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ORIGINAL PAPER

Glutaraldehyde-crosslinked cells from *Aspergillus oryzae* IPT-301 for high transfructosylation activity: optimization of the immobilization variables, characterization and operational stability

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Abstract

Cells of *Aspergillus oryzae* IPT-301 rich in fructosyltransferase (FTase) were successfully immobilized by crosslinking with glutaraldehyde and used for the transfructosylation reaction of sucrose. The glutaraldehyde concentration and pH used in the immobilization process were optimized for maximizing the transfructosylation activity (A_T) and minimizing the hydrolytic activity (A_H). Also, the operational stability and the influence of temperature, pH and sucrose concentration on the enzymatic activities of the free and crosslinked cells were evaluated. Both the maximum A_T and minimum A_H were obtained for cells immobilized with glutaraldehyde concentration of 2.1% (v/v) and pH 7.9. Crosslinked cells showed considerably higher A_T/A_H ratio than free cells at several temperatures, pH and sucrose concentrations in the reaction media. Kinetics data suggested that crosslinked cells present higher substrate-enzyme affinity and transfructosylation rate than free cells. Furthermore, after 12 batch reaction cycles the FTase present in the immobilized cell kept 88.9% of its initial A_T, demonstrating a considerably higher operational stability than the FTase present in the free cell, which showed 50.3% of its initial A_T. These results suggest the potential use of crosslinked cells of *Aspergillus oryzae* IPT-301 for the large-scale production of fructooligosaccharides (FOS).

Keywords Fructosyltransferase · Crosslinked cells · Immobilization · *Aspergillus oryzae* · Fructooligosaccharides · Operational stability

Introduction

Fructosyltransferases (FTase, E.C. 2.4.1.9) are enzymes which catalyze transfructosylation reactions in sucrose molecules for the production of fructooligosaccharides (FOS) (Antosová and Pokovic 2001; Antosová et al. 2008; Muniz-Márques et al. 2016). They are industrially produced by fermentation from fungi belonging to the genera *Aspergillus*, *Penicillum*, *Aureobasidium*, *Fusarium* and *Rhodotorula*, being excreted to the culture medium (extracellular FTase) and/or remaining adhered to the microbial cells (mycelial FTase) (Wang 2015; L'Hocine et al. 2000; Ghazi et al. 2005; Aguiar-Oliveira and Maugeri 2010). FOS are fructose oligomers, whose fructosyl (F) units are bound to the terminal sucrose molecule (GF) by β-(2 → 1) glycosidic bonds, in which their main constituents are the sugars 1-kestose (GF2), nystose (GF3) and 1-β-fructofuranosyl nystose (GF4)

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(Yun 1996). The consumption of FOS, with low degree of polymerization, presents benefits to the human health for being low-calorie prebiotic sugars, non-cariogenic, for increasing the absorption of calcium and magnesium by the human organism, for reducing the levels of total cholesterol in the blood and for promoting the selectivity of bifidobacteria in the gut microbiota, helping in the elimination of pathogenic microorganisms and in the prevention of colon cancer (Vanková et al. 2008; Nobre et al. 2018).

One of the main parameters of evaluation associated to the enzymatic production of FOS refers to the ratio between the transfructosylation and hydrolytic activities (A_T/A_H) on the sucrose molecules (Hidaka et al. 1988). Route of FOS production consists in immobilizing extracellular FTase, present in the fermented broth or extracted from the microbial cells, in supports such as polymethacrylate (Ghazi et al. 2005), Amberlite IRA 900® (Platková et al. 2006) and a mixture of gelatin, sodium alginate and calcium chloride (Kamimura et al. 2009). Enzyme immobilization allows its separation from the reaction medium and the increase in its operational stability. The transfructosylation reaction can also be performed using microbial cells (Nobre et al. 2018; Sheu et al. 2013). Studies performed by Cuervo-Fernandez et al. (2007) showed that the mycelial FTase present in the microbial cells of *Aspergillus oryzae* IPT-301 presented a higher transfructosylation activity and, therefore, a higher potential for FOS production, among seventeen filamentous fungal strains investigated, with a ratio between activities ($A_{T/}A_H$) equal to 10.86. Ottoni et al. (2012) reported an FTase production with maximum transfructosylation activity of 950 U g^{-1}, respectively, employing the same microorganism, cultivated under optimized concentrations of sucrose, urea and yeast extract. Perna et al. (2018), cultivating *Aspergillus oryzae* IPT-301 by submerged fermentation, obtained a maximum ratio between activities (A_T/A_H) equal to 17, operating a 10 L bioreactor with a synthetic culture medium at 50 °C and pH 5.0. The immobilization of microbial cells is an alternative which enables the increase in operational stability (López-Gallego et al. 2005) and, furthermore, can be more advantageous than the extracellular enzymes immobilization, since it is not necessary to extract the enzyme from the microorganism and the natural structure of the biomass itself, in which the enzyme is adhered, can be used as support (Canilha et al. 2006). Chien et al. (2001) immobilized mycelial FTase from *Aspergillus japonicus* by encapsulation in gluten, obtaining a stable biocatalyst during 24 h of reaction. Ganaie et al. (2014) immobilized the mycelial FTase from *Aspergillus flavus* NFCCI 2364 by encapsulation in chitosan and alginate. The immobilization with alginate produced the highest transfructosylation activity (45 U mL^{-1}), and allowed the FTase reuse for 15 consecutive cycles, however, with a drop of 40% in FOS yield between the first and the last cycle. Similarly, the immobilization of *Aspergillus aculeatus*, in alginate, showed a FOS yield of 65.47% w/w, and it was reused during 15 cycles without significant drop in the enzymatic activity (Huang et al. 2016). Mussatto et al. (2009) and Castro et al. (2017) reported the immobilization of cells with mycelial FTase from *Aspergillus japonicus* ATCC 20,236 and *Aureobasidium pullulans*, in polyurethane foam, plant fibers, molecular sieves and glass foams. The highest FOS yields were reported for the cells immobilized in high porosity reticulated polyurethane foam.

Despite the increase in operational stability resulting from microbial cells immobilization, the use of external supports might impose diffusional restrictions to the reagents and products, limiting the enzymatic activity, or causing its total deactivation (Krasnan et al. 2016; Shuler and Kargi 2002). One of the techniques proposed to circumvent this problem is cells immobilization by reticulation, in which the cell structure itself is used as support (without the addition of an external support) by the addition of a chemical agent, which promotes cell crosslinking (Migneault et al. 2004a). The reticulation with glutaraldehyde of *Aspergillus flavus* with lipase adhered to the mycelium enabled an increase in operational stability, allowing biomass reuse for 13 consecutive cycles (Long et al. 1996). Sun et al. (2010) used whole *Rhizopus oryzae* cells reticulated with glutaraldehyde in the methanolysis reaction for biodiesel production from renewable oils. The reticulation promoted an increase in the operational stability of the cells from 10 to 15 cycles without significant drop in activity, which was attributed to the reduction in lipase desorption.

An increase in FOS production from mycelial FTase depends on an elevated transfructosylation activity, a high A_T/A_H and enough operational stability to enable its reuse in several cycles and, also, its application in continuous processes. Hence, cells rich in mycelial FTase must be immobilized so that the diffusion limitations and the loss of enzymatic activity are minimized. Few are the works in the literature on cell reticulation to obtain bioproducts and there are no studies on the immobilization by reticulation of cells of *Aspergillus oryzae* IPT-301 for FOS production. Therefore, the purpose of this work was to study the influence of the immobilization variables (glutaraldehyde concentration and pH) for glutaraldehyde-crosslinked cells from *Aspergillus oryzae* IPT-301, aiming at maximizing the transfructosylation activity, and minimizing the hydrolytic activity. Also, the operational stability and the influence of the process parameters (temperature, pH and concentration of sucrose of the reaction medium) on enzymatic activities of free and crosslinked cells were evaluated.

Materials and methods

Materials

All chemical reagents used were of analytical grade. Yeast extract, sucrose, KH_2PO_4, $MnCl_2.4H_2O$ and $FeSO_4{\cdot}7H_2O$

were acquired from Labsynth® (Diadema, Brazil). Glycerin and phenol were obtained from Isofar® (Duque de Caxias, Brazil). Glucose, $NaBH_4$, $NaNO_3$, $MgSO_4{\cdot}7H_2O$, NaOH, $Na_2S_2O_5$, $C_7H_4N_2O_7$ and $KNaC_4H_4O_6.4H_2O$ were purchased from Dinamica® (Diadema, Brazil). Potato dextrose agar was obtained from Kasvi® (São José dos Pinhais, Brazil) and the solution of glutaraldehyde Grade I (25% in water) was acquired from Sigma-Aldrich® (São Paulo, Brazil). The enzymatic colorimetric kit GOD-PAP for glucose determination was purchased from Laborlab® (Campinas, Brazil).

Microorganism and culture conditions

The fungus *Aspergillus oryzae* IPT-301was provided by the Institute for Technological Research (IPT/SP). The strain was cultivated in dishes with solid medium composed of (in %, m/v): potato dextrose agar 2.0, glycerin 2.5, yeast extract 0.5 and glucose 2.5 at 30 °C for 7 days. The inoculum was prepared by the suspension of spores in a 0.95% NaCl solution (m/v) and 0.1% Tween-80 (v/v) and dilution in glycerin solution at 20% (m/v) to obtain the concentration of 1×10^7 spores.mL^{-1}.

The experiments were performed in Erlenmeyer flasks containing 50 mL of culture medium with the following composition (in % m/v): sucrose 15.0, yeast extract 0.5, $NaNO_3$ 0.5, KH_2PO_4 0.2, $MgSO_4{\cdot}7H_2O$ 0.05, $MnCl_2{\cdot}4H_2O$ 0.03 and $FeSO_4$ $7H_2O$ 0.001. The pH of the medium was adjusted to 5.5 before sterilization. The flasks were inoculated with 0.5 mL of the spore suspension and incubated in an orbital shaker at 30 °C and 200 rpm for 64 h. At the end of the experiment, the samples were collected and filtered in filter paper (Whatman n°1) and the microbial cells (mycelium) was stored at 4 °C for further immobilization assays.

Assays of microbial cells crosslinking

Experimental design and statistical analysis

The experimental design of the type rotational central composite design 2^2 was chosen for the study of two factors: glutaraldehyde concentration (GLU) and pH of the reaction medium in microbial cells crosslinking assays, each one in five levels (Table 1). The values for the factors were chosen after a series of preliminary assays. The factors effects on the transfructosylation activity (A_T), on the hydrolytic activity (A_H) and on the ratio between the activities (A_T/A_H) were investigated for the crosslinked cells. The experimental matrix and the statistical analysis (Analysis of Variance—ANOVA) were obtained employing the software Statistica® version 7.0 (StatSoft. Inc. 2007, USA).

The response surface model was adjusted for two response variables, Y, named transfructosylation activity (U g^{-1}) and hydrolytic activity (U g^{-1}) of the crosslinked cells containing mycelial FTase. The enzymatic activity assays were performed as described in Sect. 2.4.1. The second-order response functions adjusted for the two factors were given by Eq. (1) and the differences were considered significant for p values ≤ 0.05.

$$Y = \beta_0 + \beta_1 A + \beta_2 B + \beta_{12} AB + \beta_{11} A2 + \beta_{22} B2, \quad (1)$$

in which A and B represent the levels of the factors GLU (%, v/v) and pH of the reaction medium, respectively, while β_0, β_1, β_2, β_{12}, β_{11} and β_{22} represent the estimated coefficients.

Table 1 Experimental design matrix

Runs	Coded values		Actual values	
	Glutaraldehyde concentration	pH	Glutaraldehyde concentration (%, v/v)	pH
1	−1	−1	1.20	6.40
2	+1	−1	3.00	6.40
3	−1	+1	1.20	9.40
4	+1	+1	3.00	9.40
5	−1.414	0	0.83	7.90
6	+1.414	0	3.37	7.90
7	0	−1.414	2.10	5.78
8	0	+1.414	2.10	10.01
9	0	0	2.10	7.90
10	0	0	2.10	7.90
11	0	0	2.10	7.90
12	0	0	2.10	7.90

Assays of cells immobilization by reticulation

The microbial cells (mycelium) immobilization assays were performed according to the adaptation of the methodology reported by Sun et al. (2010) and Vescovi et al. (2016). Immobilization was done in Erlenmeyer flasks containing 0.1 g of cells in 10 mL of reaction medium composed of a 25% glutaraldehyde solution (v/v) and a tris–acetate buffer solution at 0.2 mol L^{-1}. The pH of the buffer solution and the glutaraldehyde concentration were defined by the experimental design (Sect. 2.3.1). The immobilization reaction was performed in an orbital shaker at 25 °C and 200 rpm for 1 h and terminated, for further 30 min, by the addition of 0.1 mL of $NaBH_4$ 100 g L^{-1} previously dissolved in a NaOH solution at 1×10^{-3} mol L^{-1}. At the end of the reaction, the samples were collected and filtered in filter paper (Whatman no. 1). The immobilized mycelium (crosslinked cells) was abundantly washed with distilled water and stored at 4 °C for further characterization studies of the process parameters (temperature, pH and concentration of sucrose from the reaction medium) and operational stability assays.

Analytical methods

Enzymatic activity assays

The free and crosslinked cells transfructosylation (A_T) and hydrolytic (A_H) activities were determined as follows: 0.05 g of mycelium (free or crosslinked cells) were added to 3.7 mL of sucrose at 480.2 g L^{-1} and 1.2 mL of a tris–acetate buffer at 0.2 mol L^{-1} pH 5.5. The reaction was performed in a Dubnoff bath at 50 °C and 190 rpm for 1 h and interrupted by boiling (boiling water) for 10 min and ice bath for 5 min (Cuervo-Fernandez et al. 2007; Ottoni et al. 2012; Cunha et al. 2019). The reaction medium was filtered in filter paper (Whatman no. 1) and the concentrations of reducing sugars and glucose were quantified by DNS and GOD-PAP® methods (Sect. 2.4.2), respectively. One unit of transfructosylation and hydrolytic activity was defined as the amount of enzyme which transfers 1 µmol of fructose (transfructosylated fructose) or releases 1 µmol of fructose, respectively, per minute at the experimental conditions assayed (Cuervo-Fernandez et al. 2007; Ottoni et al. 2012; Ganaie et al. 2014; Cunha et al. 2019).

Analysis of carbohydrates

The glucose [G] and reducing sugars [RS] concentrations were obtained by the enzymatic colorimetric GOD-PAP® (glucose oxidase peroxidase 4-aminoantipyrine) method (Vega and Zúniga-Hansen 2011; Ganaie et al. 2014; Cunha et al. 2019) and DNS (3,5-dinitrosalicylic acid) (Miller 1959), respectively. The fructose [F] and transfructosylated fructose [F_T] concentrations in the reaction medium were determined by Eqs. (2) and (3) (Chen and Liu 1996; Cunha et al. 2019).

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

$$[F_T] = [G] - [F]. \tag{3}$$

Temperature-activity and pH-activity profiles

Enzymatic activities of free and crosslinked cells were measured in the temperature range of 30 °C–65 °C (pH 5.5) and at pH 3.5–7.5 (50 °C), using sucrose solution (480.2 g L^{-1}) as substrate. Transfructosylation (A_T) and hydrolytic (A_H) activities were determined under conditions as described in Sect. 2.4.1. The experiments were conducted in triplicate.

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

Enzymatic activities of free and crosslinked cells were determined using 3.7 mL of sucrose p.a. solution at different concentration (75.5, 151.0, 264.3, 377.5, 480.2 and 604.1 g L^{-1}) plus 1.2 mL tris acetate buffer 0.2 mol L^{-1} at pH 5.5. Transfructosylation (A_T) and hydrolytic (A_H) activities were determined under conditions as described in Sect. 2.4.1. The experiments were conducted in triplicate. For enzymatic kinetics evaluation, the sets of experimental data obtained for the A_T activities were fitted to the Michaelis–Menten model and the Hill model. Kinetic parameters such as maximum reaction rate (*Vmax*) and Michaelis constant (*Km*), and Hill constant (*n*) were obtained using a nonlinear regression analysis.

Operational stability assays of the free and crosslinked cells

Assays of operational stability of the free and crosslinked cells at the optimal conditions were performed. For this, 0.05 g of cells, free or cross-linked, containing mycelial FTase, was added to the reaction medium containing 3.7 mL of sucrose 480.2 g L^{-1} and 1.2 mL of tris–acetate buffer at 0.2 mol L^{-1} pH 5.5. The enzymatic reaction assays were conducted according to the method described in Sect. 2.4.1. At the end of each cycle, corresponding to 1 h of reaction, the free or crosslinked cells was removed from the reaction medium by vacuum filtration, washed with 100 mL of distilled water for the reagents molecules removal and/or products of its microenvironment. After washing, the microbial cell was introduced again in a new and similar reaction medium. The transfructosylation activity was evaluated considering the number of reaction cycles. The experiments were performed in triplicate.

Results and discussion

Optimization of the variables for crosslinked cells by experimental planning

In Table 2, the transfructosylation (A_T) and hydrolytic (A_H) activities values of crosslinked cells are presented in relation to the immobilization variables (glutaraldehyde concentration and pH), according to the experimental design described in Sect. 2.3.1. The values of A_T varied between 357.38 and 844.08 U g^{-1}, whereas the values of A_H varied between 113.26 and 282.38 U g^{-1}. On the other hand, the values of A_T and A_H, obtained in the central point, presented low variation, which indicates a good reproducibility of the reticulation process.

As described in Table 3, for the transfructosylation activity (A_T) the linear and quadratic terms of the glutaraldehyde concentration effects and the quadratic effect of the pH were significant, for a level of significance of 5% ($p < 0.05$). These variables were used to describe the quadratic model (Eq. 4).

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

Factors		Transfructosylation (A_T) and hydrolytic (A_H) activities		
Glutaraldehyde concentration [GLU] (%, v/v)	Immobilization pH	A_T (U g^{-1})	A_H (U g^{-1})	A_T/A_H
− 1 (1.2)	− 1 (6.4)	558.54	176.46	3.17
1 (3)	− 1 (6.4)	464.46	150.08	3.09
− 1 (1.2)	1 (9.4)	554.85	199.23	2.78
1 (3)	1 (9.4)	357.38	156.15	2.29
− 1.414 (0.83)	0 (7.9)	675.85	282.38	2.39
1.414 (3.37)	0 (7.9)	594.54	199.31	2.98
0 (2.1)	− 1.414 (5.78)	420.69	149.00	2.82
0 (2.1)	1.414 (10.01)	363.46	83.46	4.35
0 (2.1)	0 (7.9)	785.85	118.92	6.61
0 (2.1)	0 (7.9)	809.31	135.54	5.97
0 (2.1)	0 (7.9)	844.08	134.46	6.28
0 (2.1)	0 (7.9)	843.31	116.23	7.26

Table 3 Estimated effects, standard error and p value for the evaluation of glutaraldehyde concentration (GLU) and pH effects on transfructosylation activity after the crosslinked cells

Variables	Estimated effects	Standard error	p value
Mean	820.64	18.47	0.000
[GLU] (L)*	**− 101.63**	**26.12**	**0.008**
[GLU] (Q)**	**− 200.36**	**29.21**	**0.0005**
pH (L)*	− 47.93	26.12	0.116
pH (Q)**	**− 443.48**	**29.21**	**0.000005**
[GLU] × pH	− 51.69	36.94	0.21

The significant values are given in bold

*L refers to the linear term of the statistical model

**Q refers to the quadratic term of the statistical model

The pH linear and the interaction effects between the two variables were not significant.

$$A_T = 820.63 - 50.81([GLU]) - 100.17([GLU])^2 - 221.73(pH)^2, \quad (4)$$

in which A_T, [GLU] and pH are the crosslinked cells transfructosylation activity, glutaraldehyde concentration and the pH employed in the immobilization, respectively.

Table 4 presents the analysis of variance (ANOVA) for the transfructosylation activity (A_T) quadratic model of the crosslinked cells (Eq. 4). The coefficient of error determination (R^2) obtained indicates that 95.84% of the variability of the responses observed can be explained by this model. By the *F Test*, it was verified that the model explains a significant amount of variation of the experimental data, since, for the level of significance of 5%, the calculated *F* value was higher than the tabulated *F* value of a reference frequency distribution (F; degrees of freedom of the model; degrees of freedom of the deviation; level of significance) (Rodrigues and Iemma 2009). The high R^2 and the *F test* value indicate that the amount of variation due to the model is higher than the unexplained variation and, therefore, the model can be considered valid.

According to the response surface (Fig. 1a) and the contour curve (Fig. 1b), obtained from the A_T model (Eq. 4), there is an optimum region of values of glutaraldehyde concentration, between 1.65 and 2.3% (v/v), and pH, from 7.5

Table 4 Results of the analysis of variance for the quadratic model with interaction for the evaluation of the glutaraldehyde concentration (GLU) and pH effects on transfructosylation activity after the crosslinked cells

Source	Sum of squares	Degree of freedom	Mean square	F_{value}	p value
Model	356,113.50	3	118,704.5	61.44	< 0.0001
Residues	15,455.60	8	1931.95		
Lack of fit	13,053.60				
Pure error	2402.10				
Total	371,569.10	11			
$R^2 = 0.958$	$F_{3;8;0.05} = 4.07$				

to 8.3, which enable the A_T maximization of the crosslinked cells.

For the A_H of the crosslinked cells, only the linear and quadratic effects terms of the glutaraldehyde concentration were significant, for a level of significance of 5% ($p < 0.05$) (Table 5) and, therefore, they were used in the interaction model (Eq. 5). The effects of the immobilization pH and the interaction between the two variables were not significant:

$$A_H = 120.65 - 23.367([GLU]) + 56.67([GLU])^2, \quad (5)$$

in which A_H and [GLU] are the crosslinked cells hydrolytic activity and the glutaraldehyde concentration used in mycelium immobilization, respectively.

Table 6 presents the analysis of variance (ANOVA) for the quadratic model with interaction applied to the hydrolytic activity of the crosslinked cells (Eq. 5). The coefficient of error determination (R^2) indicates that 88.14% of the variability of the responses observed can be explained by this model. The value of the calculated F (33.44) for the model was higher than the value of the tabulated F (4.26) at a level of significance of 5%. The high value of the R^2 and the *F test* indicate that the model is valid to represent the experimental data of the hydrolytic activity of crosslinked cells.

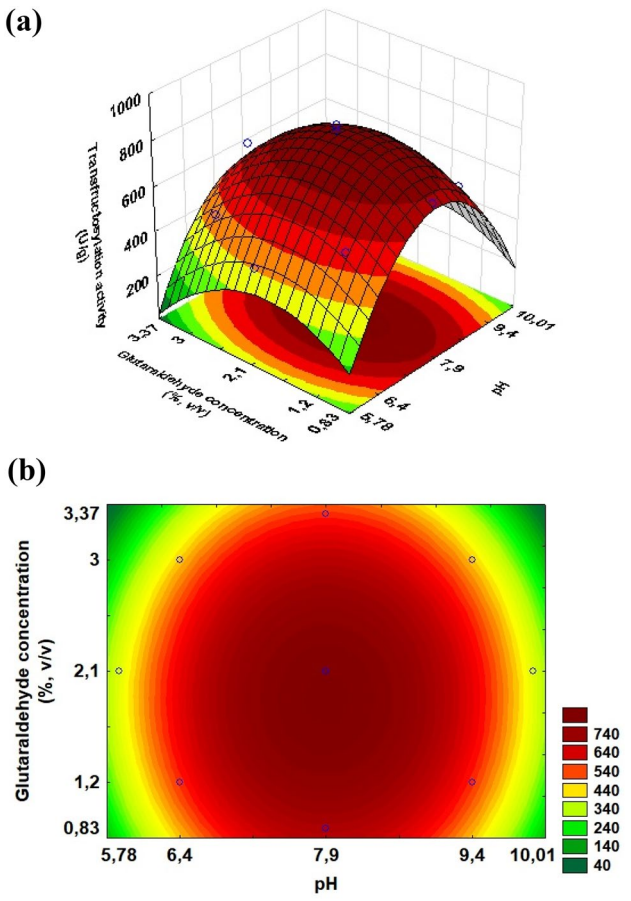

Fig. 1 Transfructosylation activity as a function of the glutaraldehyde concentration (in %, v/v) and immobilization pH: **a** response surface and **b** contour curves

Table 5 Estimated effects, standard error and p value for the evaluation of glutaraldehyde concentration (GLU) and pH effects on hydrolytic activity after the crosslinked cells

Variables	Estimated effects	Standard error	p value
Mean	126.2875	10.35	0.00002
[GLU] (L)*	**−46.7347**	**14.64**	**0.019**
[GLU] (Q)**	**110.5287**	**16.37**	**0.0005**
pH (L)*	−15.9619	14.64	0.32
pH (Q)**	−14.0862	16.37	0.42
[GLU] × pH	−8.35	20.70	0.7

The significant values are given in bold

*L refers to the linear term of the statistical model

**Q refers to the quadratic term of the statistical model

The response surface (Fig. 2a) and the contour curve (Fig. 2b), obtained from the model described by Eq. 5, indicate that there is an optimal region of glutaraldehyde concentration, between 2.0 and 2.5% (v/v), for which the cells hydrolytic activity reaches its lowest values, regardless of the immobilization pH. It is worth highlighting that, for this same region of glutaraldehyde concentration, the transfructosylation activity reached its highest values (Fig. 1), which represents a simultaneous gain in the ratio A_T/A_H.

Correlating the transfructosylation (A_T) and hydrolytic (A_H) activities values of the crosslinked cells in relation to the immobilization variables (Table 2), it was observed that the highest values of the ratio A_T/A_H were obtained for the glutaraldehyde concentration of 2.1% (v/v) and pH 7.9, conditions in which the transfructosylation and hydrolytic activities were maximized (Fig. 1) and minimized (Fig. 2), respectively. The high values of the ratio A_T/A_H obtained suggest a rise in the affinity between the mycelial FTase (enzyme adhered to the microbial cells) and the sucrose molecules (acceptor substrate), caused by possible alterations of the enzyme structure after crosslinked cells with glutaraldehyde. As reported by Antosová and Polakovic (2001), Perna et al. (2018), Cunha et al. (2019) and Huang et al. (2016), FTase presents, concomitantly, transfructosylation and hydrolytic activities. The low affinity of FTase by the water molecules present in the reaction medium, which reduces the hydrolytic activity (Antosová and Polakovic 2001), and the transfructosylation activity predominance for a sucrose concentration higher than 200 g L^{-1} favor high A_T/A_H ratios (Kim et al. 2000). The results obtained suggest that the reticulation of the microbial cell wall with glutaraldehyde can affect its hydrophilicity, limiting its interaction with water and, therefore, reducing the hydrolytic activity because of the lack of water molecules in the cellular

Table 6 Results of the analysis of variance for the quadratic model with interaction for the evaluation of the glutaraldehyde concentration (GLU) and pH effects on hydrolytic activity after the crosslinked cells

Source	Sum of squares	Degree of freedom	Mean square	F_{value}	p value
Model	25,780.46	2	12,890.23	33.44	< 0,0001
Residues	3468.78	9	385.42		
Lack of fit	3160.95				
Pure error	307.83				
Total	29,249.24	11			
$R^2 = 0.8814$	$F_{2;9;0.05} = 4.26$				

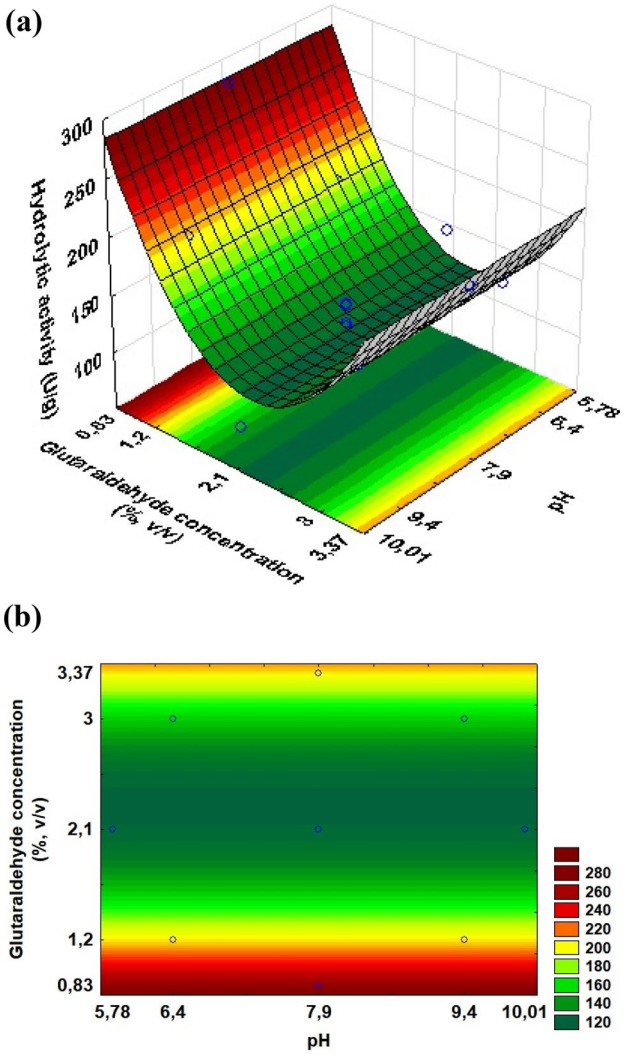

Fig. 2 Hydrolytic activity as a function of the glutaraldehyde concentration (in %, v/v) and immobilization pH: **a** response surface and **b** contour curves

microenvironment. On the other hand, the gain in the transfructosylation activity might be associated to the rise in the sucrose concentration molecules on the fungal cell wall, favored by the lower disaccharide polarity when compared to the water molecule. Therefore, an increase in the ratio A_T/A_H is observed and, consequently, a favorable situation for FOS production with higher yields (Aguiar-Oliveira and Maugeri 2010; Hidaka et al. 1988; Maresma et al. 2010).

It must be emphasized that it was not possible to directly analyze the behavior of the ratio (A_T/A_H) by experimental design, since, when analyzing the values obtained for the response ratio (A_T/A_H), by means of the Shapiro–Wilk adherence test regarding population normality (Rodrigues and Iemma 2009), it is observed that the results did not constitute a normal distribution (Gauss distribution). Since the experimental design is based on parametric statistics and, therefore, is strongly dependent on the normality of the data analyzed, results from an analysis performed by a rotational central composite design would not be trustworthy for the response ratio A_T/A_H.

Effect of reaction temperature on the enzymatic activities of the free and crosslinked cells

Figure 3a, b show the influence of reaction temperature on the enzymatic activities of the free and crosslinked cells. The free and immobilized cells showed the highest A_H at 65 °C and showed a rise of the A_T and A_H at higher temperatures, which can be attributed to the rise of collisions between substrate molecules and active sites (Fields 2001; Shuler and Kargi 2002). Also, these results are in agree with the optimum temperature ranges reported for the transfructosylation reaction of sucrose (Almeida et al. 2005; Ganaie et al. 2014; Schuurmann et al. 2014). Free cells from *Aspergillus niger* ATCC 20,611, *Aspergillus* sp. and *Xanthophyllomyces dendrorhous* also showed the highest A_T at 60 °C (Hirayama et al. 1989; Cuervo-Fernandez et al. 2004; Ning et al. 2010). In the present work, the free and crosslinked cells showed the highest A_T at 65 °C and 60 °C, respectively (Fig. 3a, b). Also, the A_T obtained from crosslinked cells at 60 °C was higher than the A_T showed by free cells at the same temperature. The opposite effect of reaction temperature on the A_T was reported by Ganaie et al. (2014) for cells from *Aspergillus flavus* NFCCI 2364 encapsulated in sodium alginate. In their work the free cells showed the highest A_T at 50 °C while immobilized cells showed the highest A_T at 60 °C. In the present work the highest A_T/A_H ratio of the free cell

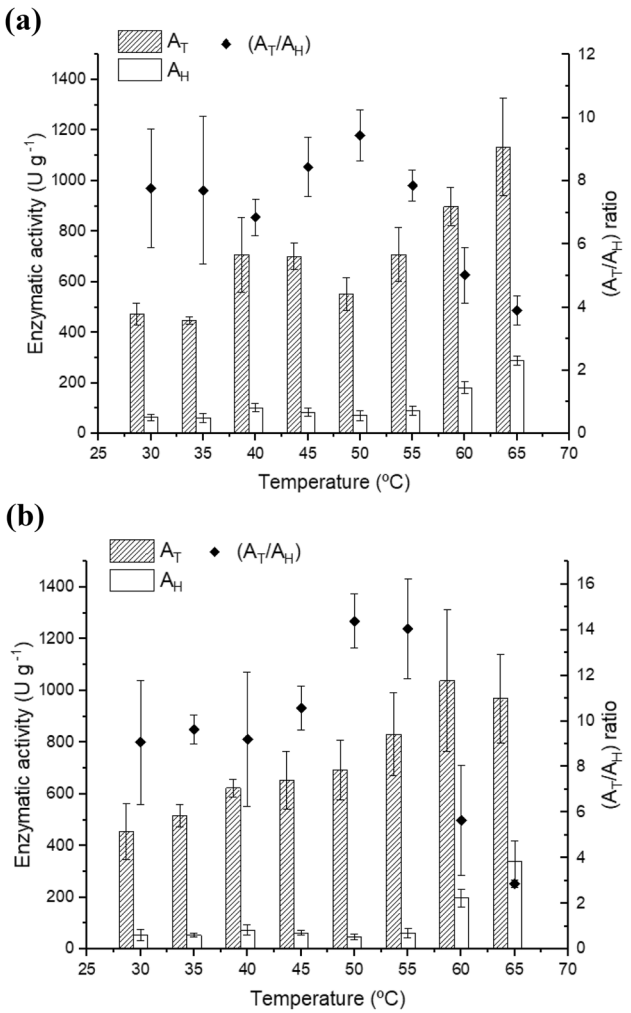

Fig. 3 Effect of reaction temperature on transfructosylation (A_t) and hydrolytic (A_h) activities, and activities (A_t/A_h) ratio. **a** Free cells and **b** crosslinked cells. Reaction condition: 48.02% (w/v) sucrose solution, 0.2 mol L^{-1} tris acetate buffer, pH 5.5

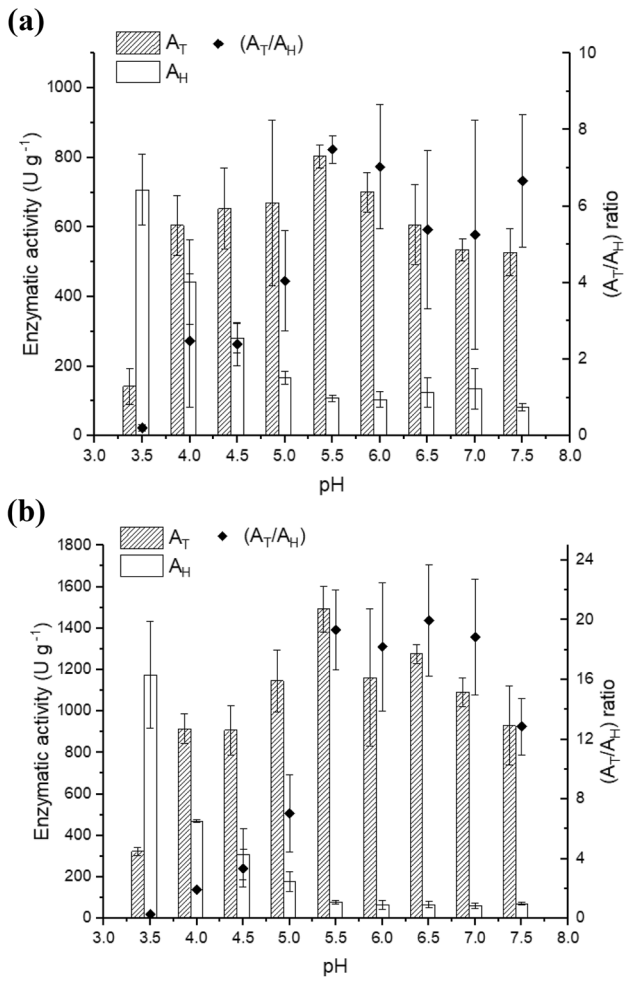

Fig. 4 Effect of reaction pH on transfructosylation (A_t) and hydrolytic (A_h) activities, and activities (A_t/A_h) ratio. **a** free cells and **b** crosslinked cells. Reaction condition: 48.02% (w/v) sucrose solution, 0.2 mol L^{-1} tris acetate buffer at 50 °C

(9.44 ± 0.82) was obtained at 50 °C (Fig. 3a), in accordance with Cuervo-Fernandez et al. (2007). The crosslinked cells of *Aspergillus oryzae* IPT-301 showed a considerable increase in the highest A_T/A_H ratio (14.38 ± 1.19) at the same temperature (50 °C), suggesting that the cell immobilization by crosslinking with glutaraldehyde is favorable for FOS production.

Effect of reaction pH on the enzymatic activities of the free and crosslinked cells

Figure 4a, b show the influence of the reaction pH on the enzymatic activities of the free and crosslinked cells. The free and crosslinked cells showed the highest A_T (803 ± 34 U g^{-1} and 1492 ± 111 U g^{-1}, respectively) at pH 5.5. However, the crosslinked cells showed higher A_T than the free cells at all the reaction pHs, suggesting that the crosslinking process made the enzyme more resistant to variations of ionic forces in the reaction medium (Long et al. 1996; Shuler and Kargi 2002). Similarly, Ganaie et al. (2014) reported changes in the effect of the reaction pH after immobilization of cells from *Aspergillus flavus* NFCCI 2364 by encapsulation in sodium alginate. In that study the free cells showed the highest A_T at pHs between 5.0 and 7.0, and maximum A_T (40 U mL^{-1}) at pH 6.0, while the immobilized cells showed the highest A_T at pHs between 5.0 and 8.0 with maximum A_T (45 U mL^{-1}) at pH 7.0. Ning et al. (2010) also reported the highest A_T (15 U g^{-1}) at pH 7.0 for enzyme present in cells from *Xanthophyllomyce dendrorhous*. In the present work the free and crosslinked cells showed the highest A_H (707 ± 102 U g^{-1} and 1174 ± 257 U g^{-1}, respectively) at pH 3.5 (Fig. 4a, b), in agree with the optimum pH reported for acid hydrolysis of sucrose (Aguiar-Oliveira and Maugeri

2010). The free cells showed the highest A_T/A_H ratios at pHs between 5.5 and 7.5, and the highest A_T/A_H ratio (7.49 ± 0.36) at pH 5.5. The crosslinked cells showed the highest A_T/A_H ratios at pHs between 5.5 and 7.0, and its highest value (19.97 ± 3.74) at pH 6.5. Also, at pH 5.5 the A_T/A_H ratio of the crosslinked cells was considerable higher (19.33 ± 2.68) than that showed by the free cells (Fig. 4a), suggesting that the immobilization of cells of *Aspergillus oryzae* IPT-301 by crosslinking changed the influence of the reaction pH on the A_T and A_H, leading to the increase of the A_T/A_H, which is favorable for FOS production.

Kinetic parameters of the transfructosylation reaction of sucrose on free and crosslinked cells

Figure 5a, b show the effect of the sucrose concentration on the enzymatic activities of the free and crosslinked cells, respectively. The highest A_T of the free and crosslinked cells were obtained at sucrose concentration of 377.5 g L^{-1}. However, the crosslinked cells showed higher A_T than the free cells at all the sucrose concentrations. The crosslinked cells showed a reduction of the A_H as the sucrose concentration increased, while the free cells showed the opposite behavior. Similarly, Zeng Kang et al. (2016) reported a reduction of the A_H as the sucrose concentration increased. The crosslinked cells showed the highest A_T/A_H ratio (15.1 ± 3.3) at 480.2 g L^{-1}.

The kinetics of the transfructosylation reaction of sucrose on free and crosslinked cells of *Aspergillus oryzae* IPT-301were fit to the Hill and Michaelis–Menten models (Fig. 6a, b). The kinetic of the free cells showed an error determination coefficient (R^2) of 0.940 and 0.910 for the Hill and Michaelis–Menten models, respectively. The kinetic of the crosslinked cells showed a R^2 of 0.957 and 0.954 for the Hill and Michaelis–Menten models, respectively. Hence, the Hill model showed the best fit for free and immobilized cells. Ghazi et al. (2005) reported that the Hill model is applicable for enzymes that transfer fructose groups. Also, this model is suitable for enzymes that show more than one catalytic site and multiple subunits such as FTases, which are found in dimeric form (Weiss 1997; Aguiar-Oliveira and Maugeri 2010; Luscher et al. 1996).

The kinetic parameters obtained for the Hill and Michaelis–Menten are listed in Table 7. The Hill coefficients (*n*) obtained for the free and crosslinked cells suggest that there is a positive cooperative behavior, in which the first sucrose molecule that reacts on the enzyme causes an increase in affinity between the rest of active sites and substrate molecules. Also, according to this coefficient the crosslinked cells showed less cooperativity than the free cells. Furthermore, the crosslinked cells showed higher $K_{0,5}$ than the free cells, while the free cells showed higher Km than the crosslinked cells, suggesting an increase in the substrate-enzyme affinity after immobilization (Shuler and Kargi 2002). Besides

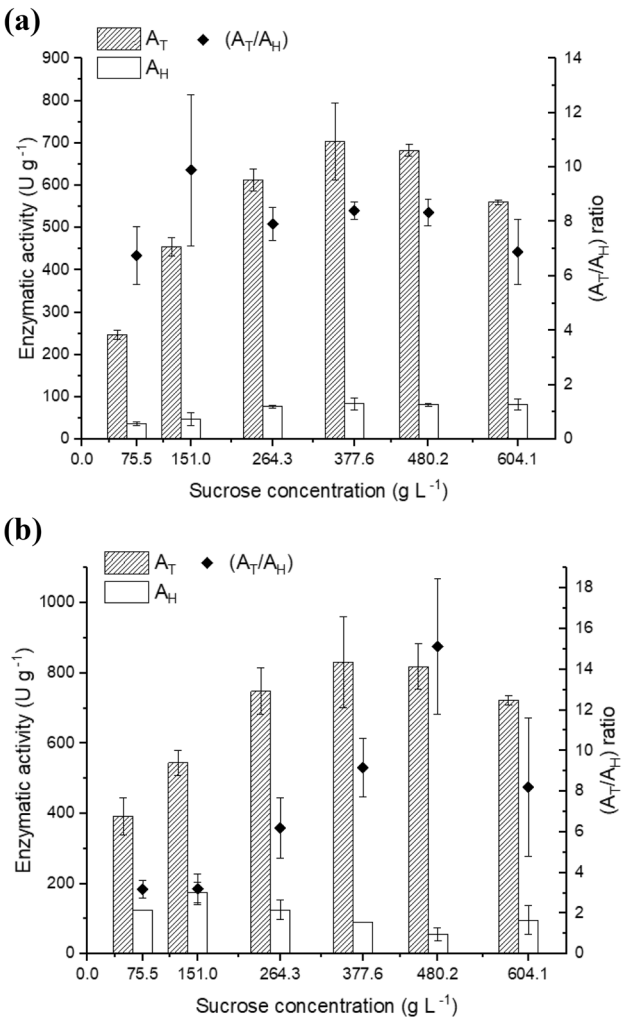

Fig. 5 Effect of substrate concentration on transfructosylation (A_T) and hydrolytic activities (A_H) and (A_T/A_H) ratios. **a** Free cells and **b** crosslinked cells. Reaction conditions: 0.2 mol L^{-1} tris–acetate buffer solution pH 5.5 at 50 °C

that the crosslinked cells showed higher V_{max} than the free cells. These results are in agreement with the increase of A_T/A_H ratios obtained after immobilization, suggesting that the crosslinking process improved the transfer of fructose groups to the sucrose molecule and decreased the hydrolytic activity.

Operational stability of the free and crosslinked cells

Figure 7 presents the results for the operational stability assays for the free and crosslinked cells, characterized by the relative transfructosylation activity along sequential reaction cycles, determined according to Sect. 2.7.

It is observed that after 12 reaction cycles, the free cells suffered a dramatic fall in activity, with final relative activity

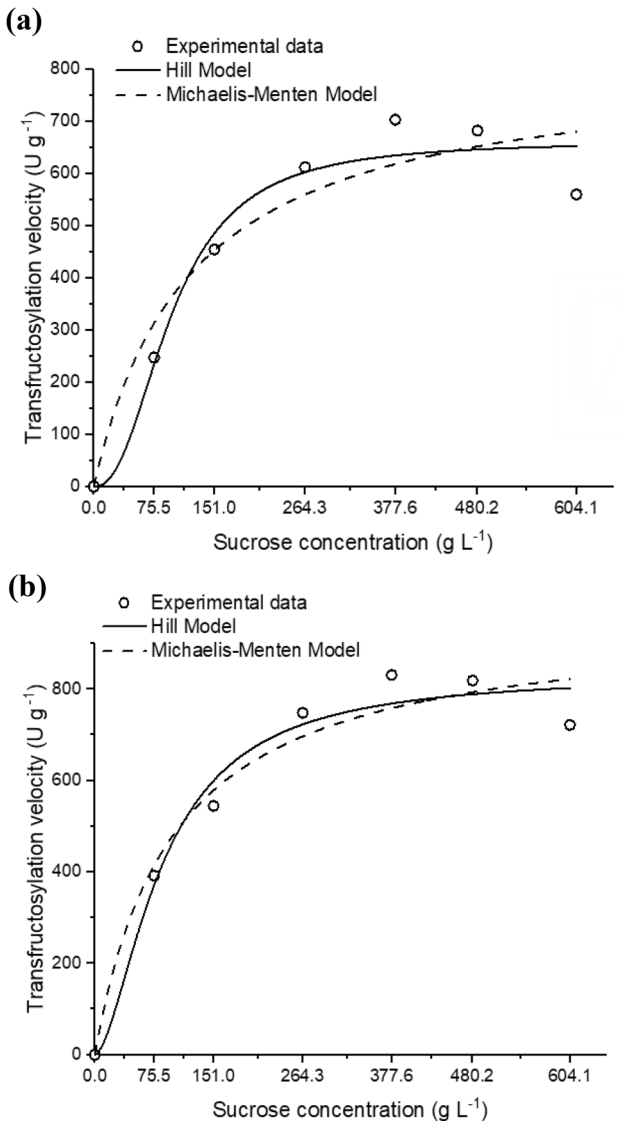

Fig. 6 Michaelis–Menten and Hill models for **a** free cells and **b** crosslinked cells at 50 °C and 0.2 mol L^{-1} tris–acetate buffer pH 5.5, comparing theorical results with experimental results

Table 7 Estimated kinetic parameters for free and crosslinked cells

Kinetic parameters	Free cells		Crosslinked cells	
	Hill model	Michaelis–Menten model	Hill model	Michaelis–Menten model
V_{max} (U g^{-1})	662.34	817.50	833.08	956.06
$K_{0,5}$ (g L^{-1})	97.80	–	85.90	–
K_m (g L^{-1})	–	121.50	–	98.50
n	2.33	–	1.67	–

of 50.3 ± 3.9%, whereas the crosslinked cells presented a relative activity of 88.9 ± 2.2%. This result indicates that there was a significant extension in the mycelial FTase operational stability after the immobilization by reticulation with glutaraldehyde. Currently, there are no studies reported on crosslinked cells, containing mycelial FTase, for FOS production. The main applications reported on the reticulation of enzymes adhered to the mycelium aimed at the immobilization of whole lipase cells for biodiesel production. Nevertheless, it is important to highlight that, similarly to the results obtained in this work, Sun et al. (2010) reported an increase in the operational stability of *Rhizopus oryzae* whole cell, containing mycelial lipase, after reticulation with glutaraldehyde. The free and crosslinked cells presented 10% and 90% of their initial activities after ten reaction cycles in batch, respectively. Likewise, Long et al. (1996) reported an increase in thermal and operational stability of *Aspergillus flavus* mycelial FTase after reticulation with glutaraldehyde, maintaining the enzymatic activity for 13 sequential batch cycles. Additionally, Szczesna-Antczak et al. (2004) presented that the process of reticulation with glutaraldehyde was more efficient than the technique of calcium alginate and polyvinyl alcohol encapsulation for the immobilization of pellets of *Mucor circinelloides* mycelial lipase used in the conversion of caprylic acid for five reaction cycles.

The whole cells with mycelial FTase have been mainly immobilized by encapsulation or absorption during fermentation, but few studies have reported the effect of these

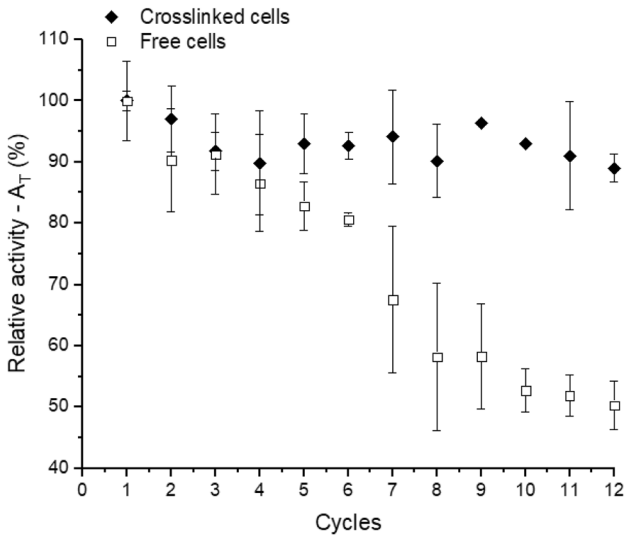

Fig. 7 Operational stability of the free and crosslinked cells along 12 batch cycles, totalizing 12 h of transfructosylation reaction. Reaction condition: 48.02% (w/v) sucrose solution, 0.2 mol L^{-1} tris acetate buffer, pH 5.5 at 50 °C

immobilizations on the operational stability of the whole cell. Ganaie et al. (2014) reported the operational stability of the mycelial FTase from *Aspergillus japonicus* ATCC 20,236, immobilized by encapsulation with alginate and chitosan. After 12 reaction cycles, FOS yield decreased from 65 to 50% using alginate, and from 42 to 2% using chitosan. These results indicate that encapsulation provided an inferior operational stability to that obtained by reticulation in this work, since FOS yield is directly associated to the transfructosylation activity.

The rise in the operational stability of the microbial cells, after reticulation with glutaraldehyde, can be related to a higher resistance to the drag of mycelial enzymes by water during the washing after each cycle, provided by the bonds imposed by the reticulation between the aldehyde and the amino groups of the protein and microbial cell, which strengthen the interaction between enzyme and mycelium (Long et al. 1996; Sun et al. 2010; Barbosa et al. 2012; Monsan 1978). Crosslinked cells with glutaraldehyde occurs by bonds between the amino groups of the cells and the aldehyde groups by the formation of Schiff bases (imine groups) (Monsan 1978; Migneault et al. 2004b). These bonds can still extend to the amino groups of lysine residues present in several proteins, so that the reticulation of the enzyme and cells ensemble can increase enzymatic stability, providing an increase in enzyme resistance to the extraction by water during the process of mycelium washing (Long et al. 1996).

The rise in operational stability of the microbial cells containing mycelial FTase can allow its reuse as biocatalyst in sequential batches for FOS production (Ganaie et al. 2014). This is a crucial factor for cost reduction in the industrial FOS production, since it reduces the production of catalytic cells. The high operational stability, together with the high value of the A_T/A_H ratio, obtained after reticulation, indicate great operational advantages of *Aspergillus oryzae* IPT-301 crosslinked cells with glutaraldehyde, increasing its potential of application in FOS production.

Conclusion

The variables of immobilization by reticulation of *Aspergillus oryzae* IPT-301 cells (pH and glutaraldehyde concentration) were optimized, enabling the maximization of the transfructosylation activity and minimization of the hydrolytic activity and, therefore, the maximization of the ratio between the activities A_T/A_H, which represents a favorable scenario for FOS production. Crosslinked cells provided higher A_T/A_H ratios at several temperatures, pHs and sucrose concentrations in the reaction media, as well as an expressive gain in the operational stability in comparison with the free cells. These results demonstrate the potential use of the cross-linked cells with glutaraldehyde in FOS production during sequential reaction cycles, also enabling its application in continuous processes.

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