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Extracellular β-D-fructofuranosidase from *Aspergillus parasiticus*: Optimization of the production under submerged fermentation and biochemical characterization

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The β-D-fructofuranosidases are enzymes with biotechnological potential that can be used in different industrial sectors as food and beverage. In this context, microorganisms are important producers of these biomolecules, especially filamentous fungi. The production of extracellular β-D-fructofuranosidase from *Aspergillus parasiticus* using sugarcane bagasse as a carbon source under submerged fermentation was optimized by factorial design and high levels of enzyme were obtained in 24 h-old cultures at 30°C using 1.5% sugarcane bagasse under agitation. The extracellular β-D-fructofuranosidase was purified 119-fold using diethyl aminoethyl (DEAE)-cellulose and Sephacryl S-200 chromatographic columns with recovery of 16%. The native molecular mass was estimated as 136 kDa with two subunits of 63 kDa determined by 7% sodium dodecyl sulfate poly-acrylamide gel electrophoresis (SDS-PAGE) and 64% carbohydrate content. The purified enzyme showed optimal temperature of activity from 38-56°C and optimum pH from 4.5 to 6.2 determined by experimental design (CCRD), with half-life of 25 min at 50°C. It was stable from pH 5.0-10.0. The extracellular enzyme activity was stimulated by Ba^{2+} and Mg^{2+}, and it was not affected by urea, silver and ethylenediaminetetraacetic acid (EDTA). The $K_{0.5}$ and V_{max} values were 10 mM and 1565 U/mg of protein, and 19 mM and 1965 U/mg of protein for sucrose and raffinose, respectively.

Key words: Invertase, fructofuranosidase, *Aspergillus parasiticus*, sugarcane bagasse, factorial design.

INTRODUCTION

Microorganisms are recognized as sources of different molecules with biotechnological potential. Among these microorganisms, the filamentous fungi deserve attention. They are able to degrade many organic and inorganic substrates by action of secreted enzymes. According to this, in the last years, the interest in the use of agro-industrial residues as substrates in bioprocess has been increased as, for example, for the production of molecules with aggregate value using different fermentative processes. As carbon source, agro-industrial residues

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Abbreviations: FOS, Fructooligosaccharides; **PB,** Plackett and Burman; **CCRD,** central composite rotatable design; **DEAE,** diethyl aminoethyl; **DNS,** 3',5'-dinitrosalisilic acid; **BSA,** bouvine serum albumin; **SDS-PAGE,** sodium dodecyl sulfate poly-acrylamide gel electrophoresis; **EDTA,** ethylenediaminetetraacetic acid.

have been used for enzyme production as, for example, β-D-frutofuranosidases (EC 3.2.1.26) also known as invertase (Pandey et al., 2000).

These enzymes can be obtained from different animals and plant tissues as well as from microorganisms as bacteria (Awad et al., 2013), yeast (Kumar and Kesavapillai, 2012; Sainz-Polo et al., 2013) and filamentous fungi as *Aspergillus niger* (Taskin at al., 2013), *Rhizopus delemar* (Orikasa and Oda, 2013) and *Aspergillus niveus* (Guimarães et al., 2009). The β-D-fructofuranosidases catalyze the hydrolysis of the sucrose molecule to produce an equimolar mixture of D-glucose and D-fructose, known as invert sugar, which has important properties that are interesting for food and beverage (Alberto et al., 2004). In addition, some microbial β-D-fructofuranosidases are able to perform transfructosilation reaction to produce fructooligosaccharides (FOS). FOS have no considerable caloric value and can be used by diabetic people because they are not metabolized by the human organism. These saccharides also collaborate to reduce the cholesterol and triglycerides levels, and they are beneficial for intestine microorganism (Mussato and Mancilha, 2007).

Production of FOS by filamentous fungi has been mentioned as, for example, by *Aspergillus phoenicis* (Aziani et al., 2012) and *Penicillium expansum* (Prata et al., 2010). Taking into account the importance of the β-fructofuranosidases and the filamentous fungi as source of enzymes for biotechnological application, the search for new fungal strains that are able to produce enzymes with attractive properties is interesting, especially if the biodiversity is con-sidered. The knowledge on fungal diversity and biotechno-logical potential is reduced and deserves attention. Thus, this manuscript describes the optimization, using factorial design approach, of the production process of an extracellular β-D-fructofuranosidase by the filamentous fungus *Aspergillus parasiticus* under submerged fermenta-tion using agro-industrial residues, as well as some enzyme properties of the purified enzyme.

MATERIALS AND METHODS

Microorganism and culture conditions

The filamentous fungus *A. parasiticus* was isolated from Brazilian soil, identified by the Laboratory of Microbiology from Universidade Federal de Pernambuco using morphological analysis and maintained on PDA (Potato Dextrose Agar) slants, at 4°C, in the culture collection from the Laboratory of Microbiology from Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto, University of São Paulo.

The submerged fermentation cultures were obtained by adding 1 mL of aqueous spore suspension (10^5 spores/mL) in 25 mL of Khanna medium (Khanna et al., 1995) in 125 mL Erlenmeyer flasks containing sugarcane bagasse as carbon source, pH 6.0 previously autoclaved at 120°C, 1.5 atmosphere for 30 min. The cultures were maintained at 30°C, under orbital agitation (100 rpm) for different periods as determined for each experiment.

Optimization of the culture condition for extracellular β-D-fructofuranosidase

The influence of different independent variables (Table 1) on the production of the extracellular β-D-fructofuranosidase by *A. parasiticus* was analyzed using the Plackett and Burman (PB) factorial design considering the high (+1) and low (-1) levels for each variable. The matrix was composed by 15 assays (12 factorial assays and 3 central point assays). The independent variables selected using the PB design (temperature and period of cultivation, and proportion of carbon source) were used to perform a central composite rotatable design (CCRD). The matrix was composed by 17 assays (8 factorial assays, six axial assays and three central point assays) (Table 3). For both design analysis, the results were submitted to variance analysis (ANOVA) with *p* value fixed at 0.2 and 0.05 for PB and CCRD, respectively. The analysis and the response surface were obtained using the software Statistica 8.0 (StatSoft).

Obtainment of enzyme extract

The cultures were filtered using filter paper Whatmann no 1 with a vacuum pump. The filtrate without cells was named as extracellular crude extract and it was dialyzed overnight against distilled water at 4°C and used for enzymatic assay and purification procedure.

Determination of the β-fructofuranosidase activity and protein quantification

The β-D-fructofuranosidase activity was determined using sucrose (1%, m/v) as substrate in 100 mM of different buffers (sodium acetate pH 4.0-5.5; MES pH 5.5-7.0; Tris-HCl pH 7.0-9.0; and McIlvaine pH 4.0-7.0) and different temperatures (40-60°C). The reducing sugar was quantified using 3',5'-dinitrosalisilic acid (DNS) (Miller et al., 1959). The absorbance was determined at 540 nm. One unit of enzymatic activity was determined as the amount of enzyme necessary to produce 1 μmol of reducing sugar per minute under the assay condition. The protein was quantified according to Lowry et al. (1951), using bouvine serum albumin (BSA) as standard. The absorbance was determined at 660 nm, and the results expressed as mg of protein per mL of sample.

Purification

The extracellular crude extract was loaded in diethyl aminoethyl (DEAE)-cellulose (1.2 x 11.0 cm) chromatographic column previously equilibrated with 10 mM Tris-HCl buffer, pH 7.0. The 2 mL fractions containing β-D-fructofuranosidase were eluted using a linear gradient of NaCl (0-1 M) in the same buffer at a flow rate of 1.54 mL min^{-1}. These fractions were pooled, dialyzed against distilled water overnight at 4°C, lyophilized, ressuspended in 2 mL of 20 mM Tris-HCl, pH 7.0 plus 100 mM NaCl and loaded in Sephacryl S-200 (1.0 x 80 cm) chromatographic column previously equilibrated with the same buffer. Fractions of 1.0 mL were collected at the flow rate of 0.37 mL min^{-1} and those with activity were pooled, dialyzed against distilled water overnight at 4°C and used for biochemical characterization.

Molecular mass and carbohydrate content determination

The native molecular mass of the extracellular β-D-fructofuranosidase from *A. parasiticus* was determined by gel filtration in Sephacryl S-200 chromatographic column using the same conditions described above. We used β-amylase (200 kDa), alcohol

Table 1. PB matrix with real values and encoded values using eight independent variables and the responses (enzyme activity).

Trial	Independent variable								Response	
	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	U/mL	U/mg of protein
1	7 (+)	30 (-)	150 (+)	1 (-)	0 (-)	0 (-)	1 (+)	96 (+)	13.1	47.5
2	7 (+)	40 (+)	80 (-)	3 (+)	0 (-)	0 (-)	0.001 (-)	96 (+)	7.6	22.2
3	5 (-)	40 (+)	150 (+)	1 (-)	1.5 (+)	0 (-)	0.001 (-)	48 (-)	2.1	7.5
4	7 (+)	30 (-)	150 (+)	3 (+)	0 (-)	1.5 (+)	0.001 (-)	48 (-)	4.7	2.9
5	7 (+)	40 (+)	80 (-)	3 (+)	1.5 (+)	0 (-)	1 (+)	48 (-)	8.7	19.6
6	7 (+)	40 (+)	150 (+)	1 (-)	1.5 (+)	1.5 (+)	0.001 (-)	96 (+)	6.2	3.7
7	5 (-)	40 (+)	150 (+)	3 (+)	0 (-)	1.5 (+)	1 (+)	48 (-)	10.6	6.8
8	5 (-)	30 (-)	150 (+)	3 (+)	1.5 (+)	0 (-)	1 (+)	96 (+)	16.3	25.1
9	5 (-)	30 (-)	80 (-)	3 (+)	1.5 (+)	1.5 (+)	0.001 (-)	96 (+)	6.6	5.1
10	7 (+)	30 (-)	80 (-)	1 (-)	1.5 (+)	1.5 (+)	1 (+)	48 (-)	13.5	10.2
11	5 (-)	40 (+)	80 (-)	1 (-)	0 (-)	1.5 (+)	1 (+)	96 (+)	9.3	5.6
12	5 (-)	30 (-)	80 (-)	1 (-)	0 (-)	0 (-)	0.001 (-)	48 (-)	8.8	29.6
13	6 (0)	35 (0)	115 (0)	2 (0)	0.8 (0)	0.8 (0)	0.5 (0)	72 (0)	18.7	32.1
14	6 (0)	35 (0)	115 (0)	2 (0)	0.8 (0)	0.8 (0)	0.5 (0)	72 (0)	18.9	27.1
15	6 (0)	35 (0)	115 (0)	2 (0)	0.8 (0)	0.8 (0)	0.5 (0)	72 (0)	20.2	29.4

X_1, pH; X_2, temperature (°C); X_3, rpm; X_4, sugar cane bagasse (%, m/v); X_5, KH_2PO_4 (%, m/v); X_6, peptone (%, m/v); X_7, spores (10^7); X_8, time of cultivation (h).

dehydrogenase (150 kDa), BSA (66 kDa), egg albumin (43 kDa) and carbonic anhydrase (29 kDa) as molecular markers. The molecular mass was also determined under denaturing condition (7% sodium dodecyl sulfate poly-acrylamide gel electrophoresis (SDS-PAGE). Macroglobulin (169 kDa), β-galactosidase (112 kDa), lactoferrin (92 kDa), piruvate kinase (67 kDa), fumarase (60 kDa), lactic dehydrogenase (36 kDa) and triose phosphate isomerase (31 kDa) were used as mass molecular markers. The carbohydrate content was determined according to Dubois et al. (1956) using mannose as standard.

Determination of optimum of temperature and pH of activity and stability

The best conditions of temperature and pH of activity for extracellular β-D-fructofuranosidase from *A. parasiticus* were determined using an experimental design 2^2, where temperature and pH were considered as independent variables. We performed 11 assays (four factorial assays, four axial assays and three at the central point assays). The analysis of variance was carried with *p* value fixed at 0.05 using the software Statistica 8 (StatSoft).

The thermal stability was determined by the incubation of enzyme in aqueous solution at different temperatures (50, 60 and 70°C) and periods (0 - 60 min). The stability to pH was determined using different buffers (100 Mm) (KCl-HCl - pH 2.0; citrate - pH 3.0 and 4.0; sodium acetate - pH 5.0; phosphate - pH 6.0 and 7.0; Tris-HCl - pH 8.0 and 9.0; glicine-NaOH - pH 10.0) and periods (0, 30 and 60 min). Samples were taken off at a pre-determined time, kept in ice bath and after assayed for enzymatic activity as described previously using 200 µL of purified enzymatic sample (10 µg/mL).

Influence of different compounds and salts on enzyme activity

The influence of different compounds (β-mercaptoethanol, ethylenediaminetetraacetic acid (EDTA) e urea) and salts ($AgNO_3$, $BaCl_2$, $CaCl_2$, $CoCl_2$, $CuSO_4$, $FeCl_3$, $FeSO_4$, KCl, $MgCl_2$, $MgSO_4$, NaCl, NaF NH_4Cl, $ZnCl_2$ e $ZnNO_3$) on the extracellular β-D-fructofuranosidase activity was investigated by adding of 1 mM of each in the enzymatic assay and the activity was determined as described previously using 200 µL of purified enzymatic sample (10 µg/mL).

Determination of kinetic parameters

The kinetic parameters V_{max}, $K_{0.5}$ and $V_{max}/K_{0.5}$ were determined using both sucrose and raffinose (0.25 - 100 mM) as substrates in 100 mM sodium acetate buffer pH 5.5, at 50°C. A sigmoid plot was obtained using the software Origin 8 (Origin Lab).

RESULTS AND DISCUSSION

Optimization of culture conditions for β-D-fructofuranosidase production

The enzyme production by microorganisms is directly influenced by the culture conditions, for example, temperature and period of cultivation, pH of the culture medium and carbon source, among others. The statistic methodology, as experimental design, is a powerful tool to optimize the culture conditions for enzymatic production by filamentous fungi. The PB matrix obtained for optimization of the extracellular β-D-fructofuranosidase production by *A. parasiticus* is presented in Table 1. Considering the enzyme production, the best condition was obtained at the assay 15, with 20.2 U mL^{-1}. According to the statistical analyses with *p* value fixed at 0.05, the influences of independent variables temperature (X_2), proportion of KH_2PO_4 (X_5) and peptone (X_6) and number of spores used (X_7), were significant ($p < 0.05$)

Table 2. Effects analyses for the β-fructofuranosidase activity (U/mg of protein).

Variable	Effect	Standard error	t(21)	*p*-value
Media	18.7	1.5	12.8	0 [A]
X_1	3.7	3.3	1.1	0.272
X_2	-11	3.3	-3.4	0.003 [A]
X_3	0.2	3.3	0	0.964
X_4	-5.2	3.3	-1.6	0.127 [B]
X_5	-7.5	3.3	-2,3	0.032 [A]
X_6	-19.7	3.3	-6	0.000 [A]
X_7	7.2	3.3	2.2	0.039 [A]
X_8	4.1	3.3	1.3	0.221

A, Significant parameters ($p < 0.05$); B, significant parameters ($p < 0.15$).

Table 3. Matrix and responses for 2^3 experimental design with real and encoded values.

Trial	Independent variable			Response	
	X_2	X_4	X_8	U/mL	U/mg of protein
1	27 (-)	0.9 (-)	43 (-)	5.7	35.2
2	27 (-)	0.9 (-)	101 (+)	6.9	48.0
3	27 (-)	2.1 (+)	43 (-)	7.5	35.8
4	27 (-)	2.1 (+)	101 (+)	9.0	32.0
5	33 (+)	0.9 (-)	43 (-)	6.1	42.9
6	33 (+)	0.9 (-)	101 (+)	6.8	39.5
7	33 (+)	2.1 (+)	43 (-)	6.9	26.2
8	33 (+)	2.1 (+)	101 (+)	9.0	26.1
9	25 (-1.68)	1.5 (0)	72 (0)	7.7	30.3
10	35 (+1.68)	1.5 (0)	72 (0)	6.7	25.9
11	30 (0)	0.5(-1.68)	72 (0)	5.2	43.2
12	30 (0)	2.5 (+1.68)	72 (0)	8.7	31.3
13	30 (0)	1.5 (0)	24 (-1.68)	7.1	27.9
14	30 (0)	1.5 (0)	120 (+1.68)	9.6	30.5
15	30 (0)	1.5 (0)	72 (0)	7.7	29.9
16	30 (0)	1.5 (0)	72 (0)	7.7	30.6
17	30 (0)	1.5 (0)	72 (0)	7.7	31.4

X_2, Temperature (°C); X_4, sugar cane bagasse (% m/v); X_8 - time of cultivation (h).

with R^2 of 0.75 (Table 2). Only the variable X_7 showed a positive effect, suggesting that an increase in the number of spores would be favorable to the enzyme production. The variables X_2, X_5 and X_6 showed negative effect. Then, the variables X_2 (temperature) and X_4 (sugar cane bagasse) were selected for CCRD despite the effect of the latter to be considered not significant. In addition, the variable X_8 (period of cultivation) was also included because the production of metabolites by microorganisms is influenced by this aspect, what has been demonstrated by a lot of works. For CCRD the variables X_5 (KH_2PO_4), X_6 (peptone) and X_7 (number of spores) were not considered.

The matrix obtained for CCRD is presented in Table 2. Taking into account the R^2 value of 0.92, the influences of all variables were significant. The variable sugarcane bagasse (X_4) was significant in both levels, linear and quadratic while the temperature (X_2) was only in the quadratic level and the period of cultivation (X_8) was only in linear level (Table 4). The main interactions as well as the second-order interactions observed for experimental design are very important for the understanding of the behavior of the process (especially, in this case, the fermentative process).

Considering the variance analysis, the *F*-test showed that the model is predictive and the calculated *F* value (34.7) was 10-fold higher than the tabled *F* value (3.26). According to that, a global model of second order for β-D-fructofuranosidase production was established as function of these three variables (Equation 1). The reduced model

Table 4. Determination of coefficients of regression for the β-fructofuranosidase production.

Variable	Coefficient	Standard error	t(7)	*p*-value
Media	7.7	0.18		
X_2 (L)	-0.15	0.09	-1.8	0.1122 [B]
X_2 (Q)	-0.2	0.09	-2.4	0.0482 [A]
X_4 (L)	0.95	0.09	11	0 [A]
X_4 (Q)	-0.3	0.09	-3.4	0.0111 [A]
X_8 (L)	0.7	0.09	8.1	0.0001 [A]
X_8 (Q)	0.2	0.09	1.9	0.0974 [B]
$X_2.X_4$	-0.1	0.11	-1	0.3364
$X_2.X_8$	0.05	0.11	0.2	0.8214
$X_4.X_8$	0.2	0.11	1.8	0.1149 [B]

A, Significant parameters ($p < 0.05$); B, significant parameters ($p < 0.15$).

Table 5. Purification of the extracellular β-fructofuranosidase from *A. parasiticus*.

Step	Volume (mL)	Activity (Total U)	Protein (Total mg)	U/mg of protein	Yield (%)	Purification factor (fold)
Crude extract	350	1521	123	12.4	100	1
DEAE-cellulose	43	828	13	63.7	54.4	5.1
Sephacryl S-200	16	237	0.16	1481.2	15.6	119.4

was obtained using only the significant level of each variable (equation 2).

$$U/mL = 7.7 - 0.15X_2 - 0.2X_2^2 + 0.95X_4 - 0.3X_4^2 + 0.7X_8 + 0.2X_8^2 - 0.1X_2X_4 + 0.05X_2X_8 + 0.2X_4X_8 \quad (1)$$

$$U/mL = 7.7 - 0.2\,X_2^2 + 0.95\,X_4 - 0.3\,X_4^2 + 0.7\,X_8 \quad (2)$$

Using the reduced model, the surface response was obtained (Figure 1). The high level of enzyme production (> 8 U mL^{-1}) was obtained when we used 1.5-2.5% (m/v) sugar cane bagasse and temperature from 27 to 30°C (Figure 1A). When the period of cultivation was considered, the best results were obtained between 101 and 120 h in the same temperature interval (Figure 1B). Considering the proportion of the sugar cane bagasse and the period of cultivation (Figure 1C), the best production was obtained in the same intervals cited above. According to these observations, the reduced model obtained was validated using the encoded levels 0 (30°C) for temperature, 0 (1.5 m/v) for sugar cane bagasse and -1.68 (24 h) for period of cultivation.

Under these conditions, we obtained 6.92 U mL^{-1} while the calculated value (using the equation) was 7.08 U mL^{-1}. Then, the temperature of 30°C, proportion of sugar cane at 1.5% (m/v) and period of cultivation of 24 h were selected for enzyme production by *A. parasiticus* aiming at the puri-fication and characterization. Many studies determined the best culture conditions for β-D-fructofuranosidase production using one-factor analysis as presented by Alegre et al. (2009), which observed a temperature of 40°C and period of 72 h for cultivation of *Aspergillus caespitosus*. The same was observed for enzyme pro-duction by *A. niveus* and *A. phoenicis* (Rustiguel et al., 2010; Guimarães et al., 2009). The use of experimental design allows one to observe the interaction of factors, what does not occur when one factor analysis is used.

The use of agro industrial residues as sugar cane bagasse to produce biomolecules is interesting and has attracted the attention from different sectors. Many authors have mentioned the use of soybean meal, wheat bran and sugar cane bagasse as carbon sources for β-D-fructofuranosidase production by filamentous fungi (Guimarães et al., 2009; Alegre et al., 2009; Giraldo et al., 2011).

Purification

The extracellular crude extract obtained under the optimized condition was submitted to two chromategraphic steps. The β-fructofuranosidase was purified 119-fold with 16% recovery (Table 5). According to this procedure, it is possible to obtain around 10 mg/L of β-fructofuranosidase. The native molecular mass estimated by Sepha-cryl S-200 was 136 kDa, while a molecular mass of 63 kDa was obtained under denaturating condition

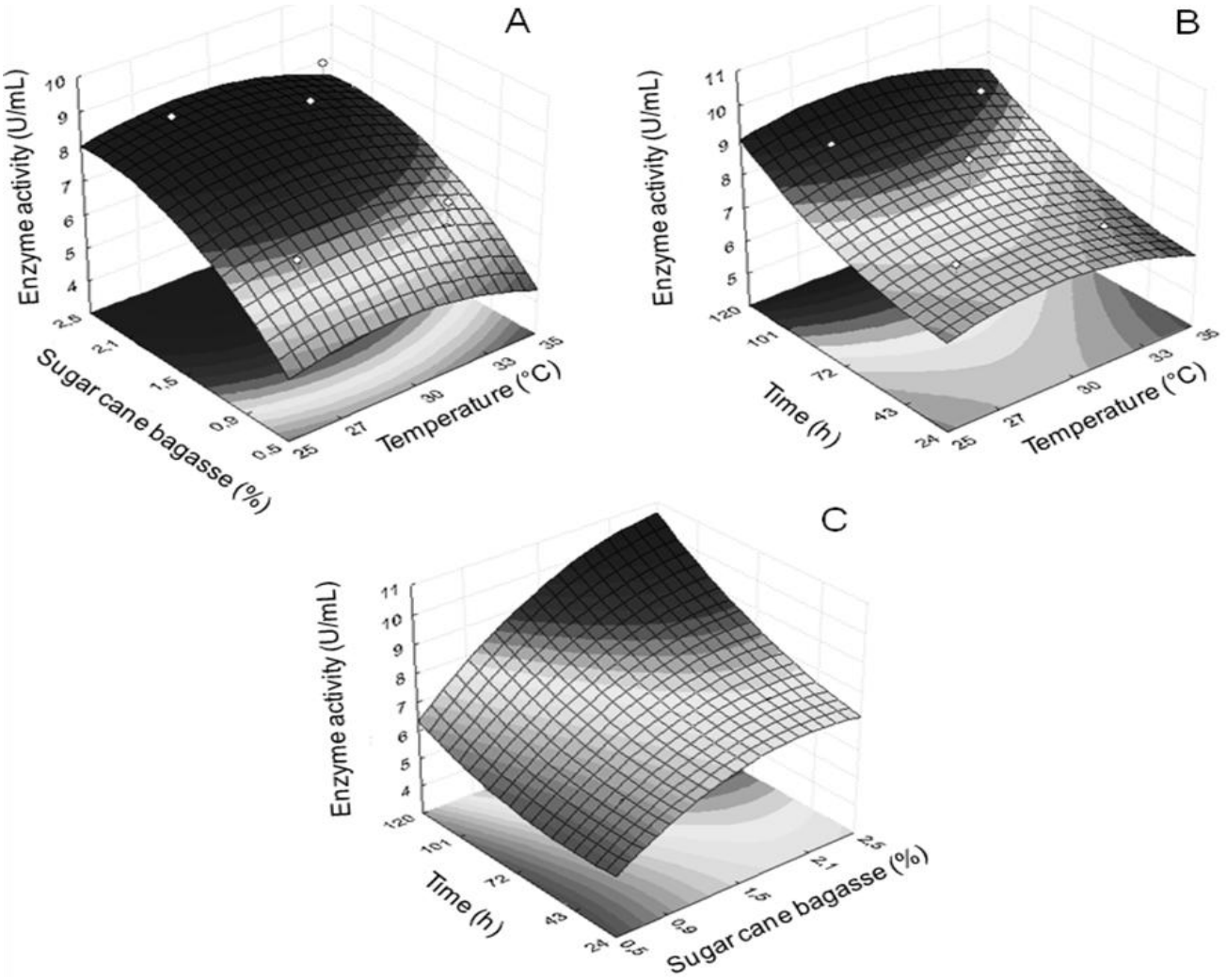

Figure 1. Surface responses for the extracellular β-fructofuranosidase production by *A. parasiticus* as function of temperature and proportion of sugar cane bagasse (A), temperature and time of cultivation (B), and proportion of sugar cane bagasse and time of cultivation (C).

(SDS-PAGE) (Figure 2), indicating a homodimeric structure with 64% carbohydrate content. The β-fructofuranosidases from *Aspergillus ochraceus* and *A. phoenicis* were also characterized as homodimers (Rustiguel et al., 2010; Guimarães et al., 2007). The carbohydrate content was higher than that observed for the enzymes produced by other filamentous fungi as, for example, *A. niger* (17%) (Nguyen et al., 2005) and *A. ochraceus* TS (30%) (Gosh et al., 2001), among others.

Optimization of the temperature and pH of reaction and stability

The influence of independent variable temperature (X_1) and pH (X_2) on the extracellular β-fructofuranosidase activity was analyzed using experimental design (Table 6). The highest levels of enzymatic activity were obtained when the reaction was conducted at 45°C and pH 5.0, which were defined as central point levels. The influence of both variables was significant in the linear and quadratic levels with *p* value fixed at 0.05 and with R^2 value of 0.96 (Table 7). According to the F-test, the calculated *F* value (135.7) was 52-fold higher than the tabled *F* value (2.57), allowing the obtainment of the second-order model, that was significant and predictive for *A. parasiticus* β-fructofuranosidase activity (equation 3).

$$\text{Relative activity (\%)} = 99.3 + 4.2X_1 - 21.9X_1^2 + 13.1X_2 - 37X_2^2 + 21.6X_1X_2 \quad (3)$$

The analysis of the response surface plot shows that the highest enzyme activity can be obtained between 38 and

Table 6. CCRD matrix using real and encoded values for relative β-fructofuranosidase activity.

Trial	X_1 (°C)	X_2 (pH)	Relative activity (%)
1	34 (-)	3.6 (-)	46.9
2	34 (-)	6.4 (+)	33.8
3	56 (+)	3.6 (-)	11.3
4	56 (+)	6.4 (+)	90.5
5	30 (-1.41)	5 (0)	22.2
6	60 (+1.41)	5 (0)	54.4
7	45 (0)	3 (-1.41)	5.6
8	45 (0)	7 (+1.41)	35.1
9	45 (0)	5 (0)	99
10	45 (0)	5 (0)	99.5
11	45 (0)	5 (0)	100

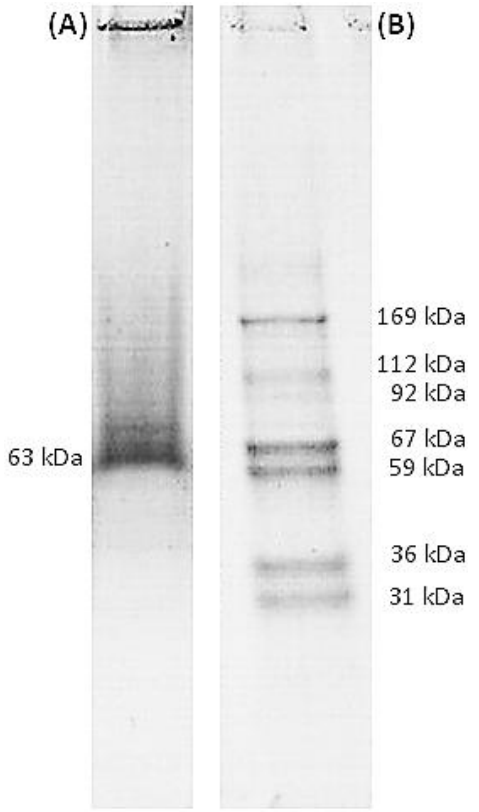

Figure 2. 7% Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Lane A, β-fructofuranosidase; Lane B, molecular mass markers.

56°C, and pH from 4.5 to 6.2 (Figure 3). Generally, the influence of temperature and pH on enzyme activity has been determined using the one-factor approach, but the experimental design is a powerful approach that also allows the analysis of interaction of these both variables. The values of temperature obtained were similar if compared to the enzymes from *A. caespitosus* (Alegre et al., 2009) and *A. niger* PSSF21 (Reddy et al., 2010), but higher than that observed for the enzymes produced by *Fusarium solani* (Bhatti et al., 2006) and *A. niger* IMI303386 (Nguyen et al., 2005). In addition, the pH values were similar to the most fungal β-fructofuranosidases as, for example, from *Termitomyces clypeatus* (Chowdhury et al., 2009).

The extracellular β-fructofuranosidase produced by *A. parasiticus* had a half-life (t_{50}) of 25 min at 50°C, but when the temperatures of 60 and 70°C are considered, the t_{50} was reduced to 6 min (Figure 4). On the other hand, the enzyme was fully stable at temperatures below 50°C. This aspect is very interesting because the hydrolytic process can be conducted in mild conditions allowing the energy economy. Additionally, the enzyme was stable to wide pH range (5.0 to 10.0) as also observed for enzymes from *A. niger* IMI303386 (Nguyen et al., 2005) and *A. niger* AS0023 (L'Hocine et al., 2000). Modifications in the pH values of the enzyme environment can promote modifications in the lateral chains of the amino acids interfering with the protein conformation and consequently with the catalytic activity. Thus, an enzyme that is more resistant to pH variation is a good option for different biotechnological application.

Influence of different compounds on enzyme activity

The extracellular β-fructofuranosidase activity from *A. parasiticus* was increased in the presence of Ba^{2+} (+29%) and Mg^{2+} (+22%) (Data not shown). On the other hand, when $CuSO_4$ and $CoCl_2$ were used, the enzyme activity was reduced around 77 and 40%, respectively. Interesting, the enzyme activity was preserved in the presence of β-mercaptoethanol that are able to act on disulfide bonds, EDTA that is chelant of divalent cations, urea that is responsible to denature proteins and $AgNO_3$ that is able to precipitate proteins. Increase in the enzyme activity by addition of Ba^{2+} and Mg^{2+} was also observed for *Penicillium variotti* (Giraldo et al., 2011) and *A. niveus*

Table 7. Determination of the coefficients of regression for relative β-fructofuranosidase activity.

Variable	Coefficient	Standard error	t (27)	*p*-value
Media	99.3	2.42	41	0 [A]
X_1 (L)	4.2	1.48	3	0,008 [A]
X_1 (Q)	-21.9	1.76	-12	0 [A]
X_2 (L)	13.1	1.48	9	0 [A]
X_2 (Q)	-37	1.76	-21	0 [A]
$X_1.X_2$	21.7	2.09	10	0 [A]

A, Significant parameters ($p < 0.05$).

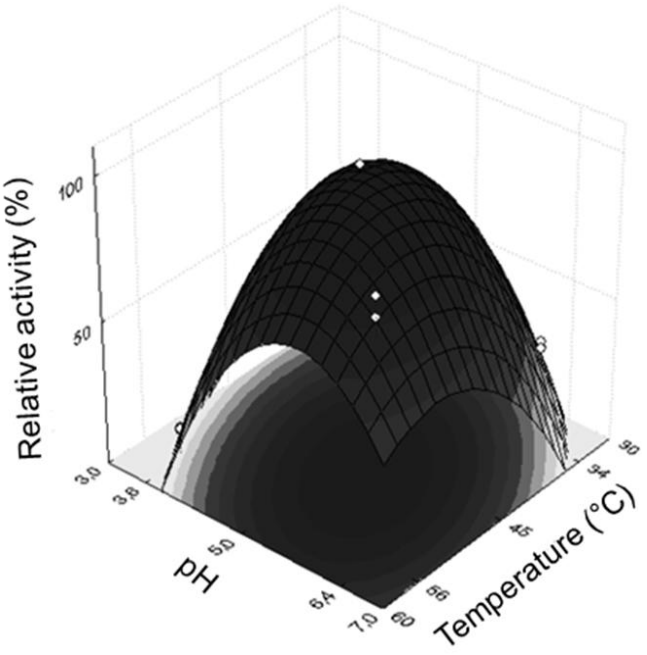

Figure 3. Response surface for the extracellular β-fructofuranosidase activity as function of temperature and pH.

enzymes (Guimarães et al., 2009). The enzyme produced by *A. phoenicis* had its activity increased by the addition of Ag^+, with suggestion of a new group of fructofuranosidases activated by silver (Rustiguel et al., 2010).

Determination of kinetic parameters

The extracellular β-fructofuranosidase from *A. parasiticus* was able to hydrolyze sucrose, inulin and raffinose, as well as the mixture of theses substrates (data not shown). The best activity was observed for the sucrose and raffinose mixture (1:1) (16.6 U/mL). The experimental values of hydrolysis obtained for the mixtures were approximately similar to that obtained for the hypothetical sum of the individual values observed for each substrate, indicating the possibility of the existence of different catalytic sites. Another aspect that should be highlighted is the hydrolysis on inulin. There is a wide discussion on the nature of the enzyme, that is, if β-D-fructofuranosidase or if inulinase. Many authors have considered the relation between the hydrolysis of sucrose on inulin (S/I value) to define the nature of the enzyme. The S/I value for *A. parasiticus* extracellular enzyme was 7.3, higher than that obtained for *A. phoenicis* enzyme (Rustiguel et al., 2010), indicating the enzyme as β-fructofuranosidase.

However, the S/I value cannot be considered as isolated for the distinction of the enzymes, but also kinetic and structural studies. The S/I value also depends on the inulin source used. Taking into account that the best hydrolytic activity was obtained on sucrose, the kinetic parameters using this substrate were determined. The purified extracellular enzyme showed $K_{0.5}$ of 10 mM, V_{max} of 1565 U/mg of protein and $V_{max}/K_{0.5}$ of 156.5 U/ mg of protein mM^{-1}. When used raffinose as substrate, the $K_{0.5}$ was 19 mM, with V_{max} of 1965 U/mg of protein and $V_{max}/K_{0.5}$ of 103.4 U/mg of protein mM^{-1}. For both substrates the coefficient of Hill (n) was higher than 1.0, indicating a positive cooperation.

Thus, the extracellular β-fructofuranosidase from *A. parasiticus* showed higher affinity by sucrose than by raffinose. The affinity by the former was higher than that observed for the enzymes produced by *A. ochraceus* (Guimarães et al., 2007), *A. phoenicis* (Rustiguel et al., 2010) and *A. pullulans* (Yoshikawa et al., 2006).

Conclusion

The filamentous fungi are important sources of bio-molecules with biotechnological potential as enzymes. The production of extracellular β-fructofuranosidase by *A. parasiticus* was optimized using an experimental design, as well as the temperature and pH of the activity. This is the first time that the experimental design was used to analyze the influence of temperature and pH on fungal β-fructofuranosidase activity.

The homodimeric glycoprotein showed attractive characteristics for application, as wide range of pH stability and mild conditions of temperature, minimizing the energetic expense.

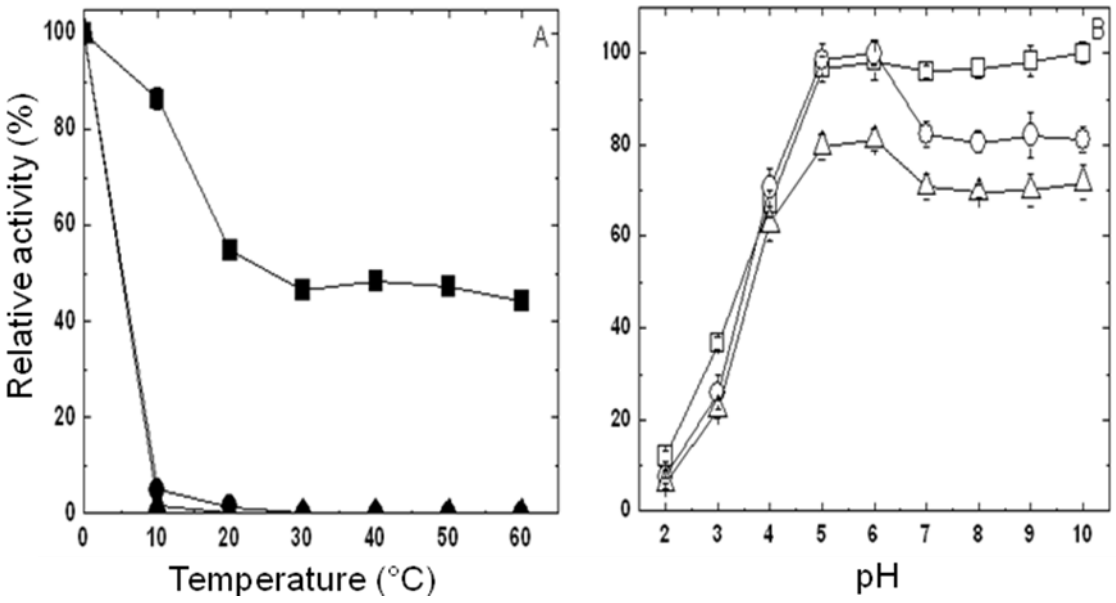

Figure 4. Thermal stability (A) at 50°C (■), 60°C (●) and 70°C (▲), and stability to pH (B) for 0 (□), 30 (○) e 60 (∆) minutes for extracellular β-fructofuranosidase activity.

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