted 20 January, 2014

Production of phytase by *Aspergillus japonicus* URM 5633 was investigated by solid state fermentation (SSF) using cassava bast as substrate. The effects of temperature, pH and initial moisture on phytase production were studied using a full factorial design 2³. The maximum phytase activity was 174 U/g dry substrate. The optimum conditions for phytase production were pH 6.5 and 40% initial moisture at 26°C with 96 h of fermentation. A factorial design 2² was created to optimise phytase production, where the initial moisture and temperature were the variables studied. The parameters for phytase production were fixed at 30% initial moisture, 30°C and pH 5.5, and the maximum activity obtained was 183.1 U/g dry substrate. The effects of glucose concentration, nitrogen source type and nitrogen source concentration in phytase production was studied using the 2³ factorial design. Nitrogen source concentration was significant for phytase activity and had a negative effect, indicating that smaller values increased enzyme activity. The optimum activity for phytase occurred at pH 3.6 and at an apparent optimum temperature of 60°C. Phytase was stable in the pH range of 2.4-3.0 at 30-70°C. The enzymatic activity increased significantly in the presence of FeSO₄.

Key words: Filamentous fungus, residue, enzyme, production, characterization.

INTRODUCTION

Brazil is one of the largest producers of cassava in the world. The industrial process for the production of cassava derivatives generates large amounts of waste in the form of bark and bast. Currently, there is a high demand for use of industrial wastes as substrates to reduce the environmental impact and generate resources (Ferreira

and Silva, 2011).

Solid state fermentation (SSF) systems have generated much interest lately because they offer economical and practical advantages, including the use of agro-industrial residues as substrates for obtaining products with added value, higher concentration and improved recovery in

*Corresponding author. E-mail: moreiralab@yahoo.com.br. Tel: +55 87 3764 5500. Fax: +55 87 3764 5520.

simple cultivation facilities with low capital investment and plant operation cost (Latifian et al., 2007). In recent years, the use of filamentous fungi in SSF for the production of commercially important products has increased and has become an attractive alternative method for the production of enzymes such as phytases (Madeira et al., 2011; Awad et al., 2014).

Phytic acid (myo-inositol 1,2,3,4,5,6-hexakis dihydrogen phosphate) and mixed cation salts of phytic acid, designated as phytate, are a group of organic phosphorus compounds found widely in nature, particularly in legumes, cereals and oilseed crops (Reddy et al., 1982). Phytic acid, which is a constituent of animal feed, is not digested by monogastric animals and hence, creates problem in the availability of phosphorus in their diet (Mittal et al., 2011). Phytases (EC 3.1.3.8 and EC 3.1.3.26) are the primary enzymes responsible for the hydrolytic degradation of phytic acid and its salts (phytates). In addition, phytase reduces the amounts of phosphorus entering the environment and problems resulting from eutrophication and chelation of nutrient factors from the soil because supplementation of the diets of monogastric animals with phytase reduces phosphate excretion in the faeces by up to 50% (Greiner and Konietzny, 2006). In the past decade, there has been a great deal of interest in the study of microbial phytase producers and in optimising media and conditions with the aim of increasing yields to commercially feasible levels (Lan et al., 2002a, b).

Phytase can be found in plants, bacteria, yeast, filamentous fungi and animals. However, among microorganisms, phytase activity has been most frequently observed in fungi, particularly in *Aspergillus* (Kim et al., 1999; Vats and Banerjee, 2002; Bhavsar et al., 2012; Cheng et al., 2012; Jafari-Tapeh et al., 2012; Akturk et al., 2013; Bhavsar et al., 2013).

Most of the studies involving enzyme production have been done using one variable-at-a-time (OVAT) approach, providing a favorable range of individual parameters for maximum productivity (Sharma et al., 2009; Mishra and Malik, 2012; Mishra et al., 2013). However, these studies completely lacks in representing the effect of interaction between different factors. Recently, factorial experimental design and response surface methodology (RSM) have been a powerful tool of statistics which simplifies the optimization by studying the mutual interactions among variables over a range of values in a statistically valid manner, while reducing the number of experimental trials (Kaushik and Malik, 2011; Mishra and Malik, 2013). Some studies have utilized these tools statistical for optimization of different processes, e.g. in the enzyme production (Herculano et al., 2011; Maciel et al., 2011), dyes and metals removal (Sharma et al., 2009; Kaushik and Malik, 2011) and control and growth of the microorganism (Mishra and Malik, 2012; Mishra and Malik, 2013). Thus, the aim of this study was to optimize phytase production by

Aspergillus japonicus URM 5633 in SSF using cassava bast as substrate and to partially characterize this enzyme.

MATERIALS AND METHODS

Microorganisms

Twenty-two isolates of *Aspergillus* (Table 1) obtained from URM Culture Collection (WCDM 604), Federal University of Pernambuco, Recife, Brazil (Micoteca URM, 2013) were inoculated on malt extract agar (MEA: 20 g/L malt, 20 g/L glucose, 1 g/L peptone, 20 g/L agar) and maintained at 28°C.

Substrates

Several solid substrates, including cassava bast, crushed sunflower seeds, oat bran, rice bran, soursop umbu, soy flour and wheat bran, were used in SSF as substrates. Substrate particle sizes were 0.8-2.0 mm. The initial moisture of substrates was determined in accordance with the standards of the Institute Adolfo Lutz (Zenebon and Pascuet, 2005). Phytase production by *Aspergillus* strains was investigated at 28°C and 50% initial moisture.

Screening of phytase-producing *Aspergillus* strains

Qualitative screening

Aspergillus isolates were screened in solid medium to select strains with higher potential for phytate degradation. For selection, Petri dishes with 6 cm in diameter, containing 7 mL of sterilised MEA medium, were inoculated with each isolate and incubated for 7 days at 30°C. A 6 mm plug was cut with a sterile cork borer from the colony periphery, transferred to the centre of the culture medium, as proposed by Couri and Farias (1995), using commercial phytate (Sigma-Aldrich-St. Louis, USA) as a carbon source and incubated for 6 days at 30°C. Phytate degradation was identified by a clear halo zone around the fungal growth.

Screening of substrate for SSF

Aspergillus isolates producing larger zones of hydrolysis around their colony were selected for enzyme production in SSF. Fermentations were conducted using 250 mL Erlenmeyer flasks with 10.0 g of residue at 50% initial moisture and 28°C. The initial moisture of substrates was determined in accordance with the standards of the Institute Adolfo Lutz (Zenebon and Pascuet, 2005). Flasks were sterilised at 65°C for 2 h in an oven and exposed to ultraviolet radiation for 2 h in a microbiological cabinet (Spier et al., 2008). The substrate was moistened with sodium citrate buffer (0.1 M, pH 4.5) supplemented with 0.5% ammonium sulphate. The Petri dishes containing 7 mL of sterilised MEA medium were inoculated and incubated for 7 days at 30°C with each strain. Then, five plugs of 6 mm were cut with a sterile cork borer from the colony periphery to suspend the spores from the plugs in 30 mL of 0.02% Tween 80 solution. Spores were counted in a Neubauer counting chamber and a concentration of 10^7 spores/g of dry substrate was used for SSF. The fermentation was conducted for 120 h and samples were withdrawn every 24 h. A portion (5.0 g) of the fermented mixture was mixed with 30 mL of 0.1 M citrate buffer (pH 4.5). After maceration, the extract was clarified by filtration and centrifugation at $5,000 \times g$ for 15 min (Spier et al., 2008). The extracts were used at a dilution suitable for phytase activity determination.

Table 1. Selection of *Aspergillus* phytase producers according to their colony diameters on selective medium.

Culture/Access number URM ^a	Diameter of colony (cm)	Presence of halo
<i>A. caespitosus</i> Raper & Thom URM 5182	4.2	-
<i>A. candidus</i> Link URM 5611	2.0	+
<i>A. duricaulis</i> Raper & Fennell URM 5608	4.9	+
<i>A. flavus</i> Link URM 5621	3.4	+
<i>A. granulatus</i> Raper & Thom URM 4641	2.5	+
<i>A. japonicus</i> Saito URM 5620	4.6	+
<i>A. japonicus</i> Saito URM 5633*	6.0	+
<i>A. niger</i> Tiegh. URM 2228	5.1	-
<i>A. niger</i> Tiegh. URM 2604	5.9	-
<i>A. niger</i> Tiegh. URM 3886	5.9	-
<i>A. niger</i> Tiegh. URM 5117	5.2	+
<i>A. niger</i> Tiegh. URM 5437	5.1	+
<i>A. niger</i> Tiegh. URM 5438	5.8	+
<i>A. niger</i> Tiegh. URM 5439	5.0	+
<i>A. ochraceus</i> G. Wilh. URM 5609	4.3	+
<i>A. sclerotiorum</i> G.A. Huber URM 5616	2.5	+
<i>A. stromatoides</i> Raper & Fennell URM 5615	5.3	+
<i>A. sydowii</i> (Bainier & Sartory) Thom & Church URM 4198	2.0	-
<i>A. sydowii</i> (Bainier & Sartory) Thom & Church URM 5612	3.0	+
<i>A. terreus</i> Thom URM 5606	4.0	+
<i>A. versicolor</i> (Vuill.) Tirab. URM 5535	2.6	+
<i>A. versicolor</i> (Vuill.) Tirab. URM 5701	2.0	+

(-): No halo; (+): presence of halo; (*) isolate selected for the production. ^aAccess number in the URM Culture Collection (URM, Recife, Brazil).

Table 2. Variable levels of the 2² experimental design for optimisation of the production of phytase by *A. japonicus* URM 5633.

Variable	Level		
	Low (-1)	Central (0)	High (+1)
T	22	26	30
Im	30	40	50

Im, Initial moisture (%) and T, temperature (°C).

Table 3. Variable levels of the 2³ experimental design for evaluating phytase production by *A. japonicus* URM 5633, using initial moisture of 30% and nutrient solution at pH 5.5 and 30°C.

Variable	Level		
	Low (-1)	Central (0)	High (+1)
Glucose (%)	0	1.0	2.0
Nt	CSL	YE	Urea
Nc (%)	0.25	0.50	0.75

Nt, Nitrogen source type; Nc, nitrogen source concentration (%); CSL, corn steep liquor; YE, yeast extract.

Experimental design and statistical analysis

The influence of pH, initial moisture and temperature on phytase production was evaluated on the basis of a 2³ factorial design (Bruns et al., 2006) and four central points. After the selection of variables that significantly influenced phytase production, enzyme production was optimised using a 2² factorial design (Table 2) maintained at a constant pH (pH 5.5), given that this did not influence phytase production.

The influence of glucose concentration, nitrogen source type and nitrogen source concentration on phytase production was evaluated (Table 3). All statistical analyses were performed with Statistic 8.0 software (Statistica, 2008).

Enzyme assay

Phytase activity was determined by quantification of the phosphate

released from phytate during an enzymatic reaction using the ammonium molybdate method with modifications described by Spier et al. (2010). Sodium acetate buffer (350 µL, 100 mM, pH 4.5) containing sodium phytate (875 nmol) was used as substrate. After pre-incubation at 37°C for 10 min, the enzymatic reaction was initiated by the addition of enzyme extract (50 µL). The homogenised solution was incubated for 30 min at 37°C and a 1.5 mL solution consisting of ammonium molybdate 10 mM : H₂SO₄ 5 N : acetone (1:1:2 v/v) and 1 M citric acid (100 mL) was added. The release of inorganic phosphorus was determined at an absorbance of 355 nm. One unit of phytase is defined as the amount of enzyme that releases 1 nmol of inorganic phosphorus per minute under the test conditions. Enzyme activity was expressed in units per gram of

dry basis (U/g dry substrate). A standard curve was constructed with dibasic potassium phosphate at 10-600 nM/mL phosphate. Protein content was determined according to Bradford (1976) at 595 nm against a bovine serum albumin standard.

Enzyme characterization

Phytase activity from the crude enzymatic extract was measured at different pH and temperature values.

Optimum pH and temperature for enzyme activity

The effect of pH on phytase activity was measured using the following buffers: glycine-hydrochloric acid buffer 0.1M (pH 2.4-3.6); sodium acetate buffer 0.1 M (pH 3.6-6.0) and Tris-HCl 0.1 M (pH 6.5-8.0). The optimum temperature within the 30-90°C range was determined by incubation of the reaction mixture at optimum pH.

pH and temperature stability

Crude enzymatic extract was diluted (1:2) in the buffers (pH 2.4-8.0), and the aliquots were withdrawn for determination of specific activities at 0, 180 and 360 min. For determination of thermal stability, the crude enzymatic extract was incubated at temperatures of 25-80°C and aliquots were withdrawn for determination of specific activities at 0, 60, 120 and 180 min.

Influence of divalent metal ions and EDTA on enzyme activity

Metal ions (Na^+ , Mn^{2+} , Cu^{2+} , Zn^{2+} , Ni^{2+} , Ag^+ , Mg^{2+} , Fe^{2+} and Ca^{2+}) and EDTA (final concentration of 10 and 100 mM) were mixed with enzyme extract solution in 2.5 mM sodium acetate buffer (pH 5.0) and incubated for 30 min at 50°C. Phytase activity was determined as described above.

RESULTS AND DISCUSSION

Fungi are important producers of extracellular enzymes and are relatively easy to grow in controlled environments (Santos, 2007). Screening is often the first step to select microorganisms with characteristics intended for Industrial applications, which allows the characterization and selection of fungal strains with optimal enzyme production (Maciel et al., 2013).

Aspergillus strains were evaluated for their ability to synthesise phytase in solid medium. Table 1 lists the strains screened for phytase production. *A. japonicus* URM 5633 was the most promising strain for fermentation studies. Since the fungal enzymes to be used in industry must not be contaminated with toxic metabolites, *A. japonicus* URM 5633 was tested for the presence of mycotoxins (ochratoxin A and fumonisin B₂), but these metabolites were not detected (data not shown).

Gargova et al. (1997) on studying 203 fungal strains belonging to the genera *Aspergillus*, *Mucor*, *Penicillium* and *Rhizopus*, observed clear zones around 91.6% of the colonies. The *Aspergillus* species, *A. oryzae* (Chantasartasamee et al., 2005), *A. ficcum* (Bogar et al.,

Table 4. Effects of different substrates on phytase production by *A. japonicus* URM 5633.

Substrate	Phytase activity (U/g dry substrate)
Bast cassava	11.3 ± 1.0
Crushed	5.3 ± 0.8
Sunflower seeds	3.5 ± 1.0
Oat bran	9.3 ± 0.3
Rice bran	6.4 ± 0.9
Soursop umbu	2.7 ± 1.1
Soy flour	4.7 ± 0.7
Wheat bran	11.0 ± 0.8

2003; Zhang et al., 2010) and *A. niger* (Casey and Walsh, 2003; Vassilev et al., 2007; Bhavsar et al., 2012; Bhavsar et al., 2013) were hypothesised to be good phytase producers.

The production of phytase by *A. japonicus* URM 5633 on different agro-industrial substrates was assayed. All the substrates tested had phytase activity (Table 4), though in varying degrees. Maximum enzyme productivity (11.3 ± 1.0 U/g dry substrate) was achieved with cassava bast, followed by wheat bran (11.0 ± 0.8 U/g dry substrate), whereas minimum productivity (2.7 ± 1.1 U/g dry substrate) was observed with soursop umbu. Roopesh et al. (2006) tested wheat bran, sesame oil cake and groundnut oil cake for phytase production. The mixture of wheat bran with sesame oil cake (1:1 ratio) resulted in better phytase activity (32.2 U/g dry substrate) using *Mucor racemosus* NRRL1994.

Ramachandran et al. (2005) also found a higher production of phytase by *Rhizopus oryzae* NRRL 1891 by mixing coconut oil cake and sesame oil cake (1:1 ratio) (35 U/g dry substrate). Maximum enzyme productivity reported by Awad et al. (2014) was achieved with corn cope (46 ± 2.8 U/g dry substrate) followed by corn bran (41 ± 4.2 U/g dry substrate), whereas minimum productivity was obtained with sesame oil cake (8 U/g dry substrate). A combination of corn cobs and corn bran in 1:1 ratio enhanced phytase production to 57 ± 3.5 U/g dry substrate. Most studies involving SSF used a mixture of substrates for increased enzyme production, but this mixture was not done in our study. However, this work contributes to the knowledge of phytase production by different agro-industrial substrates that could be used in future work.

We attempted to optimise the production of the enzyme at different pH values, initial moisture and temperature. The phytase produced in SSF was assayed during 120 h, where the maximum activity was 174 U/g dry substrate at 96 h pH 6.5, 40% initial moisture and 26°C (Table 5, Run 3). These conditions contribute to the large-scale phytase production, minimising costs and production time for the industry.

The phytase activity obtained with cassava bast was

Table 5. Results of the 2^3 design for phytase production in solid state fermentation by *A. japonicus* URM 5633 using cassava bast as substrate.

Run	pH ^a	Im ^b	T ^c	Phytase activity (U/g dry substrate)				
				24 h	48 h	72 h	96 h	120 h
1	4.5	40	26	83.3	97.2	115.3	154.0	157.2
2	4.5	60	26	32.8	89.0	80.5	73.4	67.9
3	6.5	40	26	56.8	70.7	92.1	174.0	140.1
4	6.5	60	26	61.1	61.1	36.4	31.4	27.7
5	4.5	40	34	66.2	90.5	83.7	80.1	80.5
6	4.5	60	34	33.4	68.0	13.9	10.0	8.8
7	6.5	40	34	85.7	131.1	58.6	50.8	56.2
8	6.5	60	34	29.5	33.3	35.4	50.6	33.0
9 ^d	5.5	50	30	45.5	71.2	37.1	45.0	36.0
10 ^d	5.5	50	30	51.8	51.9	65.1	38.4	46.3
11 ^d	5.5	50	30	48.1	48.9	38.4	78.5	44.4
12 ^d	5.5	50	30	46.1	54.3	52.7	32.9	30.4

The phytase activity with subscript gives the cultivation time in hours; ^apH, ^bIm- initial moisture (%), ^cT- temperature (°C) and ^dcentral points.

Figure 1. Pareto chart for the effects calculated from the responses of the 2^3 design for the phytase production over 96 h of solid state fermentation by *A. japonicus* URM 5633. (1) Initial moisture, (2) pH and (3) temperature.

higher than that reported by Spier et al. (2008). These authors obtained 51 U/g dry substrate using citric pulp as nitrogen source and 60% initial moisture after 96 h at 30°C in SSF with *A. niger* FS3 isolate. The optimum conditions for phytase production reported by Awad et al. (2014) were 27°C, pH 8.0 and 66% initial moisture content in SSF using *Penicillium purpurogenum* GE1. The use of cassava bast may be highly economical at the industrial scale for phytase production. Brazil has 10% of the world production and is the second largest cassava

producer in the world. Thus, cassava bast is an abundant residue, obtained from the production of flour, with great viability for industrial use (Sousa et al., 2011). A comparison of enzyme production by microorganisms is not easy, owing to the different growing conditions and methods used for determination of enzyme activities.

Fungi are considered the best adapted organisms for SSF, given that their hyphae can grow on the surface of particles and can penetrate to the interparticle spaces and colonise them (Santos et al., 2004). Use of waste products in SSF has become a goal of many researchers because it provides a cheap medium and resolves the problems resulting from waste accumulation (Awad et al., 2014).

The effects of the tested variables on phytase production at 96 h of SSF are described in Figure 1. At positive values of a variable, phytase production is higher at higher levels of the variable, and at negative values the phytase production is higher at lower levels. Initial moisture and temperature had significant negative effects on phytase production. Temperature has played an important role in influencing the process parameters on the enzymes production response under solid state fermentation (Ustok et al., 2007). The phytase production was enhanced at 40% initial moisture and 26°C. A similar result was reported by Awad et al. (2014), who obtained maximum activity for phytase at 66% moisture content and 27°C and observed that increased levels of these variables in the fermentation medium led to reduction in enzyme productivity.

The two significant variables (initial moisture and temperature) influencing phytase production were further optimised using the 2^2 factorial design. The pH was maintained at 5.5 for 96 h of fermentation. Initial moisture

Figure 2. Pareto chart for the effects calculated from the responses of the 2^2 design for phytase production over 96 h of solid state fermentation by *A. japonicus* URM 5633. (1) Temperature and (2) initial moisture.

Table 6. Results of the 2^2 design for phytase production at 96 h in solid state fermentation by *A. japonicus* URM 5633 using cassava bast as substrate.

Run	T ^a	Im ^b	Phytase activity (U/g dry substrate)
1	22	30	138.9
2	30	30	183.1
3	22	50	97.4
4	30	50	58.2
5 ^c	26	40	124.8
6 ^c	26	40	135.3
7 ^c	26	40	117.4
8 ^c	26	40	131.4

^aT- Temperature (°C) and ^bIm- initial moisture (%).

and the temperature and initial moisture interaction (1 x 2) exerted significant negative effects on phytase production (Figure 2). Maximum activity (183.1 U/g dry substrate) was obtained with 30% initial moisture at 30°C (Table 6, Run 2).

Initial moisture, temperature and pH were maintained at 30%, 30°C and 5.5, respectively, for assessing the interference of glucose concentration, nitrogen source type and nitrogen source concentration in phytase production. As shown in Figure 3, only varying nitrogen source concentration had significant effects on phytase production. Some interactions had significant effects, showing a dependent relationship between them. The interaction of glucose concentration, nitrogen source type

and nitrogen source concentration (1 x 2 x 3) showed a significant negative effect on phytase production, with decreased values of these variables resulting in increased enzyme activity. In contrast, the interaction of nitrogen source type and nitrogen source concentration (2 x 3) showed a significant positive effect, indicating that high levels of these variables increase enzyme activity.

Enzyme characterization

The effect of pH on phytase activity is shown in Figure 4. The optimum activity for phytase occurred at pH 3.6. Phytase was stable in the pH range of 2.4-3.0. After

Figure 3. Pareto chart for the effects calculated from the responses of the 2^3 design for phytase production over 96 h of solid state fermentation by *A. japonicus* URM 5633 at varying glucose concentrations, nitrogen source type and nitrogen source concentration. (1) Glucose (%), (2) nitrogen source type and (3) nitrogen source concentration (%).

Figure 4. Effect (□) and stability (●) of pH on the activity of phytase from *A. japonicus* URM 5633.

incubation for 360 min at pH 2.4-8.0, >50% of the activity remained (Figure 4). Maximum activity was obtained at 60°C (Figure 5). Phytase was stable at 30-70°C, but activity decreased as temperature increased. However, at 80°C, 78% of the activity remained.

The optimum temperature and stability were within the typical temperature for phytase activity. Phytases from *Aspergillus* species usually exhibit optimum temperatures between 50 and 65°C (Vats and Banerjee, 2004). Casey and Walsh (2003) observed increased activity at 65°C

Figure 5. Effect (□) and stability (●) of temperature on the activity of phytase from *A. japonicus* URM 5633.

Table 7. Influence of metal ions and EDTA on phytase activity of *Aspergillus japonicus* URM 5633 in solid state fermentation using cassava bast as substrate.

Metal ion	10 mM	100 mM
	Relative activity (%)	
Control	100	100
Sodium borate (Na ⁺)	111	97
Manganese chloride (Mn ²⁺)	96	100
Copper sulphate (Cu ²⁺)	119	125
Zinc sulphate (Zn ²⁺)	109	101
Nickel nitrate (Ni ²⁺)	122	237
Silver nitrate (Ag ⁺)	153	94
Magnesium sulphate (Mg ²⁺)	123	80
Ferrous sulphate (Fe ²⁺)	445	592
Calcium chloride (Ca ²⁺)	126	**
EDTA	111	103

**Precipitated.

and at optimum pH of 5.0 for phytase from *A. niger* ATCC 9142. However, Escobin-Mopera et al. (2012), working with phytase from *Klebsiella pneumoniae* 9-3B, observed a 15% reduction in enzyme activity at 60°C after only 10 min. These authors obtained highest enzyme activity at 50°C, which was stable even after 1 h of exposure. The maximum phytase activity was at pH 4.0, with broad pH stability from 2.0 to 7.0. Rani and Ghosh (2011), working with *R. oryzae* for phytase production by SSF, obtained a lower apparent optimum temperature (45°C) and dual pH optima at 1.5 and 5.5. Probably, the most important factor among all the physical variables affecting the production

of enzymes and metabolites is the incubation temperature, given that enzymatic activities are sensitive to temperature (Krishna, 2005). The high stability at specific temperatures suggested that the phytase is acceptable for commercial application.

Phytase activity was stimulated in the presence of Na⁺, Cu²⁺, Zn²⁺, Ni²⁺, Ag⁺, Mg²⁺, Fe²⁺, Ca²⁺ and EDTA at 10 mM and of Mn²⁺, Cu²⁺, Zn²⁺, Ni²⁺, Fe²⁺ and EDTA at 100 mM. However, Fe²⁺ (10 and 100 mM concentrations) significantly stimulated phytase activity (Table 7). Escobin-Mopera et al. (2012) observed that phytase activity was slightly stimulated by Ca²⁺ and EDTA and

inhibited by Zn^{2+} and Fe^{2+} . In contrast, Casey and Walsh (2003) observed that phytase activity was moderately inhibited by Ca^{2+} but was not significantly affected by Fe^{2+} or Fe^{3+} . These authors also showed that phytase activity was moderately stimulated in the presence of Mg^{2+} , Mn^{2+} , Cu^{2+} , Cd^{2+} , Hg^{2+} , Zn^{2+} and F^{-} ions.

Conclusions

The results demonstrated the feasibility of phytase production by the new isolate *A. japonicus* URM 5633 using cassava bast as substrate in SSF. The use of low-cost medium and a very simple technique for production of phytase, which is considered to be one of the most important enzymes, is a great benefit for food industry, chemical, pharmaceutical, leather industries, textile industries, in wastewater treatment and animal feed. The conditions of 30% initial moisture at 30°C and pH 5.5 with 96 h of incubation were the best for production of phytase using cassava bast as substrate in SSF. The optimum activity for phytase was at pH 3.6, and the apparent optimum temperature was 60°C. Phytase was stable in the pH range 2.4-3.0 at 30-70°C. Enzymatic activity was significantly increased in the presence of FeSO_4 . The optimisation of phytase production clearly demonstrated the influence of process parameters on the yield of the enzyme.

ACKNOWLEDGEMENTS

The authors acknowledge the financial support received from CNPq (Process no 150016/2009-3), CAPES/PROCAD (Process no 0921/2010) and FACEPE.

REFERENCES

- Akturk S, Güvenmez HK, Dinçer S (2013). Purification and characterization of extracellular phytase from *Aspergillus niger*. Curr. Opin. Biotechnol. 24:S85.
- Awad GEA, Helal MMI, Dania EN, Esawy MA (2014). Optimization of phytase production by *Penicillium purpurogenum* GE1 under solid state fermentation by using Box-Behnken design. Saudi. J. Biol. Sci. 21:81-88.
- Bhavsar K, Buddhiant P, Soni SK, Depan D, Sarkar S, Khire JM (2013). Phytase isozymes from *Aspergillus niger* NCIM 563 under solid state fermentation: Biochemical characterization and their correlation with submerged phytases. Process Biochem. 48:1618-1625.
- Bhavsar K, Kumar VR, Khire JM (2012). Downstream processing of extracellular phytase from *Aspergillus niger*. Chromatography process vs. aqueous two phase extraction for its simultaneous partitioning and purification. Process Biochem. 47:1066-1072.
- Bogar B, Szakacs G, Linden JC, Pandey A, Tengerty RP (2003). Optimization of phytase production by solid substrate fermentation. J. Ind. Microbiol. Biotechnol. 30:183-189.
- Bradford MM (1976). A rapid and sensitive method for the quantification of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72:248-254.
- Bruns RE, Scarminio IS, Neto BB (2006). Statistical Design-Chemometrics. Elsevier, Amsterdam, The Netherlands. p. 422.
- Casey A, Walsh G (2003). Purification and characterization of extracellular phytase from *Aspergillus niger* ATCC 9142. Biore. Technol. 86:183-188.
- Chantasartasamee K, Ayuthaya DIN, Intarareugsorn S, Dharmstithi S (2005). Phytase activity from *Aspergillus oryzae* AK9 cultivated on solid state soybean meal medium. Process Biochem. 40:2285-2289.
- Cheng C-W, Chen C-K, Chang C-J, Chen L-Y (2012). Effect of colour LEDs on mycelia growth of *Aspergillus ficuum* and phytase production in photo-fermentations. J. Photochem. Photobiol. B. 106:81-86.
- Couri S, Farias AX (1995). Genetic manipulation of *Aspergillus niger* for increased synthesis of pectinolytic enzymes. Rev. Microbiol. 26 : 314-317.
- Escobin-Mopera L, Ohtani M, Sekiguchi S, Soni T, Abe A, Tanaka M, Meevootisom V, Asano K (2012). Purification and characterization of phytase from *Klebsiella pneumonia* 9-3B. J. Biosci. Bioeng. 113:562-567.
- Ferreira MS, Silva JRB (2011). Utilização da casca, entrecasca e raspa da mandioca na alimentação de ruminantes. Rev. Bras. Agropecu. Sustent. 1:64-66.
- Gargova A, Roshkova Z, Vancheva G (1997). Screening of fungi for phytase production. Biotechnol. Technol. 11:221-224.
- Greiner R, Konietzny U (2006). Phytase for food application. Food Technol. Biotechnol. 44:125-140.
- Herculano PN, Porto TS, Moreira KA, Pinto GA, Souza-Motta CM, Porto AL (2011). Cellulase production by *Aspergillus japonicus* URM5620 using waste from castor bean (*Ricinus communis* L.) under solid-state fermentation. Appl. Biochem. Biotechnol. 165:1057-1067.
- Jafari-Tapeh H, Hamidi-Esfahani Z, Azizi MH (2012). Culture condition improvement for phytase production in solid state fermentation by *Aspergillus ficuum* using statistical method. ISRN Chem. Eng. 1-5.
- Kaushik P, Malik A (2011). Process optimization for efficient dye removal by *Aspergillus lentulus* FJ172995. J. Hazard. Mater. 185:837-843.
- Kim DS, Godber JS, Kim HR (1999). Culture conditions for a new phytase-producing fungus. Biotechnol. Lett. 21:1077-1081.
- Krishna C (2005). Solid-State Fermentation Systems - An Overview. Crit. Rev. Biotechnol. 25:1-30.
- Lan GQ, Abdullah N, Jalaludin S, Ho YW (2002a). Optimization of carbon and nitrogen sources for phytase production by *Mitsukella jalaludinii*, a new rumen bacterial species. Lett. Appl. Microbiol. 35:157-161.
- Lan GQ, Abdullah N, Jalaludin S, Ho YW (2002b). Culture conditions influencing phytase production of *Mitsukella jalaludinii*, a new bacterial species from the rumen of cattle. J. Appl. Microbiol. 93:668-674.
- Latifian M, Hamidi-Esfahani Z, Barzegar M (2007). Evaluation of culture conditions for cellulase production by two *Trichoderma reesei* mutants under solid-state fermentation conditions. Bioreour. Technol. 98:3634-3637.
- Maciel M, Ottoni C, Santos C, Lima N, Moreira K, Souza-Motta C (2013). Production of polygalacturonases by *Aspergillus* Section *Nigri* strains in a fixed bed reactor. Molecules 18:1660-1671.
- Maciel MHC, Herculano PN, Porto TS, Teixeira MFS, Moreira KA, Souza-Motta CM (2011). Production and partial characterization of pectinases from forage palm by *Aspergillus niger* URM4645. Afr. J. Biotechnol. 10 : 2469-2475.
- Madeira Jr. JV, Macedo JA, Macedo GA (2011). Detoxification of castor bean residues and the simultaneous production of tannase and phytase by solid-state fermentation using *Paecilomyces variotii*. Bioreour. Technol. 102:7343-7348.
- Micoteca URM (2013). Available online: www.ufpe.br/micoteca/ (accessed on 9 November 2013).
- Mishra S, Kumar P, Malik A (2013). Effect of process parameters on the enzyme activity of a novel *Beauveria bassiana* isolate. Int. J. Curr. Microbiol. Appl. Sci. 2:49-56.
- Mishra S, Malik A (2012). Comparative evaluation of five *Beauveria* isolates for housefly (*Musca domestica* L.) control and growth optimization of selected strain. Parasitol. Res. 111:1937-1945.
- Mishra S, Malik A (2013). Nutritional optimization of a native *Beauveria bassiana* isolate (HQ917687) pathogenic to housefly, *Musca domestica* L. J. Parasit. Dis. 37:199-207.

- Mittal A, Singh G, Goyal V, Yadav A, Aneja KR, Gautam SK, Aggarwal NK (2011). Isolation and biochemical characterization of acidothermophilic extracellular phytase producing bacterial strain for potential application in poultry feed. *Jundishapur J. Microbiol.* 4:273-282.
- Ramachandran S, Roopesh K, Nampoothiri KM, Szakacs G, Pandey A (2005). Mixed substrate fermentation for the production of phytase by *Rhizopus* spp. using oilcakes as substrates. *Process Biochem.* 40:1749-1754.
- Rani R, Ghosh S (2011). Production of phytase under solid-state fermentation using *Rhizopus oryzae*: novel strain improvement approach and studies on purification and characterization. *Bioresour. Technol.* 102:10641-10649.
- Reddy NR, Sathe SK, Salunkhe DK (1982). Phytates in legumes and cereals. *Adv. Food. Res.* 28:1-9.
- Roopesh K, Ramachandran S, Nampoothiri KM, Szakacs G, Pandey A (2006). Comparison of phytase production on wheat bran and oilcakes in solid-state fermentation by *Mucor racemosus*. *Bioresour. Technol.* 97:506-511.
- Santos MM, Rosa AS, Dal'boit S, Mitchell DA, Kriger N (2004). Thermal denaturation: is solid-state fermentation really a good technology for the production of enzymes. *Bioresour. Technol.* 93:261-268.
- Santos SFM (2007). Study of pectinase production by solid state fermentation using cashew apple as substrate. PhD Thesis, Federal University of Rio Grande do Norte, Brazil.
- Sharma S, Malik A, Satya S (2009). Application of response surface methodology (RSM) for optimization of nutrient supplementation for Cr (VI) removal by *Aspergillus lentulus* AML05. *J. Hazard. Mater.* 164:1198-1204.
- Sousa TCR, Aguiar JLP, Lôbo CF (2011). A importância da mandioca. In Fialho JF, Vieira EA. *Mandioca no cerrado*, Embrapa Cerrados, Planaltina, Distrito Federal. pp. 14-23
- Spier MR, Greiner R, Rodriguez-León JA, Woiciechowski AL, Pandey A, Soccol VT, Soccol CR (2008). Phytase production using citric pulp and other residues of the agroindustry in SSF by fungal isolates. *Food Technol. Biotechnol.* 46:178-182.
- Spier MR, Woiciechowski AL, Letti LA, Scheidt GN, Sturm W, Rodriguez-León JA, Carvalho JC, Dergint DE, Soccol CR (2010). Monitoring fermentation parameters during phytase production in column-type bioreactor using a new data acquisition system. *Bioprocess Biosyst. Eng.* 33:1033-1041.
- Statistica (Data analysis Software Systems) (2008), version 8.0, StatSoft, Inc, Tulsa, OK, USA.
- Ustok FI, Tari C, Gogus N (2007). Solid state production of polygalacturonase by *Aspergillus sojae* ATCC 20235. *J. Biotechnol.* 127:322-334.
- Vassilev N, Vassileva M, Bravo V, Fernandez-Serrano M, Nikolaeva I (2007). Simultaneous phytase production and rock phosphate solubilization by *Aspergillus niger* grown on dry olive wastes. *Ind. Crop. Prod.* 26:332-336.
- Vats P, Banerjee UC (2002). Studies on the production of phytase by a newly isolated strain of *Aspergillus niger* var teigham obtained from rotten wood-logs. *Process Biochem.* 38:211-217.
- Vats P, Banerjee UC (2004). Production studies and catalytic properties of phytase (myo-inositolhexakisphosphate phosphohydrolases): on overview. *Enzyme Microb. Technol.* 35:3-14.
- Zenebon O, Pascuet NS (2005). Métodos físico-químicos para análise de alimentos. Instituto Adolfo Lutz, São Paulo, Brazil. p.1020.
- Zhang GQ, Dong XF, Wang ZH, Zhang Q, Wang HX, Tong JM (2010). Purification, characterization, and cloning of a novel phytase with low pH optimum and strong proteolysis resistance from *Aspergillus ficuum* NTG-23. *Bioresour. Technol.* 101:4125-4131.