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SYNTHESIS AND CHARACTERIZATION OF FRUCTOSYLTRANSFERASE FROM *Aspergillus oryzae* IPT-301 FOR HIGH FRUCTOOLIGOSACCHARIDES PRODUCTION

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Abstract - Fructooligosaccharides (FOS) are mainly produced by microbial fructosyltransferases (FTase, E.C.2.4.1.9), and *Aspergillus oryzae* IPT-301 has shown high fructosyl transferring and low hydrolytic activities, which leads to high FOS production yields, but the main operating parameters for its best performance have been scarcely studied. Thus, this work aimed to evaluate the cellular growth, production and characterization of mycelial and extracellular FTases by *Aspergillus oryzae* IPT-301. Experimental design showed that the extracellular FTase performance was optimized (high transfructosylation activity and low hydrolytic activity) for reaction pH 5.5 - 6.75 and temperature of 45-50 °C and was fitted by the Michaelis-Menten model, while the mycelial FTase showed better performance at pH below 6.5 and temperature above 46 °C and was better fitted by the Hill model. The results obtained showed that the fungus represents a promising source for FOS production on a laboratorial scale.
Keywords: *Aspergillus oryzae* IPT-301; Submerged fermentation; Fructosyltransferase; Enzymatic characterization; Fructooligosaccharides.

INTRODUCTION

Nowadays, there is a continuous and growing attention for dietary prebiotics-oligosaccharides (PO), mainly due to their proven beneficial effects on human and animal health. Among the different PO described in the literature, fructooligosaccharides (FOS) have been described as an important food ingredient due to their excellent nutrition- and health-relevant properties, including low calorie, non-cariogenicity, ability to stimulate the growth of beneficial colonic lactic acid bacteria and to enhance the intestinal immune response, reduction of total serum cholesterol levels, as well as enhancement of calcium absorption (Jitonnom et al., 2018; Zhang et al., 2017).

In nature, FOS can be found in different plants such as onion, garlic, banana, Jerusalem artichoke and some grasses (Maiorano et al., 2008). However, their commercial production is predominantly based on microbial extracellular/mycelial enzymes called fructosyltransferases (FTases EC.2.4.1.9) (Muñiz-Márquez et al., 2016) in the presence of sucrose. Various FOS synthesized from sucrose include 1-kestose (GF2), nystose (GF3), and 1F -β-fructofuranosylnystose (GF4) where 1-3 units of fructose are attached via β-(2-1) linkage to the sucrose (Hernández et al., 2017;

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Gujar et al., 2018). These enzymes possess both transfructosylating and hydrolytic activities (Huang et al., 2016). The ratio between transfructosylation and hydrolytic activities (A_t/A_h) can be considered as the most important criteria when evaluating FOS production from different microbial enzymes. When high ratio values (A_t/A_h) are obtained, FTase exhibits greater transfructosylation activity in the reaction medium. Therefore, high transfructosylation activity allows a high conversion of sucrose to FOS, while a high (A_t/A_h) is required to avoid the FOS molecule hydrolysis (Hidaka et al.,1988; Cuervo-Fernandez et al. 2004; Cuervo-Fernandez et al. 2007).

Several filamentous fungal strains belonging to the *Aspergillus, Penicillum* and *Fusarium* genera have been reported to be producers of these FOS producing or FTase enzymes (Lateef et al., 2008; Lateet et al., 2012; Guo et al., 2016; Ademakinwa et al., 2017; Mano et al., 2018). Many efforts are devoted to optimize nutritional and culture parameters of fermentation processes or genetic alterations to increase enzyme yield and FOS productivity (Ademakinwa et al., 2017; Ganaie et al., 2017; Zhang et al., 2017). Lateef et al. (2012) reported the performance of FTase production by *Aspergillus niger* using agroindustrial rejects as support (ripe banana peel and cola nut pod), supplemented with yeast extract and sucrose, fermented in shake flasks (30 ± 2 °C, 144 hours), obtaining values up to 27.77 U g^{-1}, demonstrating the potential use of rejects as a culture medium for the production of FTase.

They are mainly composed of kestose (GF2), nystose (GF3), and fructosylnystose (GF4). FOS can be produced by the fructosyltransferase (EC 2.4.1.9) or β-fructofuranosidase (FFase; EC 3.2.1.26) having a transfructosylating activity. FFase catalyzes both hydrolytic and a fructosyltransferase activities; however, the latter is evidenced only under high sucrose concentrations [3]. It cleaves the β-1,2 linkage of sucrose and transfers the fructosyl group leading to a FOS formation. Efforts are being made to explore the nature of these prebiotics and to identify microorganisms that produce FFase with a desired stability and suitable activity for industrial use. They are mainly composed of kestose (GF2), nystose (GF3), and fructosylnystose (GF4). FOS can be produced by the fructosyltransferase (EC 2.4.1.9) or β-fructofuranosidase (FFase; EC 3.2.1.26) having a transfructosylating activity. FFase catalyzes both hydrolytic and a fructosyltransferase activities; however, the latter is evidenced only under high sucrose concentrations [3]. It cleaves the β-1,2 linkage of sucrose and transfers the fructosyl group leading to a FOS formation. Efforts are being made to explore the nature of these prebiotics and to identify microorganisms that produce FFase with a desired stability and suitable activity for industrial use

Nascimento et al. (2016) utilized *Penicillium citreonigrum* URM 4459 and applied response surface methodology in shake flasks to determine the fermentation conditions (25.5 ºC, 67.8 h and pH 6.5) that maximize the FTase production obtained, detecting the maximal yield of 301.84 U mL^{-1}. Farid et al. (2015) evaluated the influence of medium composition and cultivation parameters on FTase production by *Penicillium aurantiogriseum* AUMC 5605 in shake flasks. The maximum FTase enzyme activity was produced at initial cultivation pH values ranging from 6.0-6.5, at an agitation speed of 200 rpm and using vegetative fungal cells as inoculum in the presence of the optimized media. Recently, Nobre et al. (2018) established by experimental design the optimal fermentation conditions (37 °C, pH 6.2) to produce FOS from *Aspergillus ibericus* MUM 03.49. In a bioreactor operated in a one stage process, using the whole cells of the microorganism FOS production reached 0.64 ± 0.02 $g_{FOS}.g_{initial\ sucrose}^{-1}$.

In our previous group experiments (Cuervo-Fernandez et al., 2007; Maiorano et al., 2009; Ottoni et al., 2012) the FTase produced by *Aspergillus oryzae* IPT-301 presented high transfructosylation activities when concentrated sucrose solutions were used as substrate, which consequently led to high production of FOS. Despite its excellent performance, there is no previous research in the literature which aimed to study the influence of the reaction media temperature, pH and sucrose concentration on *Aspergillus oryzae* IPT-301 FTase. The present study focused on laboratory-scale production of *Aspergillus oryzae* IPT-301 extracellular and mycelial FTases by submerged fermentation using synthetic culture medium and characterization studies of the process parameters (thermal and pH stability, temperature, pH and concentration of sucrose from the reaction medium) and on enzymatic kinetic parameter determination.

MATERIALS AND METHODS

Chemicals

All chemicals used were of analytical grade. Yeast extract, sucrose, KH_2PO_4, $MnCl_2.4H_2O$, $FeSO_4.7H_2O$ were purchased from Labsynth® (Diadema, Brazil). Glycerin and phenol were obtained from Isofar® (Duque de Caxias, Brazil). Glucose, $NaNO_3$, $MgSO_4.7H_2O$, NaOH, $Na_2S_2O_5$, $C_7H_4N_2O_7$ and $KNaC_4H_4O_6.4H_2O$ were from Dinamica® (Diadema, Brazil). Dextrose Potato Agar was obtained from Kasvi® (São José dos Pinhais, Brazil) and the enzymatic kit GOD-PAP for glucose determination from Laborlab® (Campinas, Brazil).

Microorganisms and culture conditions

Aspergillus oryzae IPT-301 strain was obtained from the Institute for Technological Research (IPT/

SP). The strain was grown on solid media containing (in % w/v): glycerin 2.5, glucose 2.5, dextrose potato agar 2.0 and yeast extract 0.5, at 30 ºC for 7 days. Spore solution was preparared in glycerol 20 % (w/v) and ajusted for 10^7 spores mL^{-1}. The spore suspension was mantained in a freezer at around -12 °C.

Extracellular and mycelial enzyme production

The microbial growth and standard enzyme production was carried out in 250 mL unbaffled Erlenmeyer flasks containing 50 mL of culture medium, with the following composition (in % w/v): sucrose 15.0, yeast extract 0.5, $NaNO_3$ 0.5, KH_2PO_4 0.2, $MgSO_4.7H_2O$ 0.05, $MnCl_2.4H_2O$ 0.03 e $FeSO_4.7H_2O$ 0.001. The pH of the medium was adjusted to 5.5 before sterilization.

Flasks were inoculated with 0.5 mL of 10^7 spores mL^{-1} suspension previously prepared and incubated in rotatory shaker at 30 °C and 200 rpm for 76 h. Samples for analysis were colleted in predetermined periods until 76 h and filtered using filter paper (Whatman N° 1). In the filtered broth, pH and extracellular fructosyltransferase (FTase) activity were measured.

Mycelial FTase activity were determinated with the wet cell pellet formed and the biomass concentration was determined by dry cell weight per volume (g L^{-1}) (Perna et al., 2018). After filtration, using distilled water, the biomass was washed and dried at 60 °C for 24 h. In predetermined periods, broth was filtered and the pH values were measured with a digital pHmeter and the fructosyltransferase activities were determined as described below. The experiments were conduted in triplicate.

Analytical methods

Standard enzymatic activity assay

The enzymatic activities were determinated as follows: 0.1 mL of suitably diluted supernatant (extracellular FTase) or 0.05 g of wet mycelium (mycelial FTase) was mixed with 3.7 mL of 47.06 % (w/v) sucrose solution and 1.2 mL tris-acetate buffer 0.2 mol L^{-1} at pH 5.5. The reaction was carried out in a shaking waterbath at 50 °C, 190 rpm for 60 min and stopped by boiling for 10 min (Cuervo-Fernandez et al., 2004). The units of transfructosylation (A_t) and hydrolytic (A_h) activities were defined as the amount of enzyme that transfers a micromole of fructose or releases a micromole of fructose, respectively, per minute under the chosen experimental conditions.

Carbohydrate analysis

Glucose concentration (G) and reducing sugars (R) were estimated by an enzymatic kit Glucose (GOD/PAP) method and the 3,5-dinitrosalicylic acid (DNS) method (Miller, 1959), respectively. The concentrations of both fructose (F) and transferred fructose (F_T) in the reaction medium were computed using Equations (1) and (2) (Chen and Liu, 1996).

$$[F] = [R] - [G] \tag{1}$$

$$[F_T] = [G] - [F] \tag{2}$$

Characterization of extracellular and mycelial enzymes

Thermal and pH stability on enzymatic activity

The thermal and pH stability of the reaction media in activities of fructosyltransferase was investigated. Regarding the thermal stability, 0.1 mL of suitably diluted supernatant (extracellular FTase) or 0.05 g of wet mycelium (mycelial FTase) was incubated across a broad temperature range (30 ºC - 65 °C) at pH 5.5 for 1 h, and residual activity was assayed under standard conditions. The pH stability was determined by incubating 0.1 mL of suitably diluted supernatant (extracellular FTase) or 0.05 g of wet mycelium (mycelial FTase) samples in tris-acetate buffer 0.2 mol L^{-1} (pH range of 3.0 - 8.0) at 4 °C, for 24 h, and then the residual activity was measured under standard conditions (item 2.4.1).

Effects of sucrose concentration in the reaction media and determination of kinetic parameters

Enzymatic activities were determined with 0.1 mL of suitably diluted supernatant (extracellular FTase) or 0.05 g of wet mycelium (mycelial FTase) with 3.7 mL of sucrose solution at different concentrations (78.0, 148.0, 296.0, 370.0, 470.6 and 592.0 g L^{-1}) and 1.2 mL tris-acetate buffer 0.2 mol L^{-1} at pH 5.5. The reaction was performed as described above. For enzymatic kinetics evaluation, the sets of experimental data obtained for the transfructosylation activities (A_t) were fitted to the Michaelis-Menten model and the Hill model. Kinetic parameters such as maximum reaction rate (V_{max}) and Michaelis constant (K_m), and Hill constant (n) were obtained using a nonlinear regression analysis performed with the OriginLab® version 8.0 software.

Experimental design and statitical analysis

The experimental design was obtained employing the software STATISTICA® version 12.0 (StatSoft. Inc. 2007, USA). A full 2^2 factorial design with three assays at the center point was chosen for the study of two factors: pH and temperature of reactional media, each at five levels. Star points were added to the experimental design to compose a second-order model (Table 1). The data factors were chosen after a series of preliminary assays.

The statistical analaysis of the data was carried out using STATISTICA® version 12.0 (StatSoft. Inc.

Table 1. Experimental design matrix.

Runs	Coded values		Actual values	
	Temperature	pH	Temperature (°C)	pH
1	1	1	50	8.0
2	1	-1	50	5.5
3	-1	-1	40	5.5
4	-1	1	40	8.0
5	-1.414	0	38	6.75
6	1.414	0	52	6.75
7	0	-1.414	45	5.00
8	0	1.414	45	8.50
9	0	0	45	6.75
10	0	0	45	6.75
11	0	0	45	6.75

2007, USA). The response surface model was fitted to two response variables, Y, namely transfructosylation and hydrolytic activities for extracellular (in U mL^{-1}) and mycelial (in U g^{-1}) FTases. The enzymatic activity assay were carried out as decribed above. The second order response functions for two factors are given in Equation (3) and the differences were considered significant at p-values ≤ 0.05.

$$Y = \beta_0 + \beta_1 A + \beta_2 B + \beta_{12} AB + \beta_{11} A^2 + \beta_{22} B^2 \quad (3)$$

where A and B represent the levels of the factors temperature (ºC) and pH, respectively, while β_0, β_1, β_2, β_{12}, β_{11} and β_{22} represent the estimated coefficients with β_0 having the role of the offset term.

RESULTS AND DISCUSSION

Influence of cell growth and enzymatic activity

Figure 1 shows the growth profile of *Aspergillus oryzae* IPT 301; the peak biomass concentration, 9.35 ± 1.26 gL^{-1}, occurred in 48 h of fermentation. In addition, acidification of the culture medium from pH 5.50 to 4.82 occurred between 48 h and 72 h of the process.

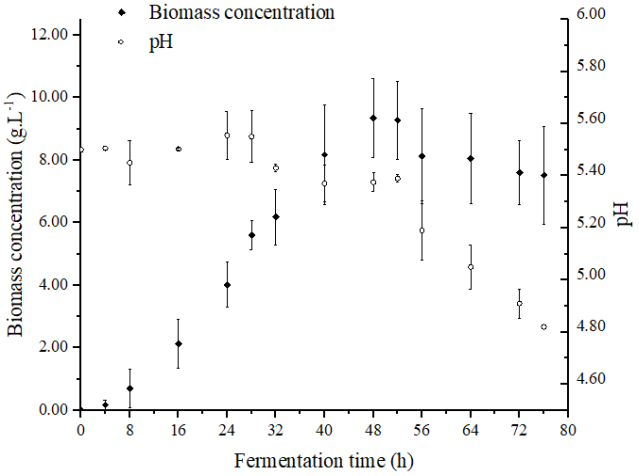

Figure 1. Concentration of the cellular biomass of *Aspergillus oryzae* IPT-301 and pH progression as a function of fermentation time. Experimental conditions: spores suspension 10^7 spores per mL, synthetic culture medium, 200 rpm and 30 ºC.

The extracellular transfructosylation (A_t) and hydrolytic (A_h) activities as a function of fermentation time are set forth in Figure 2A. The maximum production of the enzyme FTase, 19.76 ± 0.56 U mL^{-1}, occurred in 64 h of fermentation. At the same time, a low A_h (1.65 ± 0.31 U mL^{-1}) was detected and the best ratio was 12.22 ± 1.82 between A_t/A_h. The highest activity obtained in mycelium (Figure 2B), A_t (524.55 ± 177.10 U g^{-1}) and A_h (141.07 ± 53.12 U g^{-1}) were determined in 72 h of fermentation. However, the best 8.73 ± 2.02 (A_t / A_h) ratio occurred in 48 h, whereas in 72 h, the value of this ratio was 3.82 ± 0.67.

According to Maiorano et al. (2009), the maximum production of FTase using *A. oryzae* IPT-301 occurred in the range of pH 4.5 to 5.0, while the maximum biomass production was verified for fermented broths with pH 5.0. *A. oryzae* IPT 301 is a producer of acid

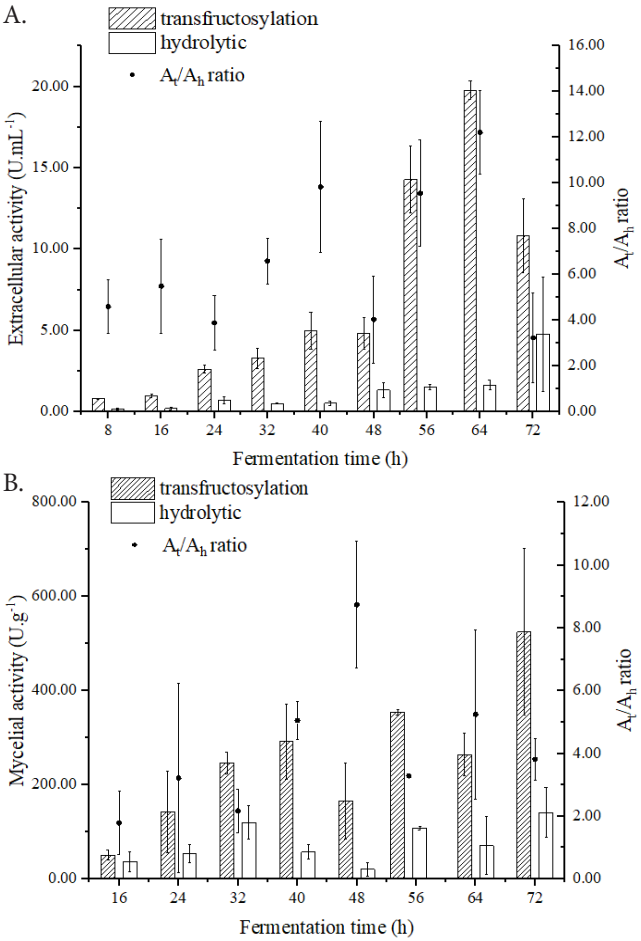

Figure 2. Influence of the fermentation time on the hydrolytic (A_h) and transfructosylation (A_t) activities of extracellular (A) and mycelial (B) FTase and the ratio between the activities (A_t/A_h) in 47.06 % (m/v) sucrose solution and 0.2 mol L^{-1} tris-acetate buffer, pH 5.5 at 50 ºC.

proteases, responsible for the cleavage of peptide bonds and release of amino acids, which causes acidification of the culture medium, also interfering with the enzymatic activities (Castro and Sato 2014; Tsujita et al., 1997). According to Ganaie and Gupta (2014), the highest productivity of FOS can be obtained in 36 h of fermentation when using the strains of the fungi *A. niger* and *A. flavus*. However, the authors point out that, when the production of FTase and FOS is carried out, a low sugar yield can be observed, since the production of biomass and, consequently, of enzyme occur in an optimal temperature range between 28 ºC to 30 °C, whereas the maximum FOS production can be checked for an optimum range of 50 ºC - 60 °C.

Effect of pH and temperature on stability enzymatic

The stability of the extracellular FTase showed the highest relative transfructosylation activity at pH 6.0 (Fig.3A), whereas the mycelial FTase occurred between pH 5.0 - 8.0 (Fig. 3B). Yang et al. (2016) using *A. niger* YZ59 recombinant expressed in *P. pastoris* GS115, detected the optimum pH of FTase at 5.5 and stability of the enzyme ranging from pH 3.0 to 10.0. The work of Lateef et al. (2007) and Sangeetha et al. (2005) reported the stability of mycelial FTases produced by *Aureobasidium pullulans* CFR 77 and *Aspergillus oryzae* CFR 202 for ranges of pH 3.5 - 10.0 and pH 5.0 - 7.0, respectively.

The thermal stability of the extracellular FTases (Fig. 4A) occurred only at temperatures below 35 °C, and attained at up to 96 %. At temperatures above 40 °C, there was a marked decrease in activity, and only 44.26 % of the initial activity was maintained at a temperature of 65 °C. Xu et al. (2015) reported that the enzyme produced by *Penicillium oxalicum* was maintained with up to 80 % of the initial A_t for a temperature range between 25 °C and 55 °C. Madlová et

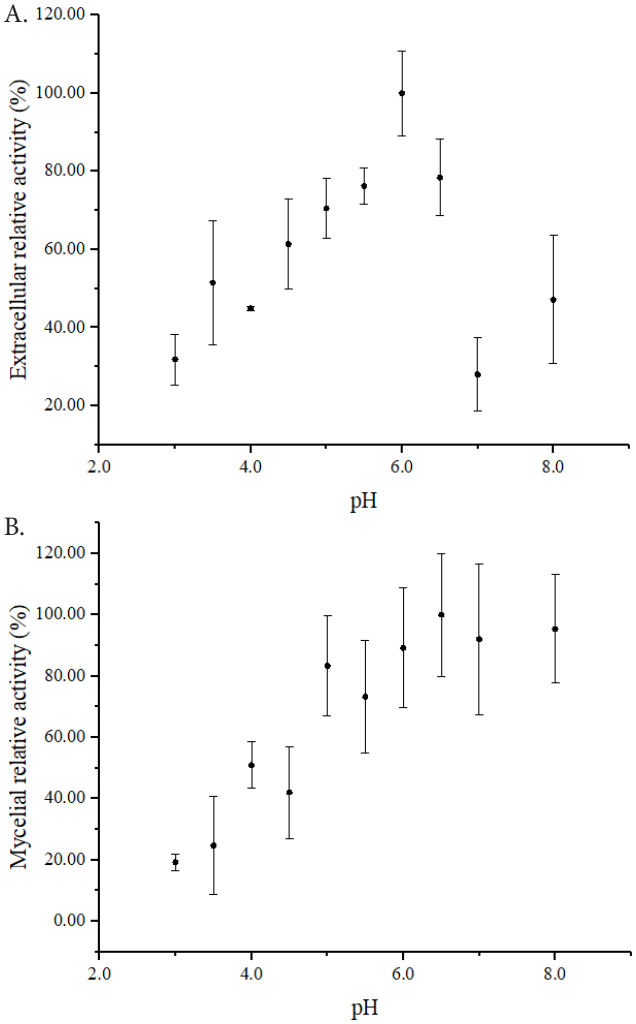

Figure 3. The stability of FTase-pH after 24 h of incubation in 0.2 mol L^{-1} tris-acetate buffer solution at 4 °C. Activity of transfructosylation (A) extracellular at 100 % (14.96 ± 1.62 U mL^{-1}) and (B) mycelial at 100 % (189.23 ± 37.82 U g^{-1}).

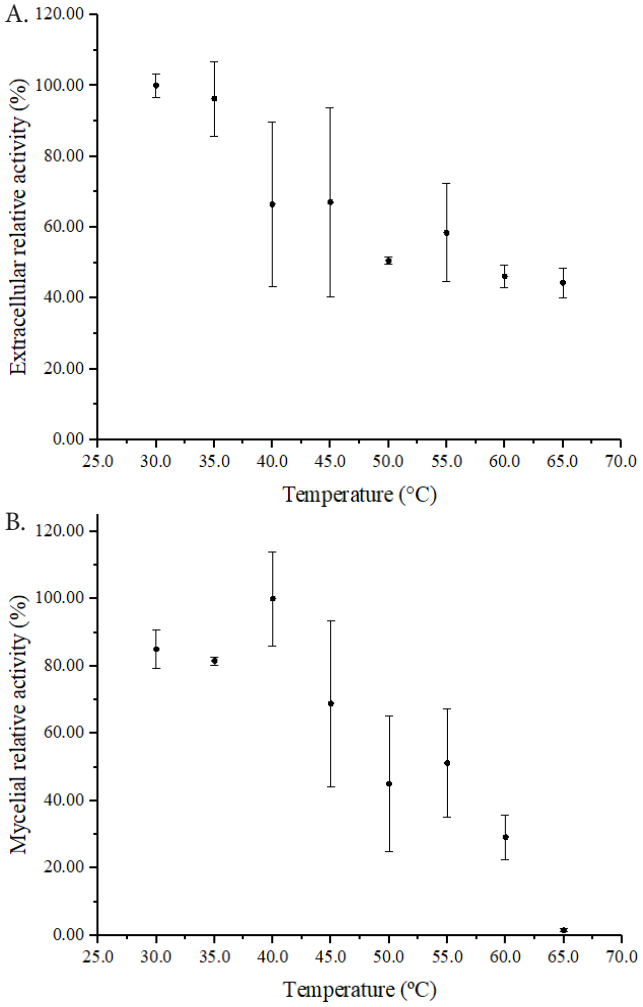

Figure 4. Thermal stability of the FTase evaluated at different temperatures a-post incubation for 1 h in 0.2 mol L^{-1}tris-acetate buffer pH 5.5. Activity of transfructosylation (A_t) at 100% (A) extracellular 100 % (22.65 ± 0.72 U mL^{-1}) and (B) mycelial 100 % (418.56 ± 58.44 U g^{-1}).

al. (2000) demonstrated that FTases of *Aureobasidium pullulans* were rapidly inactivated when subjected to temperatures higher than 60 °C, even in the presence of high substrate concentrations. Mycelial FTase (Fig. 4B) remained stable for a temperature range from 30 °C and 40 °C, where relative transfructosylation activities remained between 80 % and 100 %. At temperatures above 45 °C, a marked decrease in enzyme activity was observed until complete inactivation of the enzyme was achieved at 65 °C. Similar behavior has also been reported in Hayashi et al. (1990), in which the mycelial FTase of *Aureobasidium* sp. ATCC 20254 showed low thermal stability at temperatures above 50 °C until complete inactivation at 70 °C after 15 min incubation.

Effects of sucrose concentration in the reaction medium and determination of kinetic parameters

The sucrose concentration range of 296 g L^{-1} - 592 g L^{-1} had an average value of A_t equal to 14.58 ± 0.09 U mL^{-1}. The extracellular FTase, produced by *A. oryzae* IPT-301, was satisfactorily adjusted to the Michaelis-Menten kinetic model without being inhibited by the different concentrations of sucrose evaluated, with R^2 = 0.991 (Fig. 5A). The values of the kinetic parameters ($V_{máx}$ 16.23 U mL^{-1} and K_m 50.41 g L^{-1}) of the proposed model were obtained using the software Origin 8.0. Differently, Aguiar-Oliveira and Maugeri (2010) reported that, at substrate concentrations above 500 g L^{-1}, inhibition occurs by the substrate, with adjustment by the classic michaelian model with inhibition. In this work, it was not possible to obtain the kinetic parameters that supported the hypothesis of such inhibition with the data obtained.

The substrate concentration effect on mycelial FTase activities were also investigated. Compared with the concentration effects of extracellular FTase, the enzyme showed higher sensitivity to the sucrose concentration in the reaction medium, with a maximum value of A_t 282.57 ± 27.33 U·g^{-1} in 470.6 g·L^{-1} sucrose solution. The A_h was maintained at an average value of 63.66 ± 15.00 U·g^{-1} for the whole sucrose concentration range evaluated. The enzymatic kinetics of the transfructosylation was evaluated following the Hill model (Fig. 5B), whose error determination coefficient value R^2 = 0.926, indicating that this model accounts for 92.6 % of the variations in mycelial transfructosylation rate, with the values of the kinetic parameters ($V_{máx}$ 342.23 U g^{-1}, $K_{0.5}$ 234.73 g L^{-1}, n = 1.41). The Michaelis-Menten model was also tested; however, the low value of the determination coefficient renders it invalid for the explanation of the transfrutosilation velocities (data not shown). Hernalsteens (2008) reported that the enzyme produced by the strain *Rhodotorula* sp. also

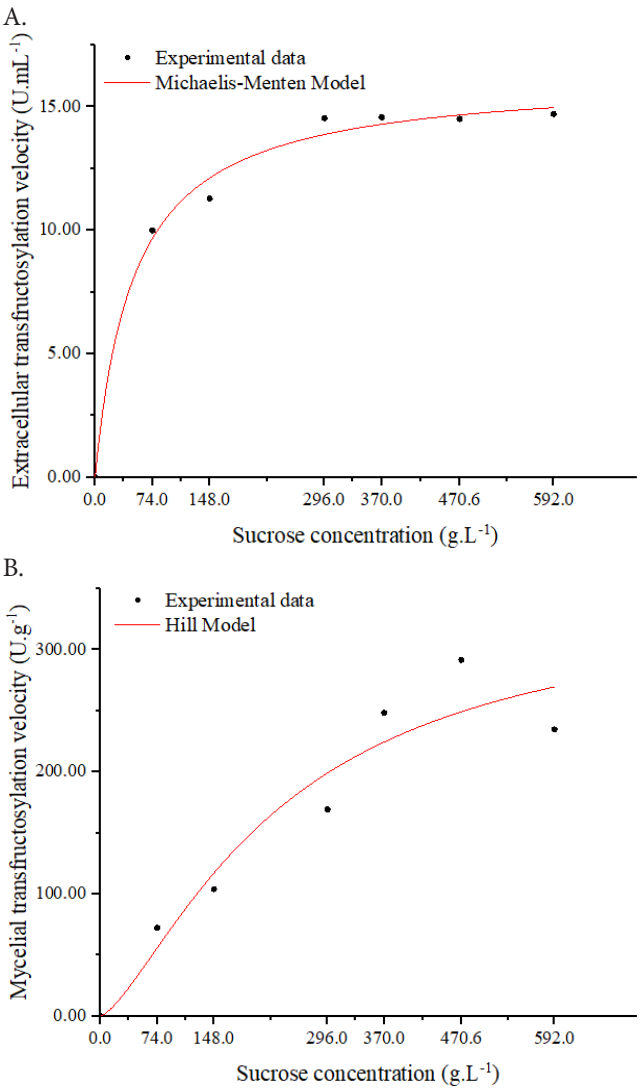

Figure 5. Michaelis-Menten model (*R^2=0.991*) for extracellular (A) and Hill model (*R^2=0.926*) for mycelial (B) FTase transfructosylation velocity at 50 °C and 0.2 mol L^{-1} tris-acetate buffer pH 5.5, comparing theorical results with experimental results.

showed cooperative kinetics according to the Hill Model for the transfructosylation rate, with values of the parameters $V_{máx}$, $Km_{0.5}$ and n of 299.0 g·L^{-1}, 0.08 g L^{-1} and 2.2, respectively.

Comparing the mycelial and extracellular FTases, an expressive increase of the Michaelis-Menten (K_m) constant of 4.7-fold was observed, indicating that the mycelial FTase exhibited lower affinity for the substrate. Hirayama et al. (1989) obtained values of K_m equal to 99.18 g L^{-1}, 273.6 g L^{-1}, 47.9 g L^{-1} and 126.54 g L^{-1}, for the enzyme purified from *Aspergillus niger* ATCC 20611 for the sugars sucrose, 1-kestose, nystose and *fructosylnystose*, respectively. Xu et al. (2015) obtained values of K_m and $V_{máx}$ equal to 56.05 g L^{-1} and 800.1 U mg^{-1} for sucrose as substrate, respectively, for the purified FTase of *Penicillum oxalicum*.

Optimization of enzymatic activities of extracelular and mycelial FTase with pH and temperature of the reaction medium

The factors and levels used, with coded and actual values, are listed in Table 1. Their respective responses to the extracellular and mycelial transfructosylation (A_t^{extra}, $A_t^{mycelial}$) and hydrolytic (A_h^{extra}, $A_h^{mycelial}$) activities are shown in Table 2. The responses were statistically analyzed and used to estimate the interaction effects with the quadratic model with interaction. According to the conditions used in the process, the A_t^{extra} values obtained varied from 3.75 U mL^{-1} to 15.85 U mL^{-1}. The central points for the three responses presented low variation, indicating, therefore, good repeatability of the process. Regarding mycelial FTase, the $A_t^{mycelial}$ ranged from 22.99 U·g^{-1} to 333.88 U·g^{-1}, while the $A_h^{mycelial}$ ranged from 28.63 U·g^{-1} to 106.40 U·g^{-1}. The obtained mycelial responses were treated statistically and, subsequently, the effects of the temperature and pH of the reaction medium were quantified.

According to Table 3, all effects were significant for the quadratic model with interaction for the influence of temperature and pH of the enzymatic reaction medium on the extracellular FTase transfructosylation activity, at a significance level of 5 % ($p < 0.05$). The adjusted model was described by Equation (4), where A_t^{extra} refers to extracellular A_t (U·mL^{-1}), T and pH represent the coded values for temperature and pH of the enzymatic reaction medium, respectively.

$$A_t^{extra} = 15.100 + 1.772.(T) - 2.591.(pH) - 3.290.(T)^2 - 4.297.(pH)^2 - 2.702.(pH).(T) \quad (4)$$

The effects of temperature and pH of the reaction medium on the quantification of A_h of extracellular FTase (A_h^{extra}) were also evaluated. According to the conditions used in the process (Table 1), A_h^{extra} values ranged from 0.65 U mL^{-1} to 10.15 U mL^{-1}, with very close values at the central points, representing good repeatability of the process. As shown in Table 3, the linear pH term (pH (L), in bold) was not significant and therefore excluded from the quadratic model with interaction (Equation 5). In the equation, A_h^{extra} refers to extracellular A_h (U mL^{-1}) and T and pH represent the coded values for temperature and pH, respectively.

$$A_h^{extra} = 0.793 - 1.573.(T) + 3.132.(T)^2 + 2.727.(pH)^2 + 2.045.(pH).(T) \quad (5)$$

Table 2. Levels of factors used in the experimental design and corresponding experimental values.

Factors		Extracellular activities		Mycelial activities	
Temperature (°C)	pH	A_t^{extra}	A_h^{extra}	$A_t^{mycelial}$	$A_h^{mycelial}$
		(U mL^{-1})		(U g^{-1})	
-1 (40)	-1 (5.5)	4.32	8.29	82.62	106.40
1 (50)	-1 (5.5)	15.86	1.08	333.88	81.17
-1 (40)	1 (8.0)	3.80	6.44	142.39	44.00
1 (50)	1 (8.0)	4.53	7.41	22.99	31.98
-1.414 (38)	0 (6.75)	8.23	10.15	31.59	28.63
1.414 (52)	0 (6.75)	9.58	5.66	222.16	40.45
0 (45)	-1.414 (5.0)	10.03	6.91	128.99	67.91
0 (45)	1.414 (8.5)	3.75	7.28	39.22	42.03
0 (45)	0 (6.75)	14.74	1.02	214.50	58.63
0 (45)	0 (6.75)	14.72	0.65	193.02	40.68
0 (45)	0 (6.75)	15.84	0.71	202.58	31.11

Table 3. Estimated effects, standard error and p-value for the evaluation of temperature and pH effects on extracellular FTase transfructosylation and hydrolytic activities.

Variables	Estimated effects	Standard error	p-value
Transfructosylation activity – Extracellular FTase			
Mean	15.10000	1.049679	0.000029
Temperature (L)	3.54480	1.285589	0.039961
Temperature (Q)	-6.58000	1.530156	0.007714
pH (L)	-5.18282	1.285589	0.010007
pH (Q)	-8.59500	1.530156	0.002475
pH x temperature	-5.40500	1.818097	0.031053
Hydrolytic activity – Extracellular FTase			
Mean	0.79333	0.720342	0.320925
Temperature (L)	-3.14745	0.882236	0.016085
Temperature (Q)	6.26417	1.050071	0.001894
pH (L)	1.25081	0.882236	0.215453
pH (Q)	5.45417	1.050071	0.003484
pH x temperature	4.09000	1.247670	0.022000

According to Rodrigues and Iemma (2009), the application of the F test to the model explains a significant amount of variation of the experimental data. The value of *F*, calculated by Equation (6), is compared with the tabulated value of a reference frequency distribution ($F_{\text{Graus of the model; degrees of freedom of the deviation; level of significance}}$).

$$\text{Test F} = \frac{\text{Regression mean squares}}{\text{Residual mean squares}} \quad (6)$$

In Table 4, the analysis of variance (ANOVA) is presented for the quadratic model with interaction applied for the transfructosylation and hydrolytic activities of extracellular FTase. The adequacy of the model was evaluated using the determination coefficient (R^2). For the transfructosylation activity, approximately 93.48 % of the variability of the observed responses can be explained by the adjusted model (Equation 4). As the coefficient of variation was high and the tabulated value of F for 95 % confidence is 5.05, inferior to the 14.303 obtained with the model, it was possible to affirm that the amount of variation due to the model was greater than the variation not explained, therefore, the model was considered valid.

The analysis of variance (ANOVA) was presented for the quadratic model with interaction applied for the hydrolytic activity of extracellular FTase (Table 4). The adjustment of the experimental responses to the model was evaluated by the coefficient of determination of error (R^2) and the F Test. It was observed that the coefficient of variation explained (R^2 = 0.9112) was high.

The results obtained by means of the response surface for the extracellular transfructosylation activity (Figure 6A) and its contour curves (Figure 6B) show an optimal zone between the temperatures of 45 °C and 50 °C and pH between 5.5 and 6.75. It should be emphasized that the results obtained by the experimental design consistently described the behavior of the enzymatic activities when evaluated, alone, under the influence of pH and temperature. While the present results indicated that the increases in temperature and pH of the reaction medium were significant, Cuervo-Fernandez et al. (2007) reported that, for the same pH and temperature ranges, only the increase in pH (5.5 to 8.0) was significant in the optimization studies, resulting in significant gains in transfructosylation activities.

Table 4. Results of the analysis of variance for the quadratic model with interaction for the evaluation of the temperature and pH effects of the enzymatic reaction medium in the transfructosylation and hydrolytic activities for the extracellular FTase.

Source	Sum of Squares	Degree of Freedom	Mean Square	F_{value}
Transfructosylation activity – Extracellular FTase				
Model	237.649	5	47.53	14.303
Residues	16.6146	5	3.32	
Lack of Fit	15.7058	3		
Pure Error	0.8216	2		
Total	254.2636	10		
R^2 = 0.93482	$F_{5;5;0,05}$ = 5.05			
Hydrolytic activity – Extracellular FTase				
Model	112.0986	4	28.02	15.408
Residue	10.9125	6	1.82	
Lack of Fit	10.8336	4		
Pure Error	0.0789	2		
Total	123.0111	10		
R^2 = 0.9112	$F_{4;6;0,05}$ = 4.53			

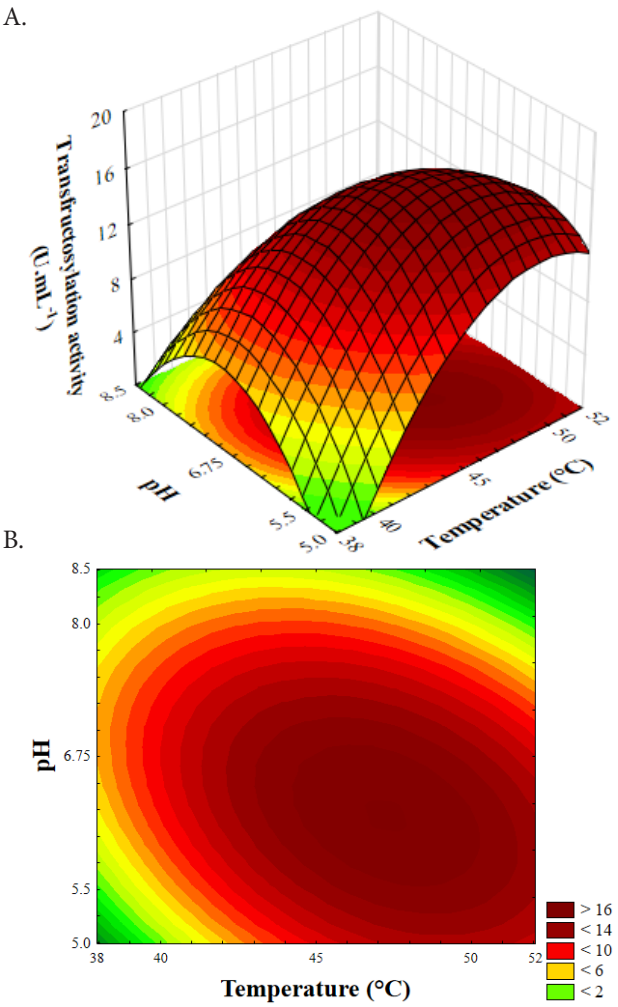

Figure 6. Response surface for the extracellular transfructosylation activity as a function of the pH and temperature of the enzymatic reaction medium (A); Contour curves for the extracellular transfructosylation activity as a function of the pH and temperature of the enzymatic reaction medium (B).

The developed model was represented by a response surface for the extracellular hydroliytic activity (Figure 7A) and the contour curves (Figure 7B), in order to analyze the interactions between the two dependent variables in the response and to obtain

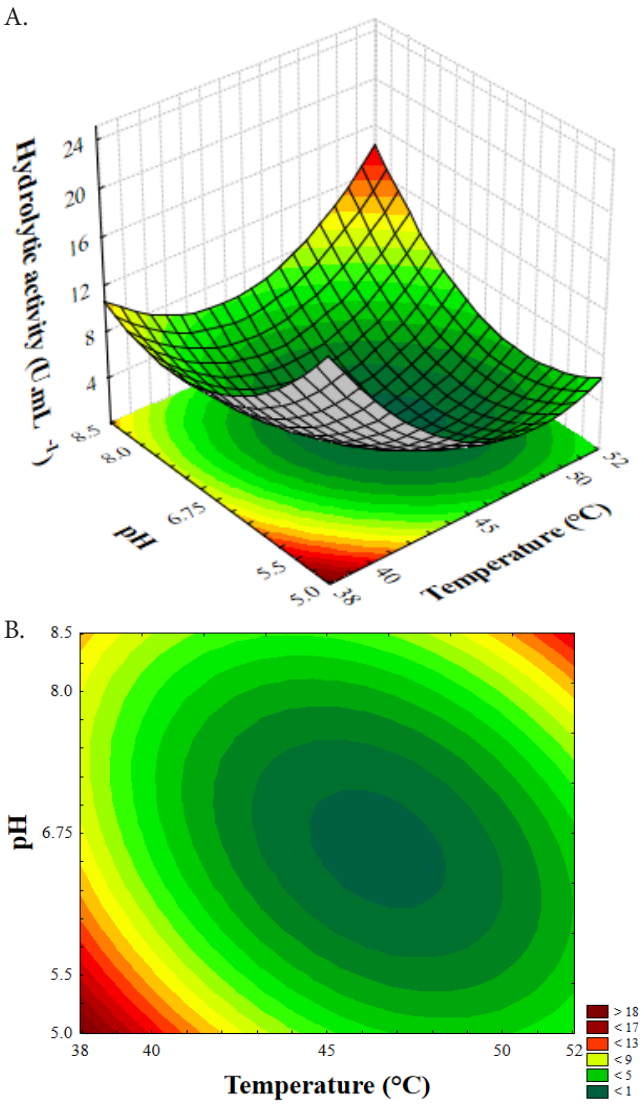

Figure 7.Response surface for extracellular hydrolytic activity as a function of pH and temperature of the enzymatic reaction medium (A); Contour curves for the extracellular hydrolytic activity as a function of pH and temperature of the reaction medium (B).

an optimal zone, where low A_h values are achieved. These low A_h values consequently result in high FOS production due to the impaired cleavage of sugar molecules with a high degree of polymerization. From the surfaces and curves obtained, the formation of an optimum zone between the temperatures of 45 ºC and 48 ºC and pH between 6.0 and 7.0 was observed.

The effects of temperature and pH of the reaction medium on the quantification of enzymatic activities of mycelial FTase ($A_t^{mycelial}$ and $A_h^{mycelial}$) were also evaluated. For the transfructosylation activity, the effects of the regression components (Table 5) and, in the 95 % confidence interval ($p < 0.05$), all were significant except the quadratic temperature term (temperature (Q), in bold). The adjusted model was described by Equation 7, where $A_t^{mycelial}$ refers to mycelial A_t ($U \cdot g^{-1}$), T and pH represent the coded values for temperature and pH of the enzymatic reaction medium, respectively.

$$A_t^{mycelial} = 203{,}366 + 50{,}170.(T) - 28{,}250.(T)^2 - 47{,}259.(pH) - 49{,}635.(pH)^2 - 92{,}665.(pH).(T) \quad (7)$$

Regarding hydrolytic activity, the high variation of the $A_h^{mycelial}$ values obtained, even at the central points where a good repetition was expected in order to reduce errors, directly reflected the calculated effects and their respective significance, in which none of the calculated terms except the mean (bold in Table 5), were significant at a significance level of 5.0 %. Thus, it was not possible to generate a model that explains the variation of mycelial hydrolytic activity as a function of pH and temperature of the enzymatic reaction medium.

The analysis of variance (ANOVA) of the quadratic model with interaction applied to the $A_t^{mycelial}$ as a function of the temperature and pH of the reaction medium (Table 6) presented the tabulated value of F for 95 % confidence of 5.05, inferior to the 11.317 obtained with the model. Another parameter analyzed was the coefficient of variation (R^2), whose value was equal to 0.9187, stating that 91.87 % of the total variation for the enzymatic activity response was due to the attribution of the mathematical model.

The results of the analysis of variance (ANOVA) showed that the quadratic model with interaction (Equation 7) was adequate for the prediction of $A_t^{mycelial}$, represented by the response surface (Figure 8A) as well as the respective contour curves (Figure 8B). The high values of $A_t^{mycelial}$ (values greater than 260.00 $U \cdot g^{-1}$) were detected in acid regions below pH 6.5 and at temperatures above 46 °C. The

Table 5. Estimated effects, standard error and p-value for evaluation of temperature and pH effects on the transfructosylation and hydrolytic activities of mycelial FTase.

Variable	Estimated effects	Standard Error	p-value
Transfructosylation activity – Mycelial FTase			
Mean	203.367	22.69200	0.000288
temperature (L)	100.342	27.79191	0.015373
temperature (Q)	-56.502	33.07899	0.148323
pH (L)	-94.518	27.79191	0.019232
pH (Q)	-99.272	33.07899	0.030063
pH x temperature	-185.330	39.30369	0.005264
Hydrolytic activity – Mycelial FTase			
Mean	43.4676	12.51806	0.017795
temperature (L)	-5.1342	15.33143	0.751339
temperature (Q)	1.6398	18.24805	0.932171
pH (L)	-37.0511	15.33143	0.060382
pH (Q)	22.0743	18.24805	0.280689
pH x temperature	6.6017	21.68191	0.772918

Table 6. Results of the ANOVA for the quadratic model with interaction described by the effects of temperature and pH on the transfructosylation activity of mycelial FTase.

Source	Sum of Squares	Degree of Freedom	Mean Square	F_{value}
Transfructosylation activity – Mycelial FTase				
Model	87414.47	5	17482.89	11.317
Residues	7723.90	5	1544.78	
Lack of Fit	7492.28	3		
Pure Error	231.62	2		
Total	95138.37	10		
$R^2 = 0.9187$	$F_{5;5;0,05} = 5.05$			

A.

B.

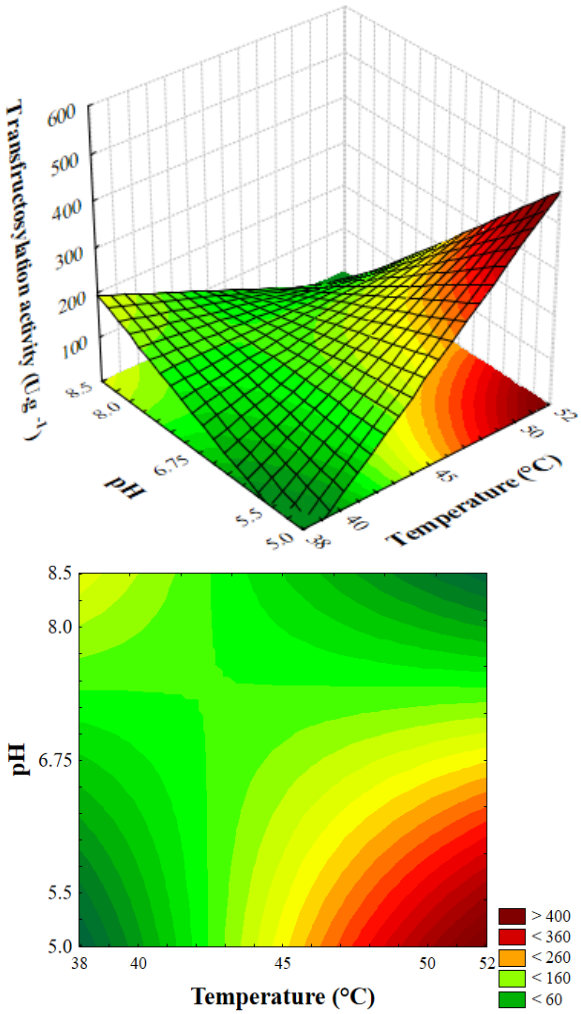

Figure 8. Response surface for the mycelial transfructosylation activity as a function of pH and temperature of the reaction medium (A); Contour curves for mycelial transfructosylation activity as a function of pH and temperature of the reaction medium (B).

behavior of mycelial FTase indicated the highest pH and temperature ranges because of greater stability due to the adhesion of the enzyme to the mycelium (Schuurmann et al., 2014).

CONCLUSIONS

Aspergillus oryzae IPT-301 showed maximum concentration of cellular biomass, 9.35 ± 1.26 g L^{-1}, in 48 h fermentation time. Extracellular FTase showed maximum A_t when produced in 64 h of fermentation and it presented stability above 80% between 30 ºC and 35 ºC and at pH 6.0. While the maximum mycelial FTase activity occurred in 72 h, indicating stability above 80% for pH ranges (6.0 - 8.0) and temperature (30 - 40 °C). The extracellular FTase showed michaelian kinetics in relation to the substrate concentration, while mycelial FTase adjusted to Hill's cooperative model. Regarding the optimization of reactional parameters for high enzymatic activities, it was proved that the optimal zone of the extracellular FTase occurred in the temperature ranges between 45 °C - 50 °C and pH between 5.5 - 6.75, large enough to resist the occurence of variation of the process parameters and keep high values of transfructosylation and low hydrolytic activities. As for mycelial FTase, the optimal zone for transfructosylation activity occurred at temperatures above 46 °C and at pH values lower than 6.5. These results will lead future research conducted on bioreactors to scale up and optimize the production of FTase on a larger scale aiming at high FOS production.

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