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β-Fructofuranosidase production by *Aspergillus versicolor* isolated from Atlantic forest and grown on apple pomace

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This study explores the production and characterization of an extracellular β-fructofuranosidase (FFase-I) by *Aspergillus versicolor* newly isolated from Atlantic Forest-Brazil. The β-fructofuranosidase production by fungus, after the optimization process using central composite design and response surface methodology, showed that 3% (w/v) apple pomace, an initial pH 7.5, and 12 days of cultivation provided the best conditions. The β-fructofuranosidase (FFase-I) was purified from the crude extract by 75% $(NH_4)_2SO_4$ precipitation followed by DEAE-Sephadex, and the molecular mass of the FFase-I was estimated to be 75 kDa by SDS-PAGE. Furthermore, the enzyme exhibited unusual tolerance to Cu^{2+}, sodium dodecyl sulfate (SDS), Tween 80, ethanol and acetone. The purified enzyme had an optimum pH of 6.0 and was stable over an acidic pH range of 3.0–6.0. The optimum temperature of the FFase-I was 55°C but was stable at 60°C for 7 h. Thus, the β-fructofuranosidase *A. versicolor* which has thermal stability and activity under acidic conditions would have potential application in sugar cane molasses hydrolysis for subsequent ethanol production.

Key words: *Aspergillus,* factorial design, fruit wastes, invertase.

INTRODUCTION

β-Fructofuranosidase (EC. 3.2.1.26), or invertase, catalyzes the irreversible hydrolysis of β-1,2-sucrose to

produce an equal mixture of glucose and fructose (Klotz and Campbell, 2004). This enzyme has been grouped in the glycoside hydrolase (GH) 32 family according to Carbohydrate-Active enZYmes Database (Lombard et al., 2014). This group of enzymes, together with the GH 68 family, is included in the GH-J clan. This family includes enzymes that catalyze the release of β-fructose from the non-reducing termini of various β-D-fructofuranoside substrates, such as invertases or β-fructofuranosidases; inulinases and levanases, which act on sucrose; raffinose; and inulin and levans, respectively (Álvaro-Benito et al., 2007; Kadowaki et al., 2013). Additionally, some β-fructofuranosidase enzymes have fructosyltransferase activity, resulting in the production of fructooligosaccharides (FOS), which are used commercially as prebiotics due to their physiological properties (Álvaro-Benito et al., 2007). This enzyme is also used extensively in lactic acid production, the fermentation of cane sugar molasses into ethanol, and calf feed production (Gehlawat, 2001; Rashad and Nooman 2009). β-Fructofuranosidase is also employed in the pharmaceutical industry in digestive aid tablets and powdered milk for infant food (Uma et al., 2012; Andrades et al., 2015). β-Fructofuranosidases from many microorganisms have been studied, including *Saccharomyces cerevisiae* (Andjelkovic et al., 2012), *Pichia anomala* (Rodriguez et al., 1995), *Aspergillus ochraceus* (Guimarães et al., 2007), *Aspergillus niveus* (Guimarães et al., 2009) and *Aspergillus niger* (Madhan et al., 2010).

Enzyme production through bioprocess technology is influenced by several variables, such as temperature, pH, cultivation time, and carbon and nitrogen sources present in the medium. Thus, optimization of culture conditions for enzyme production using a statistical approach is one of the first steps to develop a low-cost fermentation process. One of the advantages of this method is the possibility of observing the interactions between the variables and their effects on the response (Cui and Zhao, 2012).

In recent years, several lignocellulosic substrates have been explored in bioprocess technology for enzyme production or different bioactive molecules of biotechnological interest. The use of agro-industrial wastes as an alternative carbon source can reduce the cost of enzyme production by microorganisms. In general, sucrose has been reported in most studies as the best inducer for β-fructofuranosidase (FFase) production, but the use of agricultural residues, municipal solid wastes, and different sources of lignocellulosic biomass could reduce the cost of FFase production (Hayashi et al., 1992). Thus, investigations with unconventional substrates or agro-industrial waste using statistical tools can lead to the development of low cost bioprocessing applications. Hence, in this study, the apple pomace, a byproduct generated from fruit-processing industries, was investigated as a carbon source for β-fructofuranosidase production.

Brazil is among the nine biggest apple producers in the world, with a production of around 1.3 million tons of apples in 2011 (MAPA, 2013); the Brazilian apple production sector provides the raw material for juice and wine processing, releasing a significant amount of apple pomace (20–35% of the production). Generally, this waste is discharged into the soil as organic fertilizer or used as animal feed.

However, apple pomace is an interesting raw material, and it has attracted considerable attention as a potential source of sugar, dietary fiber, pectin, and citric acid flavoring, including for biotechnological applications (Canteri et al., 2012). *Aspergillus versicolor* has been reported to degrade compounds in environmental pollutants (Zhao et al., 2005) and to be a producer of xylanolytic enzyme complexes (Carmona et al., 1998). Thus, a central composite rotational design (CCRD)–based response surface methodology (RSM) approach was used to optimize cultivation conditions. Furthermore, a partial purification and characterization of an extracellular β-fructofuranosidase from *A. versicolor* isolated from the Atlantic Forest biome-Brazil was performed.

MATERIALS AND METHODS

Strain isolation and identification

A. versicolor was isolated from decaying plants obtained from the Bela Vista Biological Refuge in Foz do Iguaçu, Paraná, Brazil. Strain identification was based on an analysis of the internal transcribed spacer (ITS) regions of the ribosomal DNA gene (White et al., 1990). Genomic DNA of the fungus was extracted from mycelium obtained from submerged fermentation supplemented with 1% (w/v) glucose after 30 h of culture. The mycelium was collected by centrifugation (5,000 × *g*, 10 min), and the pellet obtained was then used for DNA extraction using the hexadecyltrimethylammonium bromide method. The primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3' forward) and ITS4 (5'-TCCTCCGCTTATTGATATGC-3' reverse) were used to amplify the ITS1, ITS2, and 5.8S regions (White et al., 1990). The sequence determined was compared with other fungi sequences deposited in the National Center for Biotechnology Information (NCBI) databank using the Basic Local Alignment Tool (BLAST; http://www.ncbi.nlm.nih.gov). A neighbor-joining phylogenetic tree was constructed from the sequence of the ITS regions of *A.*

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Table 1. Experimental design matrix and results of β-fructofuranosidase production by *A. versicolor*

Run	Variables			Enzymatic activity (U mL^{-1})	
	X1 Apple pomace (%)	X2 Cultivation time (day)	X3 Initial pH	Experimental	Predicted
1	1 (-1)	4 (-1)	4.5 (-1)	0.50	3.31
2	3 (1)	4 (-1)	4.5 (-1)	1.37	0.03
3	1 (-1)	12 (1)	4.5 (-1)	23.83	23.93
4	3 (1)	12 (1)	4.5 (-1)	64.75	65.76
5	1 (-1)	4 (-1)	7.5 (1)	1.08	8.52
6	3 (1)	4 (-1)	7.5 (1)	2.25	5.25
7	1 (-1)	12 (1)	7.5 (1)	26.58	29.14
8	3 (1)	12 (1)	7.5 (1)	77.92	70.97
9	0.32 (-1.68)	8 (0)	6 (0)	0.75	-5.89
10	3.68 (1.68)	8 (0)	6 (0)	22.92	26.50
11	2 (0)	1 (-1.68)	6 (0)	1.50	-10.15
12	2 (0)	15 (1.68)	6 (0)	65.00	62.38
13	2 (0)	8 (0)	3.5 (-1.68)	37.33	36.84
14	2 (0)	8 (0)	8.5 (1.68)	48.17	45.60
15	2 (0)	8 (0)	6 (0)	21.67	26.11
16	2 (0)	8 (0)	6 (0)	23.55	26.11
17	2 (0)	8 (0)	6 (0)	26.51	26.11
18	2 (0)	8 (0)	6 (0)	20.95	26.11

versicolor using MEGA 6 (Tamura et al., 2013).

Culture conditions

A. versicolor was grown in 125-mL Erlenmeyer flasks containing 25 mL of sterile mineral medium comprised of the following (g/L): $NaNO_3$, (3), KH_2PO_4, (1), $MgSO_4.7H_2O$ (0.5), KCl (0.5), $Fe_2(SO_4)_3$ (0.01) and yeast extract (1), supplemented with apple pomace at different concentrations. The initial pH of the medium ranged from 3.5 to 8.5 and was adjusted as shown in the experimental design CCRD- 2^3 (Table 1). The apple pomace, used as carbon source, was obtained from local markets in Cascavel, Paraná, Brazil. The fungal spores were inoculated (10^5 conidia ml^{-1}), and cultures were incubated under static conditions at 28°C for varying times (Table 1). The submerged cultures were harvested under vacuum filtration using Whatman filter paper No. 1. The supernatant obtained was used as source of extracellular β-fructofuranosidase.

β-Fructofuranosidase production from *A. versicolor* by experimental design

A CCRD 2^3 was performed using three independent variables (initial pH of the medium, cultivation time and apple pomace concentration). The CCRD contained a matrix with 18 experimental runs, which included eight runs for factorial design, indicated by lowest (-1) and highest (+1) levels, six runs for axial points (two for each variable, [-1.68] and [+1.68]) and four replicates at the central point (0). The β-fructofuranosidase activity from *A. versicolor* was determined as response (Table 1). The mathematical relationship of response *Y* and the three variables X was determined by polynomial model Equation 1:

$$Y=\beta_0+\beta_1x_1+\beta_2x_2+\beta_3x_3+\beta_{12}x_1x_2+\beta_{13}x_1x_3+\beta_{23}x_2x_3+\beta_{11}x_1^2+\beta_{22}x_2^2+\beta_{33}x_3^2 \quad (1)$$

Where, Y represents the predicted response; β_0 is the intercept; β_1, β_2 and β_3 are the linear coefficients; β_{11}, β_{22} and β_{33} are the quadratic coefficients and β_{12}, β_{13} and β_{23} are the interaction coefficients. The data obtained from response surface methodology (RSM) for the β-fructofuranosidase production were subjected to analysis of variance (ANOVA) using the statistical software Statistica, v. 10. The accuracy and general ability of the above polynomial model were evaluated using the determination coefficient (R^2).

Enzyme assay and Protein quantification

The β-fructofuranosidase activity was determined using 200 mM sucrose as the substrate in sodium phosphate buffer (100 mM, pH 6.0) incubated with the extract for 10 min at 60°C. The amounts of reducing sugars were determined using dinitrosalicylic acid, according to Miller (1959). One unit of enzyme activity was defined as the amount of enzyme necessary to produce 1 µmol of glucose per minute under the assay conditions.

The protein was quantified using the Bradford (1976) method using bovine serum albumin as a standard, and absorbance of 280 nm was used for monitoring the protein in the column eluates.

Extracellular β-fructofuranosidase purification from *A. versicolor*

The crude extracts of *A. versicolor* were concentrated with $(NH_4)_2SO_4$ at 75% (w/v) and centrifuged at 5,478 x*g* for 30 min at 4°C. Then, the pellet was resuspended in Tris-HCl buffer (20 mM, pH 7.5) and dialyzed against the same buffer for 18 h at 4°C, loaded onto a DEAE-Sephadex chromatographic column (10 × 2.0 cm) and eluted using a linear gradient of NaCl (0 - 1.0 M). Three-milliliter fractions were collected at flow rate of 1 mL min^{-1}, and those with the highest β-fructofuranosidase activity were pooled, dialyzed overnight using distilled water at 4°C, lyophilized and used for biochemical characterization.

SDS-PAGE and zymogram

FFase-I from DEAE-Sephadex was analyzed using 10% SDS-PAGE, according to Laemmli (1970). The gel was stained with Coomassie blue R250 using the molecular mass markers (GE®), consisting of phosphorylase b (97 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa) and α-lactalbumin (14.4 kDa). Zymogram was obtained using the same SDS-PAGE procedure and stained according to methodology of Rehm et al. (1998). The gel was soaked for 30 min in Triton X-100 (0.5 %) at room temperature, rinsed with distilled water and incubated in 0.3 M sucrose dissolved in phosphate buffer (50 mM, pH 6.0) for 30 min at 40°C. Then, the gel was stained with 1% triphenyltetrazolium chloride dissolved in 0.25 M NaOH and incubated at 100°C. Acetic acid (5% solution) was added to stop the enzymatic reaction.

Effect of pH and temperature on enzyme activity and stability

The effect of pH was evaluated under standard assay conditions with 0.2 M sucrose dissolved in different pH values ranging from 3.0 to 7.5 of McIlvaine buffer. pH stability was determined by measuring the residual activity after the enzyme maintained for 48 h in different range of McIlvaine buffer (3.0, 4.0, 5.0 and 6.0).

The effect of temperature on the FFase-I activity was determined by performing a standard assay at temperature ranging from 40 to 80°C. In the thermal inactivation experiments, the enzyme was incubated at different temperatures (40, 50, 55, 60, 65 and 70°C) for up to 12 h. Residual enzyme activities were determined under standard assay conditions. All of these assays were repeated three times, and the results are expressed as relative percentages when compared with the highest value.

Effects of metal ions and chemical reagents on FFase-I activity

The effects of the following salts on FFase-I activity were tested: KCl, $MgSO_4$, NaCl, $SnCl_2$, $BaCl_2$, $(NH_4)_2SO_4$, $CaCl_2$, $HgCl_2$, $CuSO_4$ and $FeSO_4$. Additionally, the effects of the following compounds on FFase-I activity was tested: EDTA, sodium dodecyl sulfate (SDS), Tween 80, ethanol and acetone. The different compounds (1 mM or 5 mM) were pre-incubated for 15 min with the enzyme. After incubation, an aliquot was withdrawn and chilled on ice, and the hydrolytic activity was determined using the standard assay with sucrose as the substrate.

Kinetic parameters (K_m and V_{max}) determination

The kinetic parameters of the FFase-I were determined using sucrose as the substrate ranging from 5 to 70 mM. The K_m and V_{max} values were determined using the Lineweaver–Burk plot.

RESULTS AND DISCUSSION

Strain identification

The fungus was isolated from the Atlantic Forest biome in Brazil. The strain was identified both by its morphological characteristics and the sequencing of the ITS regions of the ribosomal DNA. The 508-bp fragment of the sequence showed 100% identity with other *A. versicolor* strains. This sequence was deposited in the GenBank of the National Center for Biotechnology Information (NCBI) under accession number KM396917. A phylogenetic tree was constructed based on the alignment of the sequences from the ribosomal genes from some *Aspergillus* species. This isolate (*Aspergillus versicolor* FT-5) is grouped with other *A. versicolor* strains (Figure 1).

Optimization of β-fructofuranosidase production using experimental design

The design matrix (cultivation time, carbon source, and initial pH) selected for the screening of the significant variables for β-fructofuranosidase production by *A. versicolor*, and the corresponding responses are shown in Table 1. Optimization of the culture conditions was carried out using CCRD 2^3 and RSM. Based on the previous results in the laboratory, apple pomace was chosen as the carbon source because it induces the production of β-fructofuranosidase in *A. versicolor* (unpublished data). In this study, CCRD showed that the best culture conditions for β-fructofuranosidase production by *A. versicolor* were 3% (w/v) apple pomace, 12 days of cultivation time, and an initial pH of 7.5, which resulted in 77.92 U mL^{-1} (Table 1). In addition, the enzyme production improved 150-fold between Run 1 and 8 (Table 1).

Cost-efficient industrial production of commercially important enzymes largely depends on cheap media as well as inexpensive substrates. Agro-industrial byproducts, such as sugar cane molasses and soybean meal, and other wastes have been reported as carbon sources for the production of invertases (Ohara et al., 2015).

Several studies describe the production of β-fructofuranosidase by fungi of the genus *Aspergillus* using agro-industrial waste or alternative low-cost, renewable carbon sources. For example, *A. niveus* was used to produced extracellular invertase (20 U mL^{-1}) with bagasse sugarcane supplemented with sucrose and glucose as the carbon sources (Guimarães et al*.,* 2009).

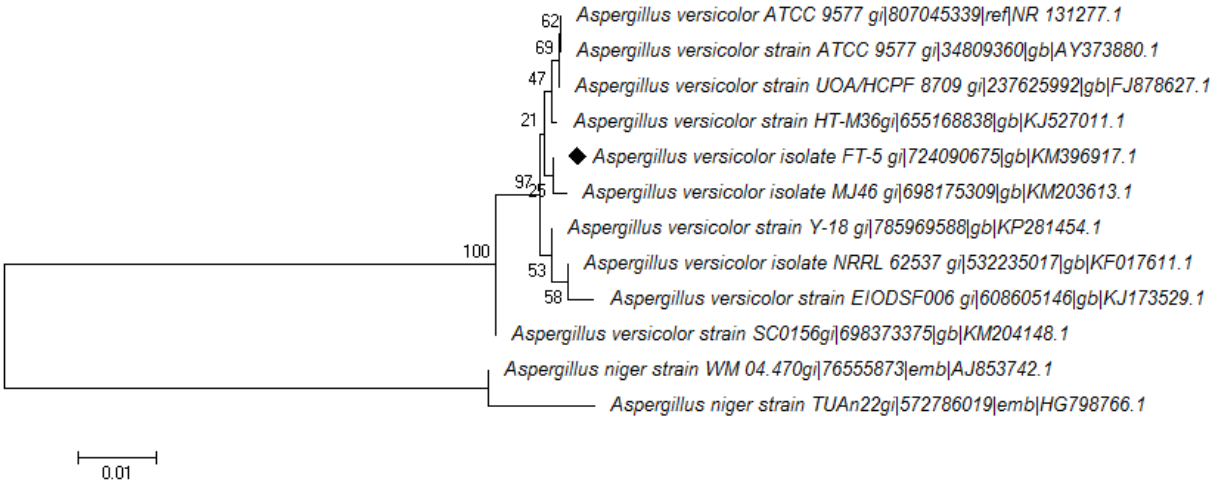

Figure 1. Phylogenetic tree constructed from sequences of the ITS regions of the ribosomal DNA from *A. versicolor* FT-5 and compared to sequences of other *A. versicolor* strains obtained from the NCBI GenBank database. *Aspergillus niger* strain WM04.47 and *A. niger* strain TUAn22 were used as out-groups. Fungus species is in bold symbol followed by Genbank accession number.

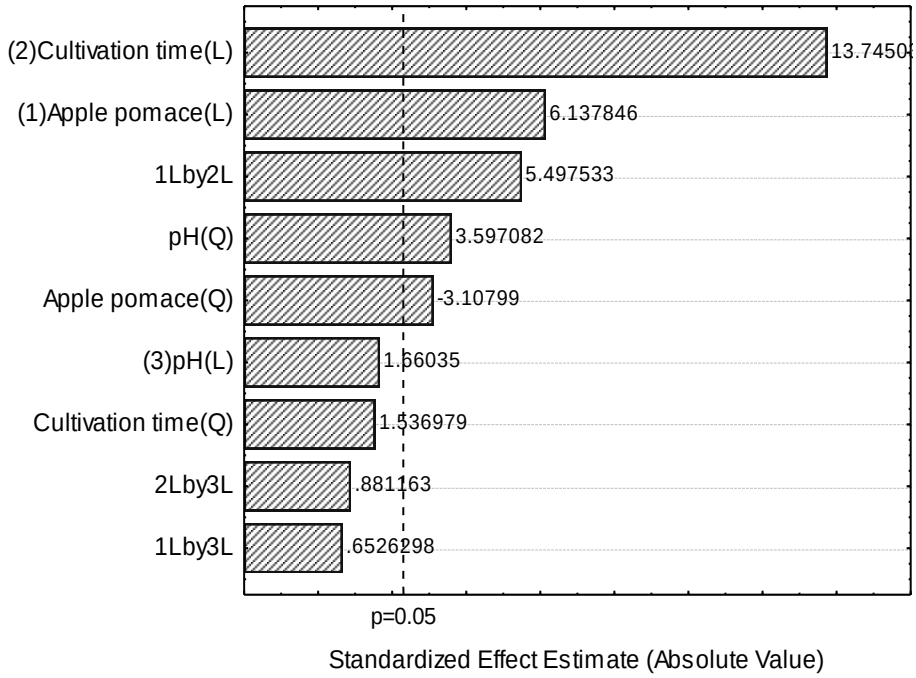

Figure 2. Pareto chart of the variable effects on β-fructofuranosidase activity using 2^3 factorial design.

Reddy et al. (2010) also reported the production of β-fructofuranosidase by *A. niger* PSSF21 using sugarcane molasses as the carbon source (19.1 U mL^{-1}).

The variables that most influenced β-fructofuranosidase production in this study were the apple pomace concentration (linear and quadratic term), cultivation time (linear term), and pH (quadratic term), which all had p values <0.05, as shown in Figure 2. The apple pomace

Table 2. Coefficient estimates by the regression model.

Parameter	Regression	Standard error	t(8)	p
Mean/Interc.	23.416	2.896	8.085	0.000 **
(1) Apple pomace (L)	9.640	1.570	6.138	0.000 **
Apple pomace (Q)	-5.077	1.634	-3.108	0.014 **
(2) Cultivation time (L)	21.587	1.571	13.745	0.000 **
Cultivation time (Q)	2.511	1.634	1.537	0.163
(3) pH (L)	2.607	1.571	1.660	0.135
pH (Q)	5.877	1.634	3.597	0.007 **
1L by 2L	11.276	2.051	5.497	0.001 **
1L by 3L	1.338	2.051	0.653	0.532
2L by 3L	1.807	2.051	0.881	0.404

**Statistically significant at 95% of confidence level.

Table 3. ANOVA of variable effects of β-fructofuranosidase production.

Source	Sum of squares	Degree of Freedom	Mean Square	F-value	F-listed	p-value
Regression	9497.20	6	1582.87	44.73	3.09	<0.001
Residual	389.22	11	35.38			
Lack of fit	370.79	8	46.35	7.54	8.85	0.062
Pure error	18.43	3	6.14			
Total	9886.42	17				

Determination coefficient (R^2= 0.9606).

concentration and cultivation time had a significant impact on the response (Table 2). Regression analysis of The experimental data yielded the following quadratic equation for β-fructofuranosidase production:

$$Y = 23.41+9.63X_1 -5.08 X_1^2 + 21.59 X_2 + 2.6 X_3 +5.87 X_3^2 +11.27 X_1 X_2 \quad (2)$$

Where, *Y* is the β-fructofuranosidase activity (U mL^{-1}), X_1 is the apple pomace concentration (%), X_2 is cultivation time (day), and X_3 is the initial pH of the medium.

Based on the F-test, the calculated F-value (44.73) is 14.47-fold higher than the critical F_{listed} (3.09) with $p<0.05$; this result implies a satisfactory representation of the process by the model. The lack of fit was not significant because the F_{cal} was lower than F_{listed}, indicating that the experimental data fit the model obtained (Table 3). The determination coefficient R^2 (0.9606) suggested that the fitted model could explain 96.06% of the total variation. Therefore, this study shows that the β-fructofuranosidase production from *A. versicolor* is enhanced when the cultivation time and apple pomace concentration are higher (Figure 3a). Likewise, higher values of initial pH and a longer cultivation time resulted in increased enzyme production (Figure 3b). Therefore, the importance of optimizing the production of invertase from fungus using an experimental design were also reported for the β-fructofuranosidase by *Aspergillus niger* in submerged and solid-state fermentation (Ashokkumar et al., 2001). Similarly, Driouch et al. (2010) also reported on optimized bioprocess for the production of fructofuranosidase by recombinant *A. niger* SKAn1015.

Purification of β-fructofuranosidase

β-Fructofuranosidase from *A. versicolor* was partially purified, and two pools were separated and named FFase-I (β-fructofuranosidase-I) and FFase-II (β-fructofuranosidase-II) (Figure 4). The ability to produce β-fructofuranosidase isoforms has been reported for other fungi, such as *Aureobasidium pullullans* DSM2404, which produced five β-fructofuranosidase isoforms grown in sucrose (Yoshikawa et al., 2006). In addition, *Fusarium oxysporum* produced two extracellular isoforms of this

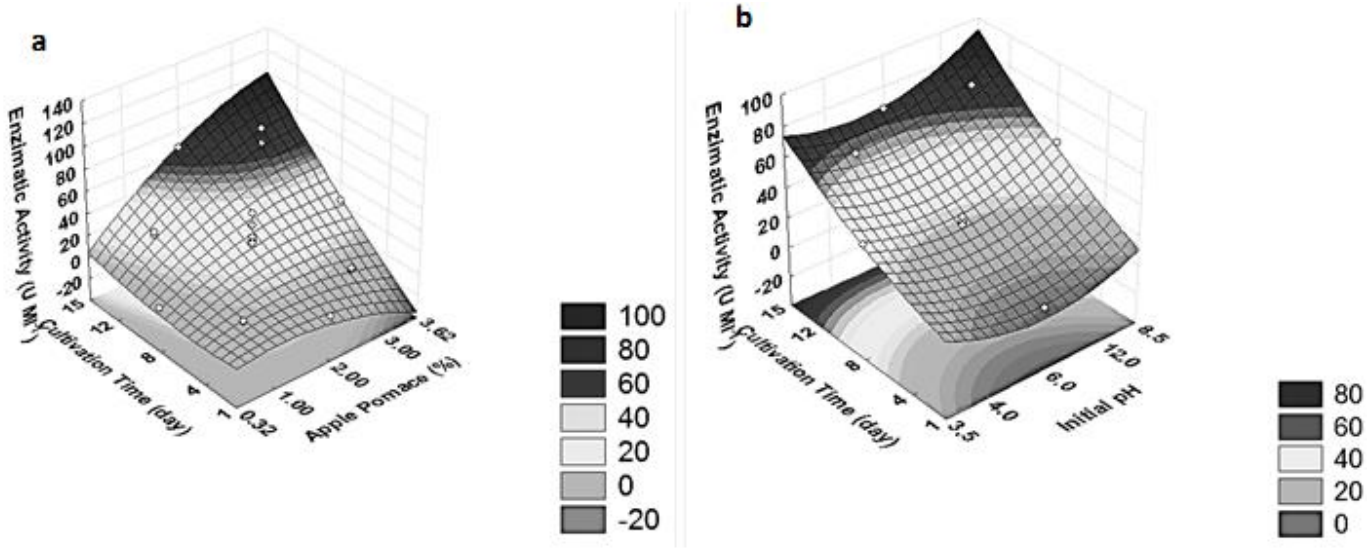

Figure 3. Three-dimensional plot representing effects of cultivation time and apple pomace on β-fructofuranosidase production by *A. versicolor* (a); cultivation time and initial pH (b).

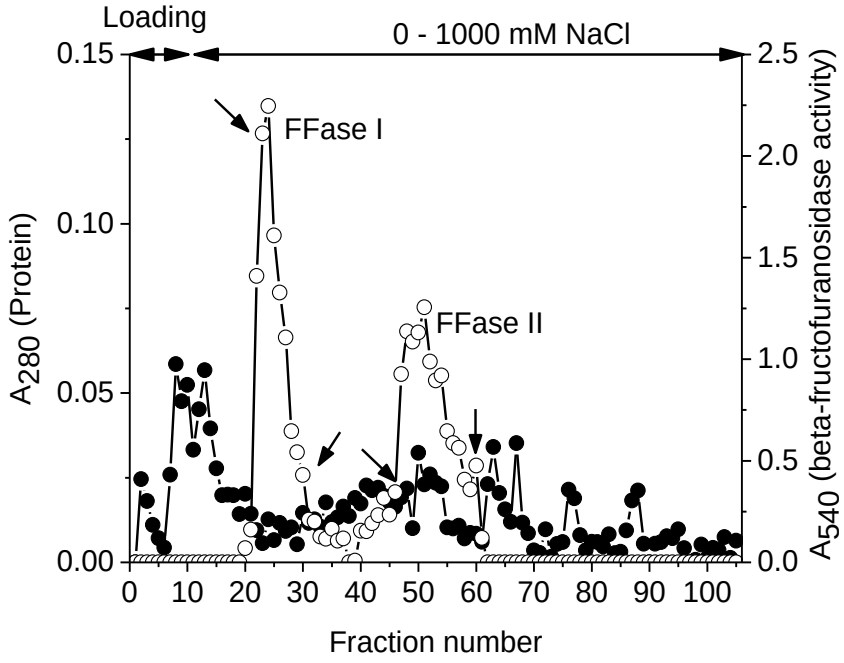

Figure 4. Chromatographic profile of β-fructofuranosidase activity from *A. versicolor* using a DEAE-Sephadex column equilibrated with Tris-HCl buffer (20 mM, pH 7.5) and eluted using a NaCl gradient. (○) absorbance 540 nm; (●) absorbance 280 nm.

enzyme using fructose as the carbon source (Wolska-Mitaszko et al., 2007). In this study, the FFase-I was purified with a yield of 22.57% after DEAE-Sephadex chromatography (Table 4). Zymogram revealed the presence of a zone of hydrolysis that corresponded with the Coomassie-stained band of purified beta-

Table 4. Summary of the purification of extracellular FFase-I from *A. versicolor.*

Step	Total activity (U)	Total protein (mg)	Specific activity (U mg^{-1})	Yield (%)	Purification fold
Crude extract	37,714.29	13,258.15	2.84	100.00	1.00
$(NH_4)_2SO_4$ precipitation	15,748.57	2932.60	5.37	41.76	1.89
DEAE Sephadex	8,510.91	815.74	10.43	22.57	3.67

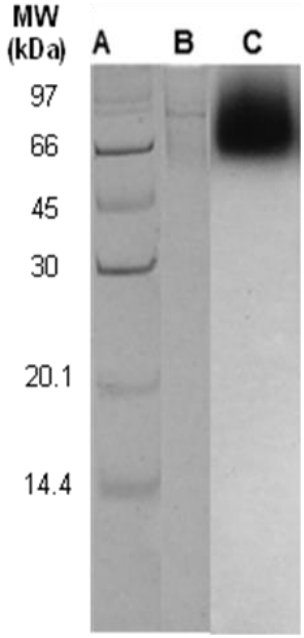

Figure 5. SDS-PAGE and zymogram of FFase-I produced by *A. versicolor*. a) Molecular mass marker; b) FFase-I after DEAE-Sephadex; c) zymogram.

fructofuranosidase-I; the molecular mass of the FFase-I was estimated to be 75 kDa by SDS-PAGE analysis (Figure 5). Some *Aspergillus* genera showed β-fructofuranosidases with different molecular masses, including *A. ochraceus* (13 kDa [Gosh et al., 2001] and 13.5 kDa invertase [Guimarães et al., 2007]), *A. niger* (75 kDa invertase [Goosen et al., 2007]), and 60 kDa invertase from *A. japonicus* (Wang and Zhou, 2006).

Effect of temperature on enzyme activity and thermal stability

FFase-I was highly active between 55 and 60°C (Figure 6a), which was higher than the β-fructofuranosidases from *Mucor geophillus* (45°C) (Quershi et al., 2012) and *A. caespitosus* (50°C) (Alegre et al., 2009), but most of the fungi show the high optimal temperature, especially between 50 and 60°C.

The thermal stability of the FFase-I was 100% for 12 h when incubated at 40, 50 and 55°C. The half-life at 60°C was 7 h, and its activity was 40 and 26% for 8 and 12 h, respectively (Figure 6b). However, at 65 and 70°C, the half-life decreased to 30 min. The FFase-I from *A. versicolor* had higher thermal stability when compared with β-fructofuranosidases produced by *A. phoenicis*, which was stable at 50°C for 1 h (Rustiguel et al., 2010), and *A. caespitosus*, which was stable at 60°C for 1 h and 20 min (Alegre et al., 2009).

Effect of pH on enzyme activity and stability

The optimum pH for the activity of FFase-I from *A. versicolor* was 6.0 (Figure 7a). Similar values were observed with extracellular β-fructofuranosidases produced by *A. caespitosus* and *S. cerevisiae* NRRLY12632 (Alegre et al., 2009; Mona and Mohamed, 2009). Most fungal β-fructofuranosidases have an optimum pH in the acidic range, as observed for *A. phoenicis* (Rustiguel et al., 2010) and *C. sitophila* PSSF84 (Patil et al., 2011) (both pH 4.5), and *M. geophillus* (pH 5.0) (Quershi et al., 2012). The FFase-I from *A. versicolor* is stable for 12 h between pH 3 and 6 (Figure 7b). This β-fructofuranosidases' property with acidic pH range is commonly found in commercial invertase microbial sources.

Effect of metal ions and chemical reagents on FFase-I activity

Among the salts tested, Fe^{+2} (5 mM) increased the activity of FFase-I by 27.32%, suggesting that the ion acts as enzyme cofactor. Furthermore, the enzyme was tolerant to ethanol, acetone and detergents (SDS and Tween 80). Enzymes that are stable in the presence of compounds such as organic solvents, surfactants and metallic ions are considered relevant for catalysis in biotechnological applications. In addition, FFase-I was tolerant to the presence of copper, which is unusual for Most enzymes because they are often inhibited by

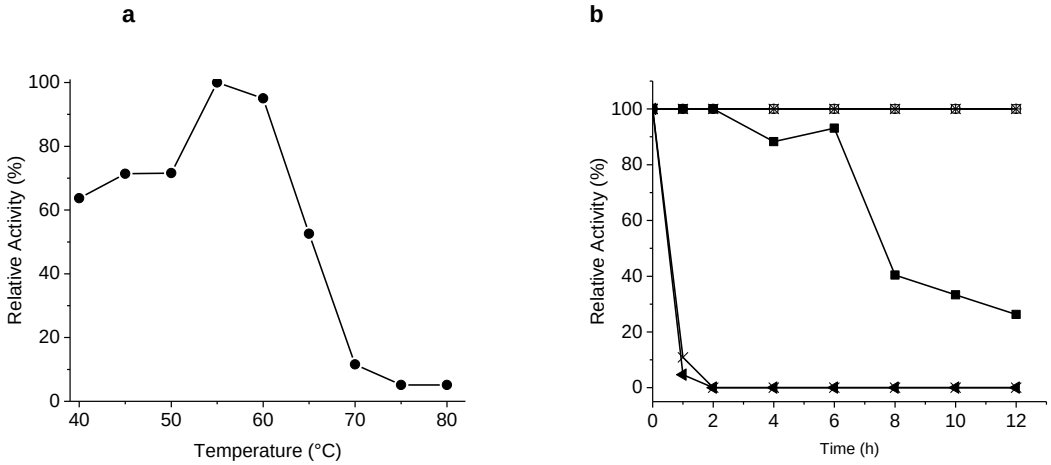

Figure 6. Effect of temperature on FFase-I activity (a) and the thermal stability of FFase-I (b) produced by *A. versicolor*; 40°C (○), 50°C (□), 55°C (*), 60°C (■), 65°C (X), 70°C (◄).

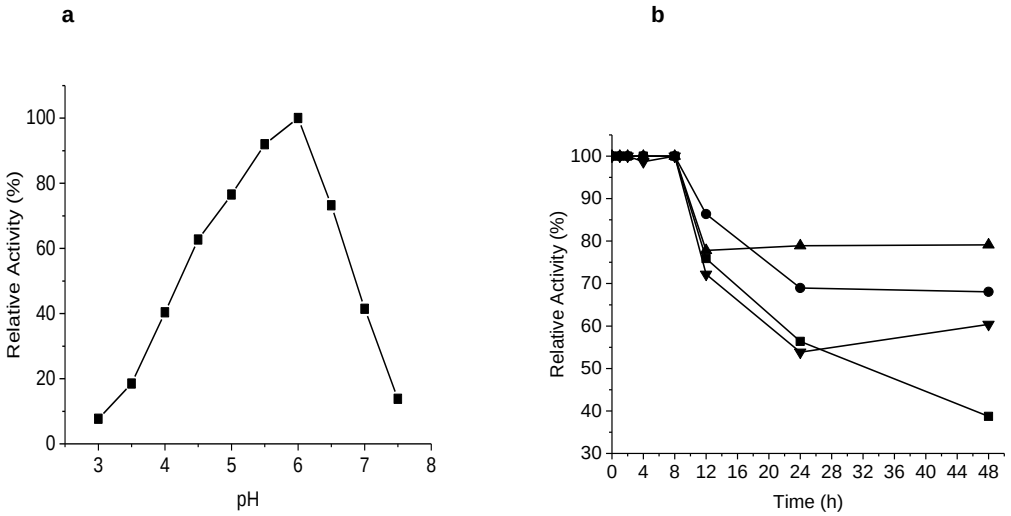

Figure 7. Influence of pH on the activity of FFase-I (a) and the thermal stability of FFase-I (b) produced by *A. versicolor* pH 3.0 (■), pH 4.0 (●), pH 5.0 (▲), pH 6.0 (▼).

copper (Guimarães et al., 2007; Uma et al., 2012). In contrast, Hg^{2+} strongly inhibited the enzyme activity at both concentrations tested (1 and 5 mM) by 86–89% (Table 5). β-Fructofuranosidases from *A. niger* IMI303386 (Nguyen et al., 2005), *Chrysonilia sitophila* PSSF84 (Patil et al., 2011) and *A. phoenicis* (Rustiguel et al., 2010) were also strongly inhibited by Hg^{2+}.

Kinetic properties

The apparent K_m and V_{max} values for the FFase-I from *A. versicolor* were 26.71 mM and 56.98 U mg^{-1}, respectively, using sucrose as the substrate. These results were very similar to those obtained with β-fructofuranosidase from *A. phoenicis,* which had K_m and V_{max} values of 25 mM and 55.679 U mg^{-1}, respectively (Rustiguel et al., 2010). However, the K_m from β-fructofuranosidase from *A. versicolor* was higher (lower affinity for the substrate) than that of *A. ochraceus*, which was 13.4 mM (Guimarães et al., 2007), as well as *A. niveus*, which was 5.78 mM (Guimarães et al., 2009).

Conclusions

β-Fructofuranosidase produced by *A. versicolor* isolated from the Parana Atlantic Forest biome showed a

Table 5. Effect of metal ions and chemical reagents on FFase–I activity.

Compound	β-Fructofuranosidase activity (%)	
	1 mM	5 mM
KCl	107.33	111.69
$MgSO_4$	108.78	110.72
NaCl	103.46	106.64
$SnCl_2$	96.46	96.32
$BaCl_2$	100.21	104.84
$(NH_4)_2SO_4$	101.13	101.70
$CaCl_2$	111.00	105.74
$HgCl_2$	11.69	14.32
$CuSO_4$	105.09	99.15
$FeSO_4$	114.04	127.32
EDTA	94.63	92.93
Ethanol	100.28	94.06
Acetone	101.41	102.40
SDS	104.24	90.95
Tween 80	101.87	103.32
Control	100.00	100.00

significant improvement (150-fold) after optimized culture conditions using apple pomace as an inexpensive and alternative carbon source. The purified FFase-I was stable under acidic pH (3–6) and was thermally stable up to 60°C. In addition, the enzyme was also tolerant to different metallic ions, organic solvents (ethanol and acetone) and detergents (SDS and Tween 80). Therefore, the FFase-I presents promising properties and is suitable for biotechnological exploitation.

Conflict of Interests

The authors have not declared any conflict of interests.

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