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SCIENTIFIC PAPER

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CROSS-LINKED WHOLE CELLS FOR THE SUCROSE TRANSFRUCTOSYLATION REACTION IN A CONTINUOUS REACTOR

Article Highlights

- Glutaraldehyde-crosslinked fungi cells were used as biocatalysts in a packed bed reactor (PBR)
- The highest activity was presented by biocatalysts prepared by cross-linking at 200 rpm and 45 min
- The behavior of the cells' activity at several operational parameters of the PBR was obtained
- The biocatalyst achieved high operational stability for 12 h of reaction in the PBR
- The biocatalyst showed a high potential for the production of fructooligosaccharides in PBR

Abstract

Fructooligosaccharides (FOS) are fructose oligomers beneficial to human health and nutrition for prebiotic sugars. Their production occurs by a transfructosylation reaction in sucrose molecules catalyzed by fructosyltransferase enzymes (FTase, E.C.2.4.1.9) adhered to microbial cells. The purpose of this work was to study the preparation, enzymatic activity, and stability of glutaraldehyde-crosslinked Aspergillus oryzae IPT-301 cells used as a biocatalyst for the transfructosylation reaction of sucrose in a packed bed reactor (PBR), aiming at FOS production. The highest transfructosylation activity (A_T) was presented by the biocatalyst prepared by cross-linking at 200 rpm and 45 min. The highest A_T in the PBR was obtained at 50 °C, with flow rates from 3 mL min^{-1} to 5 mL min^{-1} and sucrose concentrations of 473 g L^{-1} and 500 g L^{-1}. The enzymatic kinetics was described using the Michaelis-Menten model. Finally, the biocatalyst showed constant A_T of approximately 75 U g^{-1} and 300 U g^{-1} for 12 h of reaction in the PBR operating in continuous and discontinuous flow, respectively. These results demonstrate a high potential of glutaraldehyde-crosslinked A. oryzae IPT-301 cells as heterogeneous biocatalysts for the continuous production of FOS in PBR reactors.

Keywords: fructosyltransferase, whole cells, heterogeneous biocatalysts, packed bed reactors, fructooligosaccharides.

Fructooligosaccharides (FOS) are fructose oligomers produced by the transfructosylation reaction of sucrose catalyzed by fructosyltransferase (FTase, E.C.2.4.1.9), in which fructose molecules are transferred to the β-(2→1) position of the sucrose molecule, producing FOS and glucose [1,2].

These sugars are widely used in food fabrication as supplements and substitutes for sucrose because of their high sweetening power and numerous health benefits since they are low-calorie and non-cariogenic, improve intestinal function, and help reduce total serum cholesterol [1,3,4]. Nevertheless, despite the high worldwide demand for FOS, its large-scale production

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is limited by the use of soluble enzymes as biocatalysts since they have low stability and cannot be reused or applied in packed bed reactors (PBR), which can decrease the production costs of these sugars [2,5–8].

Currently, the main strategy to prepare more stable biocatalysts for the transfructosylation reaction of sucrose has been the immobilization of extracellular microbial FTases on solid matrices [9,10]. However, extracellular enzymes deactivate rapidly during immobilization, and the solid matrices can limit enzymatic activity by causing transport limitations to the molecules of reactants and products or by modifying the microenvironment of the enzyme [2,9–11]. In this sense, whole cells with transfructosylation activity can be more advantageous biocatalysts since they have mycelial enzymes naturally immobilized on their producer microorganisms, decreasing enzyme losses and operational costs associated with manipulating extracellular enzymes and matrices [3,12].

Cells from fungi like *Xanthophyllomyces* sp. and *Aspergillus* sp., and mainly from the strain *Aspergillus oryzae* IPT-301, have shown great enzymatic activity during the transfructosylation reaction of sucrose in batch reactors [1,3,4,13–16]. However, these cells' thermal and operational stability must be improved, aiming its use in PBR reactors.

The immobilization of cells by entrapment or absorption can improve their stability during FOS production in batch reactors [7,13,17]. However, these immobilization techniques can reduce the enzymatic activity of the cell because of mass transport limitations [3,6,15,18]. In this sense, immobilization of cells by cross-linking is an important alternative since it does not use external materials as support, and the cross-linked cells showed great catalytic activity and stability in reaction batches [3]. The optimal pH and glutaraldehyde concentration to maximize the transfructosylation activity of glutaraldehyde-crosslinked *A. oryzae* IPT-301 cells were reported by Garcia *et al.* [3]. However, the enzymatic activity of these cells could still be improved by studying the effect of important cross-linking variables such as cross-linking time and agitation speed aiming its use in PBR reactors.

Currently, the use of free and immobilized cells in PBR reactors for FOS production has been explored shortly [5–7]. Furthermore, the use of cross-linked cells in this process has not been reported yet. This work aimed to prepare a stable heterogeneous biocatalyst with high activity for the transfructosylation reaction of sucrose in a PBR aiming for FOS production. For this, it evaluated the effect of agitation speed and time of cross-linking on the enzymatic activity of glutaraldehyde-crosslinked cells of *Aspergillus oryzae* IPT-301, as well as the enzymatic activity and stability of these cells as a function of reaction conditions in the PBR, such as flow rate, substrate concentration, and temperature.

MATERIALS AND METHODS

Materials

All chemical products used were of analytical grade. Yeast extract, sucrose, monopotassium phosphate (KH_2PO_4), manganese (II) chloride tetrahydrate ($MnCl_2.4H_2O$), and iron (II) sulfate heptahydrate ($FeSO_4.7H_2O$) were purchased from Labsynth® (Diadema, Brazil). Glutaraldehyde solution Grade I (25% in water), sodium borohydride ($NaBH_4$), sodium nitrate ($NaNO_3$), magnesium sulfate heptahydrate ($MgSO_4.7H_2O$), 3,5-dinitrosalicylic acid ($C_7H_4N_2O_7$), potassium sodium tartrate ($KNaC_4H_4O_6.4H_2O$), and tris(hydroxymethyl)aminomethane ($NH_2C(CH_2OH)_3$) were purchased from Dinamica® (Diadema, Brazil). The enzyme kit GOD-PAP for glucose determination was obtained from Laborlab® (Guarulhos, Brazil).

Biocatalyst production

Microorganism and production of the whole cells

Whole cells of *A. oryzae* IPT-301 were provided by the Institute for Technological Research (IPT-SP). Microbial growth was conducted by submerged culture in unbaffled Erlenmeyer flasks containing 50 mL of a sterilized culture medium with the following composition (in %, w v^{-1}): 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, and $FeSO_4.7H_2O$ 0.001.[1] The pH of the medium was adjusted to 5.5 before sterilization. The flasks were inoculated with 0.5 mL of a spore suspension of 1 x 10^7 spores mL^{-1} and incubated in a rotary shaker (Tecnal® Brazil, model TE-4200) at 30 °C and 200 rpm for 64 h [1,3,5,15]. After cultivation, the culture broth was vacuum-filtered using a Whatman N° 1 filter paper to retain the whole cells with transfructosylation activity. The collected filter cake, containing the mycelial FTase, was used to prepare the biocatalyst into spherical particles with 2.58 ± 0.30 mm of diameter. The prepared biocatalyst was taken to cross-linking assays.

Cross-linking assays of the whole cells

The cross-linking assays for the biocatalyst preparation were conducted according to an adaptation of the methods reported by Gonçalves *et al.* [15] and Garcia *et al.* [3]. Initially, about 1.5 g of whole cells were added to a 250 mL Erlenmeyer flask, followed by 12.6 mL of a glutaraldehyde solution (25 wt % in water) previously dissolved in 137.4 mL of 0.2 mol L^{-1} tris-

acetate buffer (2.1% v v^{-1} of glutaraldehyde and pH 7.9). The flask was placed on an orbital shaker (Tecnal® Brazil, model TE-4200), wherein the cells were cross-linked at 25 °C and agitation speeds from 150 rpm to 250 rpm, with agitation times from 30 min to 90 min. These values were chosen according to optimal pH and glutaraldehyde concentrations reported in previous work [3] and after preliminary cross-linking assays. Afterward, 1.5 mL of sodium borohydride previously dissolved in a 1 x 10^{-3} mol L^{-1} sodium hydroxide p.a solution (100 g L^{-1}) was added, and the medium was maintained under reaction for an additional 30 min. The cross-linking cells were vacuum-filtered (Whatman n° 1 filter paper), washed with distilled water, and preserved under refrigeration at 4 °C in 0.2 mol L^{-1} tris-acetate buffer (pH 5.5) for further determination of the enzymatic activity. Subsequently, the glutaraldehyde-crosslinked cells which showed the highest activity were used in the PBR assays.

Characterization of the biocatalyst

The morphology of the cross-linked cells was determined by scanning electron microscopy (Zeiss EVO MA-10, Germany) operating with an acceleration voltage of 20 kV, a working distance of 10.1 mm, and a spot size of 390. The samples were firstly dried for 2 h at 60 °C in a Micromeritics Vap Prep 61, Sample Degas System. The BET surface area was determined by nitrogen physisorption on a Micromeritics Gemini VII Surface Area and Porosity analyzer.

Evaluation of the enzymatic activity of the biocatalyst in a packed bed reactor

Implementation of the packed bed reactor setting

The catalytic behavior of the biocatalyst was determined in a borosilicate-packed bed reactor (PBR) of 12 mm of internal diameter. The PBR was filled with biocatalyst beads (glutaraldehyde-crosslinked cells) of 2.6 ± 0.3 mm in mean diameter up to a height of 15 cm. The temperature of the PBR was controlled using a thermostatic bath with water recirculation (Tecnal® Brazil, model TE-2005), which pumped water through a borosilicate jacket coupled to the PBR. The sucrose solution was pumped through the bioreactor upwardly using a peristaltic pump (MS Tecnopon® Brazil, model LAP-101-3). The biocatalyst beads were introduced into the PBR (by the top) using a spatula, and their mass was kept constant for all experimental runs, aiming to keep enzyme loading constant. The PBR was operated in continuous and discontinuous flow (i.e., total recirculation of the exit effluent). A schematic representation of the PBR setting was shown in previous work [5].

Effect of operational parameters on the enzymatic activity of the biocatalyst

The effect of the volumetric flow rate on the transfructosylation activity of the biocatalyst was evaluated for a range from 1.0 mL min^{-1} to 5.0 mL min^{-1} at 50 °C. The effect of temperature on the transfructosylation activity was evaluated for a range from 30 °C to 60 °C at 1.0 mL min^{-1}. These tests were performed for 150 min at pH 5.5 and a sucrose concentration of 473 g L^{-1}.

The effect of substrate concentration on the transfructosylation activity of the biocatalyst was evaluated for a range from 200 g L^{-1} to 600 g L^{-1} at 50 °C, pH 5.5, and 1 mL min^{-1} for 150 min. The Michaelis-Menten model (Eq. (1)) was used to estimate the kinetic parameters by non-linear regression.

$$v = \frac{v_{max}[S]}{K_m + [S]} \quad (1)$$

where v is reaction speed (U g^{-1}), V_{max} is the maximum reaction speed reached under the condition of substrate saturation (U g^{-1}), $[S]$ is sucrose concentration (g L^{-1}), and K_m is the Michaelis-Menten constant (g L^{-1}).

Operational stability assays

The operational stability of the biocatalyst was determined for 720 min at 50 °C, pH 5.5, sucrose concentration of 473 g L^{-1}, and flow rate of 1.0 mL min^{-1}. The experiments were carried out in the PBR operating in ascendant flow in continuous and discontinuous configuration.

Determination of external mass transfer parameters

The external mass transfer coefficient (k_c) and the Sherwood (Sh), Schmidt (Sc), and particle Reynolds (Re_p) numbers were calculated using Eqs. (2), (3), (4), and (5), respectively [19,20].

$$k_c = \frac{Sh \cdot D_{AB}}{d_p} \quad (2)$$

$$Sh = 2 + 0.6 Re_p^{1/2} Sc^{1/3} \quad (3)$$

$$S_c = \frac{\nu}{D_{AB}} \quad (4)$$

$$Re_p = \frac{U \cdot d_p}{\nu} \quad (5)$$

where d_p is the biocatalyst particle diameter (m), ν is the kinematic viscosity (m^2 s^{-1}), U is reactant fluid flow velocity (m s^{-1}), and D_{AB} is the substrate diffusion coefficient (A) in the fluid (B), expressed in m^2 s^{-1}.

The mass diffusion coefficient (D_{AB}) was estimated using Eqs. (6–8), where T is the temperature of the solution (K), μ is the dynamic viscosity of the solution (cP), MM is the molar mass (g mol^{-1}), X_A is the molar fraction of the substrate, X_B is the molar fraction of the solvent, and D_{0BA} and D_{0AB}(m s^{-1}) are the diffusion coefficients under the condition of infinite dilution of the solvent in the solute (substrate) and of the solute in the solvent, respectively [5,21].

$$D_{AB} = X_A D_{0BA} + X_B D_{0AB} \quad (6)$$

$$D_{0BA} = \frac{9.40x10^{-11}T}{MM_B^{1/3}\mu_A} \quad (7)$$

$$D_{0AB} = \frac{9.40x10^{-11}T}{MM_A^{1/3}\mu_B} \quad (8)$$

The Re_p was determined using a fluid flow velocity of 2.1 x 10^{-3} m s^{-1}. The sucrose solution was considered a diluted solution since the concentration used (473.0 g L^{-1}) corresponds to a sucrose dry substance content of 32.7% (w w^{-1}), which is inside the interval defined for an ideal solution [22]. The physical properties of liquid water and a solution of sucrose at 473 g L^{-1} and 50 °C were used to calculate the external mass transfer coefficients [21,23]. The dynamic viscosity of the sucrose solution was obtained using a Brookfield viscometer, model DV-I Prime with spindle 61 and agitation of 100 rpm.

Quantification of the enzymatic activity

The enzymatic activity was determined according to the method reported by Dias *et al.* [5]. The effluent sample was collected from the bioreactor and immediately incubated in boiling water for 10 min and ice bath for 5 min. The unit of transfructosylation activity was defined as the amount of enzyme that transfers one micromole of fructose per minute per gram of dry biocatalyst under the chosen experimental condition [3,5,15]. The concentration (μmol L^{-1}) of transfructosylated fructose (F_T) present in the reaction medium was calculated by Eq. (9) from the concentrations of glucose (G) and reducing sugars (RS), which were estimated using the enzymatic Glucose kit (GOD-PAP) and 3,5-dinitrosalicylic acid (DNS) method, respectively [1,10,11].

$$F_T = 2[G] - [RS] \quad (9)$$

To obtain the dry biocatalyst, the cross-linked whole cells were abundantly washed with distilled water after each assay, then vacuum-filtered in a Whatman n°1 filter and maintained in a drying oven (ProLab®, Brazil) at 60 °C until a constant mass was obtained.

Statistical analysis

All experiments were performed in triplicate. The analysis of the means for the cross-linking assays was performed by Tukey's honest significant difference (HSD) test, with a confidence interval of 95%.

RESULTS AND DISCUSSION

Physical properties of the biocatalyst

The surface area of the biocatalyst was 2.12 ± 0.01 m^2 g^{-1}. Dias *et al.* [5] reported that free cells of *A. oryzae* IPT-301 show a surface area of approximately 2.79 m^2 g^{-1}. It suggests that the cross-linking process of the cells with glutaraldehyde did not modify their texture significantly. It is worth mentioning that although the surface area of the cross-linked cells is considerably lower than that of solid matrices, such as silica gel (approx. 320 m^2 g^{-1}) used for immobilization of extracellular FTase, the activity of mycelial enzymes present in the cells has shown to be considerably higher than the activity of extracellular FTase immobilized by adsorption or covalent bonding on solid matrices [1,3,9,10,15,24]. Additionally, the use of cross-linked cells as biocatalysts avoids the addition of external solid matrices to the reaction media.

Figure 1 shows that the biocatalyst morphology mainly consists of a network of non-septate hyphae without the presence of fruiting bodies, spores, and a homogeneous mycelium, which is characteristic of the mycelial growth of a filamentous fungus of the genus *Aspergillus*. In general, hyphae are present in filamentous fungi due to stages of spore germination, hyphal growth, branching, and fungal differentiation [25]. However, the surface of the cross-linked cells showed a more homogeneous distribution of filaments than that shown by the free cells reported by Dias *et al.* [5]. It could be attributed to the cluster formed by the hyphae network during the cross-linking process, which reduces hyphae spacing in the cells. Hyphae spacing and distribution depend on cell growth and immobilization conditions [26,27]. The formation of cross-linked multimolecular complexes of enzyme and mycelial material, promoted by glutaraldehyde, occurs through formation of Schiff bases bonds (imine groups) between glutaraldehyde aldehyde groups and amino groups of the microbial cells, which reduces the spacing between the fungus hyphae and strengthens the interaction between enzyme and mycelium, causing conformational and morphology changes at protein and cross-linked cells [28–30]. In turn, these changes may increase the exposure of biocatalyst active sites and reduce substrate molecules' transport limitations, increasing the catalytic efficiency of the mycelial enzyme [3,31].

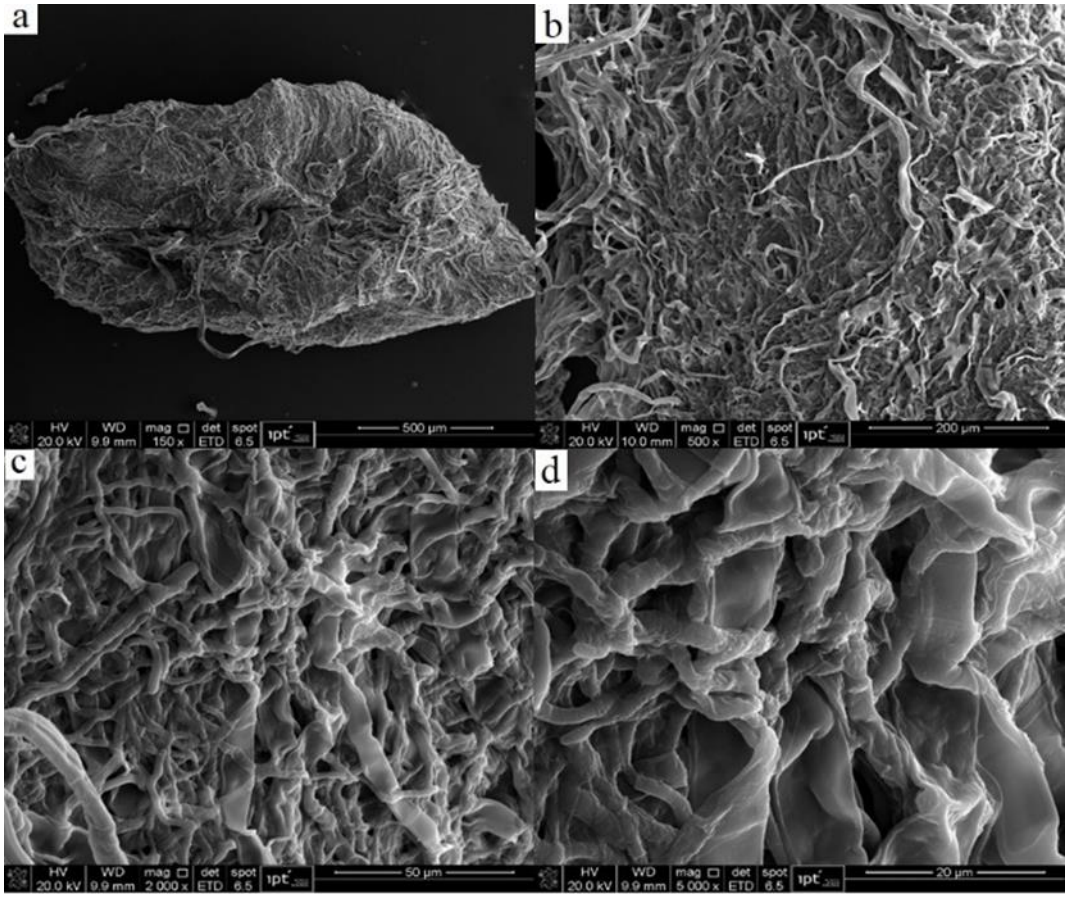

Figure 1. Micrographs of the cross-linked cells of A. oryzae IPT-301 with a magnification of (a) 150x, (b) 500x, (c) 2000x, and (d) 5000x.

Effect of cross-linking variables on biocatalyst activity

The effects of agitation speed and time during cell cross-linking on the biocatalyst activity were studied individually since preliminary statistical tests showed that both variables are independent. In the agitation speed assays, the highest transfructosylation activity (A_T) was achieved by the cells cross-linked at an agitation speed of 200 rpm, further confirmed by Tukey's HSD test (Figure 2a). Previously, Garcia *et al.* [3] had reported high A_T of *A. oryzae* IPT-301 cells cross-linked with glutaraldehyde at 200 rpm. However, the influence of agitation speed was not studied in their work. The cells cross-linked at agitation speeds of 150 rpm and 250 rpm showed the lowest A_T (below 500 U g^{-1}). The low A_T at 150 rpm could be attributed to the low mass transfer rate of the species, which reduces the effective contact between glutaraldehyde and the microbial cells, affecting the cross-linking efficiency [32,33]. On the other hand, the low A_T at 250 rpm could be attributed to an elevated shear rate on the cells caused by the high agitation speed, which can prevent cell cross-linking and the formation of bonds between enzymes and cells [34,35]. Furthermore, high agitation speeds can also produce a vortex in the vessel, which reduces the effective mixture volume and affects cell cross-linking [36].

In the agitation time assays, the highest A_T (approximately 979 U g^{-1}) was presented by the cells cross-linked for 45 min, also verified by Tukey's HSD test (Figure 2b). Similarly, Garcia et al. [3] reported that cells of *A. oryzae* IPT-301 cross-linked with glutaraldehyde for 60 min showed transfructosylation activities of approximately 986 U g^{-1}. Also, Gonçalves et al. [15] reported that cells cross-linked for 60 min and then entrapped in calcium alginate showed activity of approximately 817 U g^{-1}. Therefore, the results obtained in this work indicate an important time reduction in the cell immobilization process, which is favorable for enzymatic activity since long cross-linking times can cause enzyme denaturation because of the high glutaraldehyde reactivity [37].

Until the development of this work, only two works were found in literature about the cross-linking of whole cells with transfructosylation activity, and none of these explored the influence of agitation speed and cross-linking time on cell activity [11,15]. Gonçalves et al. [15] evaluated the stability of *A. oryzae* IPT-301 cells immobilized by cross-linking followed by entrapment in alginate. Garcia et al. [11] determined the optimal pH and glutaraldehyde concentration to maximize the activity of cross-linked *A. oryzae* IPT-301 cells. Therefore, the influence of agitation speed and cross-

linking time on the activity of cross-linked *A. oryzae* IPT-301 cells reported in this work is important original information for synthesizing novel heterogeneous biocatalysts with high enzymatic activity aiming at FOS production.

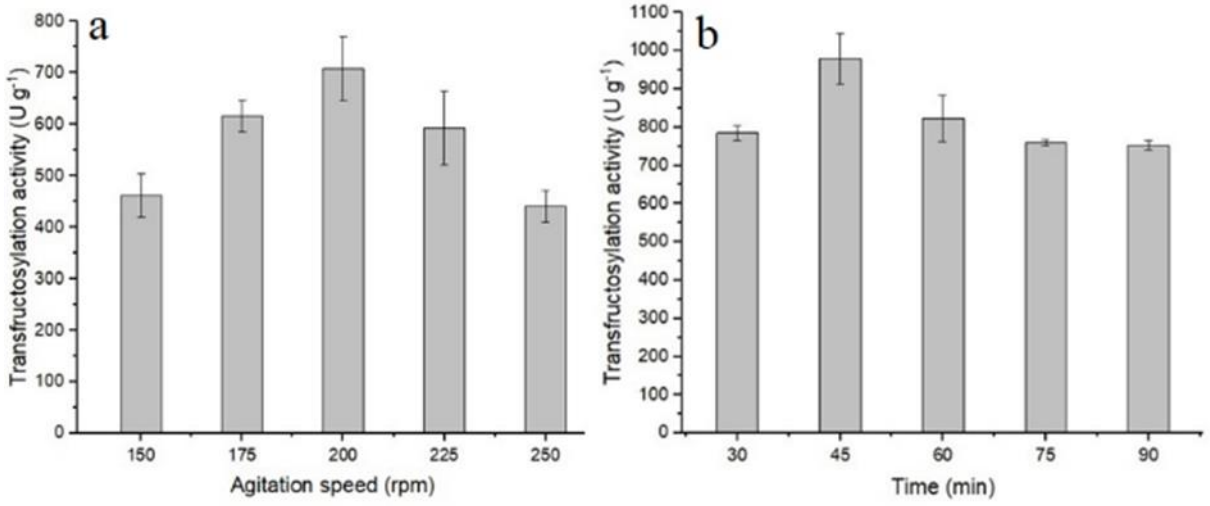

Figure 2. Effect of agitation speed (a) and effect of agitation time (b) during cell cross-linking on the transfructosylation activity of the biocatalyst.

Effect of the flow rate and temperature on the enzymatic activity of the biocatalyst

For all flow rates, the highest enzymatic activities of the biocatalyst in the PBR were obtained at the reaction time interval from 50 min to 100 min (Figure 3a). The highest A_T was obtained at the highest flow rate (5 mL min^{-1}), while the lowest A_T was obtained at the lowest flow rate (1 mL min^{-1}). Although higher flow rates provide a lower residence time in the PBR, they also allow a reduction in the transport limitations of the species on the external biocatalyst surface since higher flow velocities reduce the boundary layer in the catalyst beads, allowing faster contact between the substrate molecules and the active sites of the biocatalyst [5,20]. Conversely, the biocatalyst tested at 1 mL min^{-1} showed the lowest activity decrease, and the A_T decreased faster as the flow rate increased. This behavior could be attributed to enzyme drag at higher flow rates, as also reported by Dias *et al.* [5] for the sucrose transfructosylation reaction in a PBR catalyzed by free *A. oryzae* IPT-301 cells.

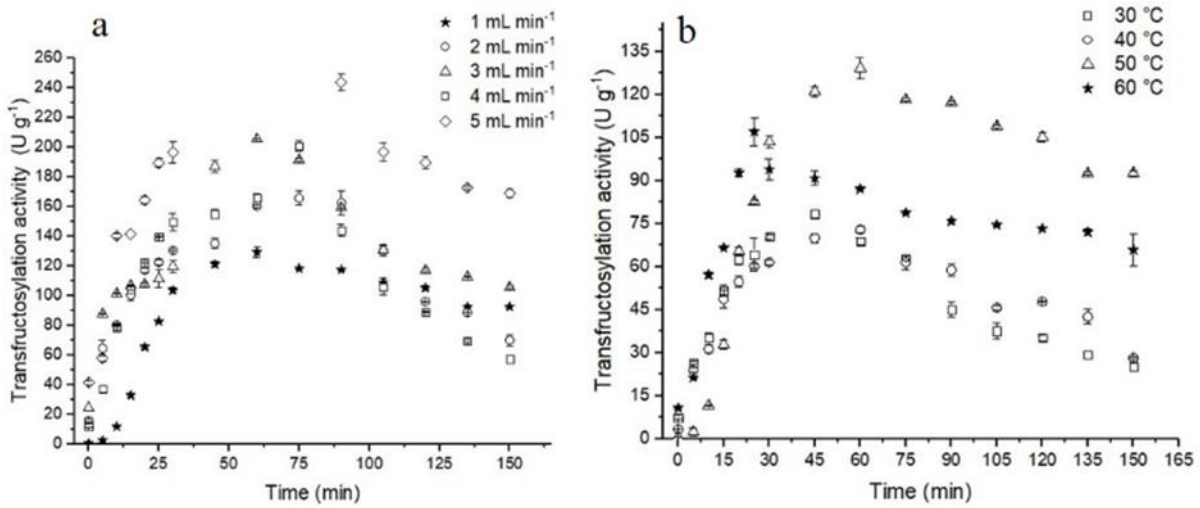

Figure 3. Effect of flow rate (a) and effect of reaction temperature (b) on the transfructosylation activity of the biocatalyst.

The highest A_T of the biocatalyst in the PBR was obtained at 50 °C, and the lowest A_T was obtained at 30 °C (Figure 3b). It can be explained by the higher frequency of collisions between substrate molecules and active sites at higher temperatures. The temperature increase enables sucrose molecules to reach the activation energy, consequently increasing the enzymatic reaction rate [3,38,39]. However, the

biocatalyst showed lower A_T for the reactor operated at 60 °C, which could be attributed to a faster thermal denaturation of enzymes at higher temperatures [24,40,41]. In thermal denaturation, the molecular interactions that maintain the force balance (hydrogen bonds, hydrophobic interactions, and van der Waals forces) of the native protein are disrupted, which affects the tertiary structure of the enzyme, causing an activity decay [24,40,41].

Additionally, for the reaction at 50 °C, proximity to the steady state was observed at about 135 min (Figure 3b). Similarly, Dias *et al.* [5] reported that free *A. oryzae* IPT-301 cells showed the highest enzymatic activity at 50 °C and steady-state at 25 min of reaction in a PBR. These results suggest that the cross-linking process did not alter the effect of temperature on the cells' activity.

Effect of sucrose concentration on the enzymatic activity of the biocatalyst and kinetics parameters

The highest A_T was presented by the reaction operated at initial sucrose concentrations of 473 g L^{-1} and 500 g L^{-1}. Similarly, Garcia *et al.* [3] reported that cross-linked cells of *A. oryzae* IPT-301 showed the highest A_T at 473 g L^{-1} in a batch reactor. It suggests that the substrate concentration effect on the A_T of the cross-linked cells was not affected by their applicability in the PBR. The lowest activities were observed for sucrose concentrations of 500 g L^{-1} and 600 g L^{-1}. Similarly, Dias *et al.* [5] reported a decrease in enzymatic activity at sucrose concentrations above 573 g L^{-1}, which can be attributed to inhibition by the substrate [42]. It suggests that A_T reduction at high substrate concentrations in the PBR was similar for both the free and cross-linked cells. Figure 4a also shows that a transition from transient to steady-state occurred at about 120 min of reaction for all tested sucrose concentrations.

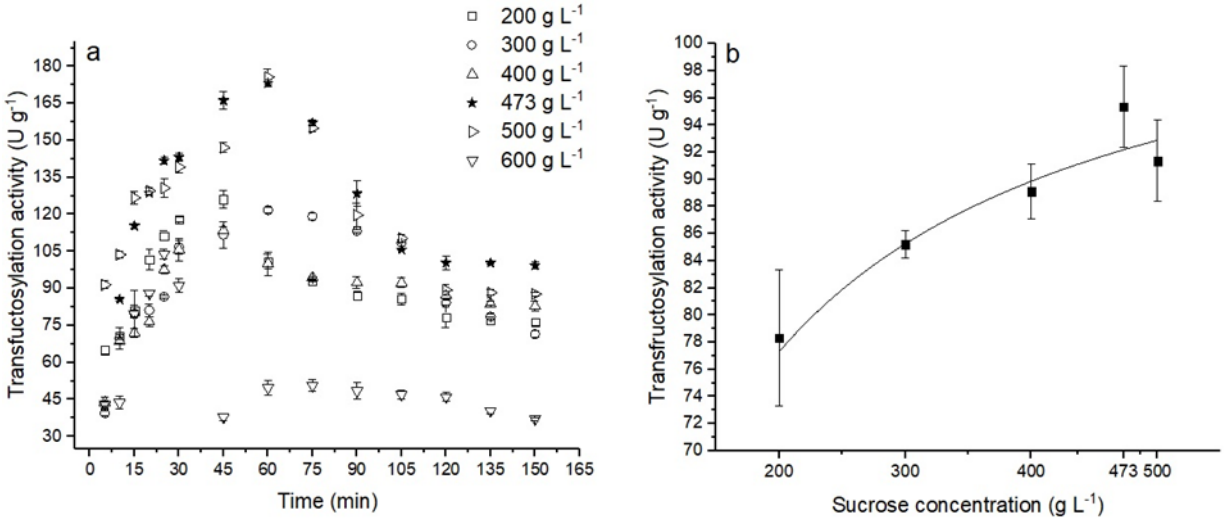

Figure 4. (a) Effect of sucrose concentration on the transfructosylation activity of the biocatalyst (b) and Michaelis-Menten model adjustment.

The A_T obtained in the assays of the substrate concentration effect was successfully adjusted to the Michaelis-Menten model (Figure 4b). This model was used because of its simplicity and wide applicability, and aimed to compare the kinetics parameters of the transfructosylation reaction on the cross-linked cells in the PBR with data reported for the same reaction on free cells in batch and PBR reactors and with the reaction catalyzed on cross-linked cells in batch reactors [3,5]. The kinetic parameters for the A_T shown by the biocatalyst are listed in Table 1. The V_{max} obtained in the PBR was lower than that reported by Garcia *et al.* [3] for a similar biocatalyst tested in a batch reactor (Table 1). This behavior was also reported for free *A. oryzae* IPT-301 cells tested in batch and PBR reactors and can be attributed to mass transport limitations in the PBR at low flow rates [3,5]. The K_m obtained for the cross-linked cells in the PBR was also lower than that obtained in a batch reactor [3]. However, it showed the opposite behavior for the free *A. oryzae* IPT- 301 cells tested in batch and PBR reactors [3,5]. It suggests that cross-linking of the cells is advantageous for application in PBR reactors. Additionally, previous work demonstrated that cross-linking of the *A. oryzae* IPT-301 cells with glutaraldehyde also increases substrate-enzyme affinity during the transfructosylation reaction in batch reactors [3]. Therefore, the kinetics parameters obtained with the Michaelis-Menten model can be significantly useful for further modeling of the transfructosylation reaction of sucrose in a PBR, catalyzed on cross-linked cells of *A. oryzae*-IPT-301, aiming FOS production.

Table 1. Kinetic parameters of the Michaelis-Menten model obtained for the transfructosylation activity of A. oryzae IPT-301 cells.

Kinect parameters	Biocatalyst/reactor			
	Cross-linked cells in a PBR	Cross-linked cells in a batch reactor	Free cells in a PBR	Free cells in a batch reactor
V_{max} (U g^{-1})	106.90 ± 4.15	956.06	634 ± 85	817.50
K_m (g L^{-1})	74.21 ± 16.14	98.50	157 ± 68	121.50
R^2	0.92	0.95	0.97	0.91
Reference	This work	[3]	[5]	[3]

Operational stability

Initially, for the PBR operating in a continuous flow, the biocatalyst showed a fast increase in A_T up to approximately 190 U g^{-1} (Figure 5). Nonetheless, after 60 min of reaction, the A_T decreased rapidly and stabilized at approximately 75 U g^{-1} after 180 min. The A_T decrease could be attributed to enzymes' drag on the cell surface. After that, the A_T stayed practically constant for up to 12 h of reaction, suggesting great operational stability of the biocatalyst. Similarly, Dias *et al.* [5] reported that free *A. oryzae* IPT-301 cells showed constant enzymatic activity for 12 h of reaction in a PBR at 50 °C and flow rate of 5 mL min^{-1}, but they presented rapid deactivation at flow rates above 11 mL min^{-1}. On the other hand, in this work, in the PBR operating with discontinuous flow, the A_T increased along the reaction time and stabilized at approximately 300 U g^{-1} after 9 h of reaction. It could be attributed to the constant recycling of dragged enzymes back to the PBR in the discontinuous flow configuration. This behavior was also reported for free *A. oryzae* IPT-301 cells used in a PBR with continuous and discontinuous descendent flow at 11.5 mL min^{-1} [5]. These results suggest that the biocatalyst prepared in this work is stable for the transfructosylation reaction of sucrose, and it has a high potential to catalyze the large-scale production of FOS in a PBR.

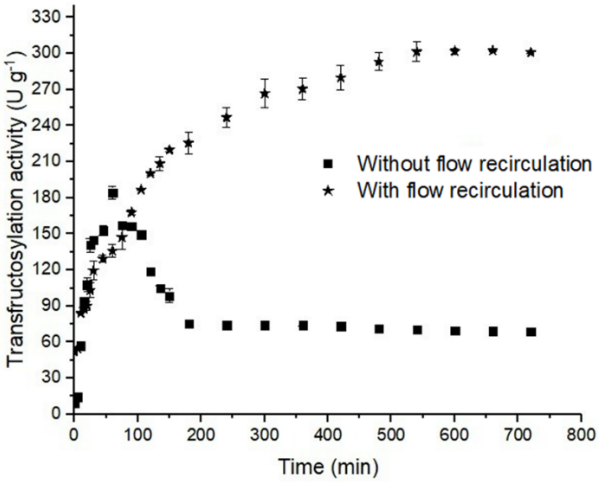

Figure 5. Operational stability of the biocatalyst in the PBR operated in continuous and discontinuous flow.

Recently, Garcia *et al.* [3] reported that cross-linked *A. oryzae* IPT-301 cells are considerably more stable than free cells in batch reactors. In that study, the cross-linked cells showed 88.9% of their initial transfructosylation activity after 12 sequential reaction batches of 1 h, while free cells only retained 50.3% of their initial activity [3]. Aldehyde groups of glutaraldehyde can act as an intramolecular crosslinker by reacting with two vicinal primary amine groups within a protein, or it can promote intermolecular cross-linking between two primary amine groups of two neighboring biomolecules by the formation of Schiff bases (imine groups) [31,43,44]. Therefore, the increase in enzymatic activity and stability after treatment with glutaraldehyde in whole cells of *A. oryzae* IPT-301 may be due to the formation of inter- or intramolecular cross-links that can cause conformational changes in the mycelial enzyme and, consequently, allow greater contact between the active sites of the protein and substrate molecules [3].

Furthermore, the higher stability of cross-linked cells can be more advantageous for FOS production when PBR reactors are used since the continuous flow offer a higher effective contact between reactants and biocatalyst [5,7]. Zambelli *et al.* [7] immobilized bacterial whole cells by entrapment in alginate and tested it in batch and PBR reactors for FOS production. The authors reported that the FOS yield was 1.7 times higher in a PBR than in a batch reactor. Similarly, Dias *et al.* [5] reported that free cells of *A. oryzae* IPT-301 showed enzymatic productivity of approximately 40 times the productivity of batch reactors. The results showed in this work are the first concerning the use of cross-linked cells as stable biocatalysts for the transfructosylation reaction of sucrose in a PBR, which is a key strategy to enable the large-scale production of FOS.

Mass transfer and kinetics regimes

Figure 6 shows that for flow rates from 1 mL min^{-1} to 4 mL min^{-1}, the transfructosylation activity of the biocatalyst in the PBR was limited by substrate diffusion [5,20]. It indicates that the sucrose molecules were transported slowly through the boundary layer at the biocatalyst surface up to the active sites, which decreases A_T. These results explain the low V_{max} obtained in the PBR (Table 1). Also, as the kinetic parameters shown in Table 1 were determined at 1 mL min^{-1}, these are apparent kinetic parameters. On

the other hand, it can be seen that the A_T stayed practically constant at flow rates of 4 mL min^{-1} and 5 ml min^{-1}, suggesting that the change in flow velocity did not alter enzymatic activity. It indicates that the reaction could be kinetics-limited or limited by internal diffusion at this flow interval. However, because of the low surface area of the biocatalyst, which is related to the absence of porosity, the reaction could be considered kinetics-limited. It is worth mentioning that although high flow rates could be used in the kinetics studies to avoid external mass transfer limitations in the PBR, the lowest flow rate was used in those assays because the A_T showed higher instability as the flow rate increased (Figure 3a).

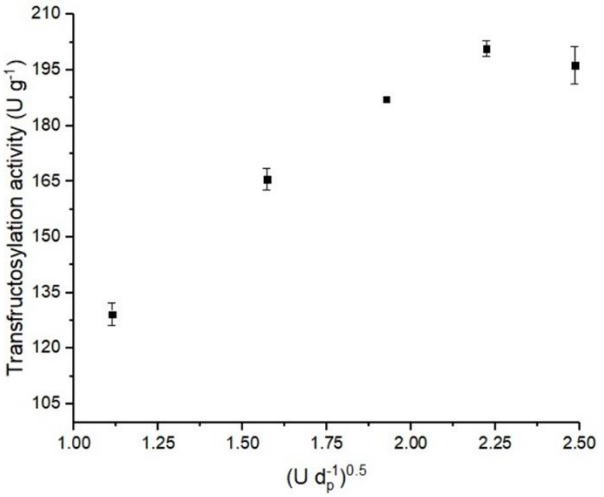

Figure 6. Regions of limitation by external mass transfer and by reaction kinetics.

The use of PBR reactors for FOS production has been explored shortly [5–7]. Therefore, there is a lack of mass transfer data in this process in the literature. However, it is known that the flow regime greatly influences the biocatalyst performance. According to the Reynolds number obtained, the PBR operated in laminar flow, suggesting a high influence of viscous forces on substrate flow (Table 2). The laminar flow is unfavorable for a proper mass transfer in the reactor. However, it is inevitable since the enzymatic reaction requires long residence times to reach high substrate conversion, and hence low flow rates must be used. Also, as shown in Figure 3a, low flow rates allow higher stability. The laminar regime was also used in the work of Dias *et al.* [5] for a PBR packed with free cells. Dias *et al.* [5] also reported lower enzyme stability at higher flow rates in their work. Furthermore, similar studies in a PBR for FOS production used flow rates below 1 mL min^{-1} [6,7]. The high Schmidt number obtained showed the great effect of viscosity on the laminar flow. Moreover, the Sherwood number indicates that the mass transfer in the PBR was predominantly convective, per the results shown in Figure 6. Finally, it was obtained a low coefficient of external mass transfer (k_c), which can be attributed to the high sucrose concentration (which results in a high fluid viscosity) and the low flow used for the transfructosylation reaction of sucrose on the cross-linked cells in the PBR. This behavior has also been reported for other enzymatic reactions tested in a PBR since enzymatic processes generally need high residence times because of their slow reaction kinetics [45,46]. These results demonstrate the importance of improving immobilization techniques, such as cross-linking of whole cells, for developing highly active and stable biocatalysts for the continuous enzymatic production of FOS at higher flow rates.

Table 2. Parameters of mass transfer and dimensionless numbers.

Parameters and dimensionless numbers	Value
Reynolds of particle – Re_p	1.058
Schmidt – Sc	6.64 x 10^6
Sherwood – Sh	118.027
Sucrose diffusion coefficient (A) in water (B) (D_{AB})	7.66 x 10^{-13} m^2 s^{-1}
Coefficient of external mass transfer (k_c)	3.51 x 10^{-8} m s^{-1}

CONCLUSION

Glutaraldehyde-crosslinked *A. oryzae* IPT-301 cells were successfully prepared and tested as biocatalysts for the transfructosylation reaction of sucrose in a PBR. The effects of the agitation speed and time of cross-linking on the biocatalyst activity were evaluated. The highest A_T was presented by cells cross-linked at 200 rpm for 45 min, which allowed the improvement of the biocatalyst preparation methodology. In the PBR operated with continuous flow, the highest A_T of the biocatalyst was obtained at 5 mL min^{-1}, 50 °C, and sucrose concentrations of 473 g mL^{-1} and 500 g mL^{-1}. Nevertheless, high flow rates should be avoided to reduce A_T loss along the time on stream. The A_T of the biocatalyst showed reaction kinetics and responses to variations in temperature and sucrose concentrations that were similar to the cross-linked cells used in the batch reactors, suggesting that it can be used in PBR systems without affecting their performance. Finally, the biocatalyst showed high operational stability for 12 hours of reaction. These results suggest a high

potential for applying the cross-linked *A. oryzae* IPT-301 cells as a biocatalyst for FOS production in PBR reactors.

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NAUČNI RAD

REAKCIJA TRANSFRUKTOZILIZACIJE U KONTINUALNOM REAKTORU

Fruktooligosaharidi su oligomeri fruktoze koji su korisni za ljudsko zdravlje i ishranu kao prebiotski šećeri. Oni se proizvode reakcijom transfruktozilacije u molekulima saharoze katalizovanom enzimima fruktoziltransferaze (FTase, E.C.2.4.1.9) prilepljenim na mikrobne ćelije. Cilj ovog rada je proučavanje pripreme, enzimske aktivnosti i stabilnosti glutaraldehidom umreženih ćelija Aspergillus orizae IPT-301 koje se koriste kao biokatalizator za reakciju ima biokatalizator pripremljen umrežavanjem na 200 o/min za 45 min. Najveća aktivnost transfruktozilacije u reaktoru sa pakovanim slojem dobijen je na 50 °C, sa protokom 3–5 ml/min i sa koncentracijama saharoze od 473–500 g/l. Enzimska kinetika je opisana Michaelis-Mentenovim modelom. Konačno, biokatalizator je pokazao konstantnu aktivnost transfruktozilacije približno 75–300 U/g tokom 12 h reakcije u reaktoru sa pakovanim slojem koji radi u kontinuiranom i diskontinuiranom režimu, redom. Ovi rezultati pokazuju visok potencijal glutaraldehidom umreženih ćelija A. orizae IPT-301 kao heterogenih biokatalizatora za kontinualnu proizvodnju fruktooligosaharida u reaktorima sa pakovanim slojem.

Ključne reči: fruktoziltransferaza, cele ćelije, heterogeni biokatalizatori, reaktori sa pakovanim slojem, fruktooligosaharidi.

Ključne reči: fruktoziltransferaze, umrežene ćelije, heterogeni biokatalizator, reaktorimi sa pakovanim slojem, fruktooligosaharidi.