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Application of an Agro-Waste for the Immobilization of Microbial Fructosyltransferase: A New Alternative for Fructooligosaccharide Production

Richard S. Pereira,[a] Ana C. Vieira,[a] Paula C. Leite,[a] Sylma C. Maestrelli,[a] Elda S. Silva,[b] Alfredo E. Maiorano,[b] Michelle C. A. Xavier,[c] Melina S. Lopes,[a] Ariela V. de Paula,[d] Sergio A. V. Morales[c] and Rafael F. Perna *,[a]

[a]*Instituto de Ciência e Tecnologia, Universidade Federal de Alfenas, Rod. José Aurélio Vilela, 11999, km 533, Cidade Universitária, 37715-400 Poços de Caldas-MG, Brazil*

[b]*Núcleo de Bionanomanufatura, Instituto de Pesquisas Tecnológicas de São Paulo, Av. Prof. Almeida Prado, 532, Cidade Universitária, 05508-901 São Paulo-SP, Brazil*

[c]*Departamento de Engenharia de Alimentos, Universidade Federal do Tocantins, Av. NS 15, ALCNO-14, 77001-090 Palmas-TO, Brazil*

[d]*Faculdade de Ciências Farmacêuticas, Universidade Estadual Paulista, Rod. Araraquara Jaú, km 01 - s/n, 14800-903 Araraquara-SP, Brazil*

This work evaluated the use of corncob as a support for the immobilization of an extracellular fructosyltransferase (E.C. 2.4.1.9) from *Aspergillus oryzae* IPT-301, aiming at the synthesis of a stable biocatalyst for the production of fructooligosaccharides. The transfructosylation activity of the immobilized enzyme was maximized via experimental design. Additionally, the biochemical properties and stability of the immobilized enzyme, as well as the reaction kinetics, were determined. The enzyme immobilized on alkali-treated corncob showed the highest transfructosylation activity at 50 °C and pH 5.5. It also presented a wide pH stability and a half-life around 1.4 times greater than the soluble enzyme. Furthermore, the immobilized enzyme showed 53% of retention of catalytic activity in a second consecutive reaction cycle, showing the potential for reuse. These results suggest that alkali-treated corncob is a promising support material for the synthesis of heterogeneous biocatalysts aiming at fructooligosaccharide production, which can contribute to the valorization of this abundant agro-waste.

Keywords: fructosyltransferase, corncob, immobilized enzyme, fructooligosaccharides

Introduction

The reuse and recycling of agro-industrial waste are essential to reduce global greenhouse gas emissions, generating valuable products in growing sectors, such as transportation, bioenergy, food and raw materials.[1] One of these materials is corncob, which is a residue generated after removing the grains from the cob, resulting in a global production of 797 million tons, which represents approximately 16.94% of the corn production of the world.[2] Corncob is a lignocellulosic material, composed mainly of 35-46% in weight of cellulose, 28-42% in weight of hemicellulose, and 11-18% in weight of lignin.[3] Recently, research works have focused on the application of this agro-industrial by-product in biocomposites,[3] biofuels, bioproducts[4] and in enzyme immobilization.[5] Since the corncob structure is thick and organized,[6] agro-waste can be a promising alternative as a support for enzymes: significant economic benefits are obtained in relation to enzyme reuse, and it is also possible to minimize the problems generated by waste disposal.[7] In addition, the application of corncob (agro-waste) on enzyme immobilization focuses on the concept of circular economy.[8]

Immobilization provides greater thermal and operational stabilities to the enzyme, slowing the denaturation of the biocatalyst and enabling its reuse in the reaction medium, which can lead to a reduction in the operational cost of the enzymatic process.[2,9-11] For enzyme immobilization, the material used as a support to the enzyme must be resistant

*e-mail: rafael.perna@unifal-mg.edu.br
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to the reaction conditions, also presenting a good affinity to the enzyme, low cost and high availability.[12] Thus, corncob is presented as a promising immobilization matrix, since it is a natural lignocellulosic material with a broad specific surface area and high porosity and resistance.[5,13-15] As an agricultural waste product, it has low cost and can be easily and abundantly found worldwide, being an economical and an efficient matrix to be utilized for the immobilization of enzymes.[5,13,16]

The immobilization of different enzymes on corncob has already been reported,[17] since this support can be reused, making it a high value bioproduct and an alternative for industrial applications. A commercial glucoamylase was immobilized in powdered corncob with 95% of yield, representing a promising strategy for future industrial applications.[5] Other authors[13] have used different methods (adsorption, cross-linking adsorption and covalent bonding) for the immobilization of lipases and peroxidases, highlighting the potential of corncob as a lignocellulosic material for the immobilization of different enzymes and the development of immobilization methods. Nevertheless, until the completion of this study, applications of agro-waste for the immobilization of the enzyme fructosyltransferase (FTase, E.C.2.4.1.9) have not been reported.

FTase is responsible for the production of fructooligosaccharides (FOS), which are low-calorie prebiotic sugars that present benefits to human health, since they are non-cariogenic and help in preventing diseases, such as colon cancer, obesity, hypertension, diabetes, renal failure, among others.[18-24] Studies[25,26] using the FTase from *Aspergillus oryzae* IPT-301 in its free form show unfavorable results for process scale-up, due to its low stability and unfeasibility for reuse. In this sense, studies[27,28] on the immobilization of this enzyme have been explored to improve its operational application, in order to increase its potential for the production of prebiotic sugars.

Therefore, this work aimed to study the biochemical properties, kinetics, and stability of an extracellular FTase from *A. oryzae* IPT-301 immobilized on alkali-treated corncob particles in order to obtain a heterogeneous biocatalyst for FOS production.

Experimental

Materials

The corncob was donated by the Farm "Posses da Serra" (Monte Belo, Brazil) and collected in the plantation field after the harvest of the corn grains. The strain *A. oryzae* IPT-301 was provided by the Institute for Technological Research (IPT, São Paulo, Brazil). Sucrose, yeast extract, KH_2PO_4, $MnCl_2.4H_2O$ and $FeSO_4.7H_2O$ were purchased from Labynth® (Diadema, Brazil). $NaNO_3$, $Mg_2SO_4.7H_2O$, NaOH, 3,5-dinitrosalicylic acid ($C_7H_4N_2O_7$) and tris(hydroxymethyl) aminomethane ($NH_2C(CH_2OH)_3$) were purchased from Dinâmica® (Indaiatuba, Brazil). The GOD-PAP® enzyme kit was purchased from LaborLab® (Guarulhos, Brazil). All chemicals used were of analytical grade.

Preparation of the immobilized support

The *in natura* corncob was dried in a drying oven at 60 °C for 24 h and shredded using a common grater. Subsequently, the corncob particles (CCP) were dry-sieved using a mechanical shaker for 10 min and sieves of 50 and 12 mesh (Bertel Indústria Metalúrgica Ltda., Caieiras, Brazil) in order to obtain support particles with a size range from 0.30 to 1.70 mm. The CCP were chemically treated according to the protocol described by Paul *et al.*,[29] and the support was submerged in a sodium hydroxide solution (10% m v^{-1}) under constant mechanical agitation for 1 h at 25 °C. Subsequently, the support was washed abundantly with distilled water, vacuum-filtered, and dried in a drying oven (TE-394/1, Tecnal®, Piracicaba, Brazil) at 60 °C for 24 h. Finally, the alkali-treated corncob particles (TCCP) were stored in a glass vacuum desiccator for 24 h for the enzyme immobilization assays.

Physicochemical characterization of the immobilization support

The morphology of the corncob particles was observed before and after the chemical treatment, by scanning electron microscopy (SEM) (Zeiss EVO MA-10, Oberkochen, Germany) operating with an acceleration voltage of 20 kV, working distance of 10 mm, and spot size of 4.8. The sample was first prepared in a Micromeritics Vap Prep 61 Sample Degas System (Norcross, USA), dried for 2 h at 60 °C, and the Brunauer, Emmett and Teller (BET) specific surface area was examined on a Micromeritics Gemini VII analyzer (Norcross, USA), 77 K nitrogen absorption, using the BET method.[30] Pore size and volume were obtained using the Barrett-Joyner-Halenda (BJH) method.[31] The chemical bonds and vibration modes of the solids were examined by Fourier transform infrared spectroscopy (FTIR) using a spectrometer (Agilent Cary 630, Santa Clara, USA) operating from 600 to 4000 cm^{-1}.

Enzyme production and immobilization

The extracellular FTase from *A. oryzae* IPT-301 was produced by submerged cultivation, according to the

method used by Cunha *et al.*,[26] and incubated in a rotary shaker (TE-4200, Tecnal®, Piracicaba, Brazil) for 64 h.[32] After the cultivation, the culture broth was vacuum-filtered and the resulting supernatant, containing an activity of 12.52 ± 2.02 U mL^{-1} of FTase, was used for the immobilization assays. The extracellular FTase was immobilized as described by Faria *et al.*,[32] and the immobilization parameters were obtained according to Araújo *et al.*,[27] by the calculation of immobilization yield (IY) and recovered activity (RA).

Biochemical characterization assays

Experimental design for the determination of the effects of temperature and pH on enzymatic activity

The elaboration of the experimental design and statistical analysis of the data was conducted using the software Protimiza Experimental Design.[33] A full 2^2 factorial design with three assays at the central point was chosen for the study of the two factors: temperature and pH of the reaction medium, each at five levels. Star points were added to the experimental design to compose a second-order model. The matrix with the values of the factors are shown in Table 1. The data for the factors were chosen after a series of preliminary assays.

Table 1. Coded values of the input variables for the statistical design

Variable		–1.41	–1	0	+1	+1.41
Temperature / °C	x_1	40	43	50	57	60
pH	x_2	4.5	4.8	5.5	6.2	6.5

x_1: temperature level; x_2: pH level.

The response surface model was fitted to one response variable (Y), namely transfructosylation activity (in U g^{-1}). The second-order response function for two factors is given in equation 1. The differences were considered significant at a *p*-value ≤ 0.10.

$$Y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_{11} x_1^2 + \beta_{22} x_2^2 + \beta_{12} x_1 x_2 \quad (1)$$

where x_1 and x_2 represent the levels of both temperature (°C) and pH, respectively, while β represents the estimated coefficients, with β_0 having the role of the offset term.

The analysis of variance (ANOVA) was presented for the quadratic model applied for the enzymatic activity of the immobilized FTase. The adjustment of the experimental responses to the statistical model was evaluated by the coefficient of determination of error (R^2) and the *F* test.[26]

Effect of substrate concentration on the enzymatic activity and determination of the kinetic parameters

The activity of the enzyme immobilized on the alkali-treated corncob particles (TCCP) was determined at 50 °C in 3.7 mL of a sucrose solution at different concentrations (200, 300, 400, 470, 500, and 600 g L^{-1}) plus 1.2 mL of tris-acetate buffer at 0.2 mol L^{-1} and pH 5.5. The Hill model was used to determine the kinetic parameters, such as maximum reaction rate (v_{max}), apparent dissociation constant ($K_{0.5}$), and Hill coefficient (n), by a nonlinear regression analysis.[32,34]

Thermal stability assays and determination of the apparent thermodynamic parameters

The soluble and immobilized enzymes were incubated in tris-acetate buffer at 0.20 mol L^{-1} and pH 5.5, in the absence of substrate, in a broad temperature range (30, 40, 50, and 60 °C) for 1, 2, 4, 6 and 24 h. After the incubation period, the samples were immediately cooled in an ice bath for 5 min, and the residual activity was determined under standard conditions. The experiments were performed in triplicate. The first-order thermal denaturation constant (k_D) was estimated adjusting the model of Sadana and Henley[35] to the experimental data of residual activities *versus* time, and by this model, it was possible to estimate the half-life ($t_{1/2}$) of the soluble and immobilized enzymes. The activation energy for thermal denaturation (E_D) of the soluble and immobilized enzymes, stability factor (SF), enthalpy of variation of the activation of denaturation (ΔH_D), Gibbs energy of the activation of denaturation (ΔG_D), and entropy of the activation of denaturation (ΔS_D) were calculated according to Araújo *et al.*[27]

pH and operational stability assays

The experiments on the stability at different pH values and the operational stability assays of the immobilized enzyme were performed according to Faria *et al.*[32] Both experiments were conducted in triplicate and under standard conditions. The enzymatic activity was evaluated considering the number of reaction cycles for the operational stability assays.

Analytical methods

The transfructosylation activity (A_t) was determined according to Faria *et al.*,[32] where the unit of A_t was defined as the amount of enzyme that transfers 1 μmol of fructose *per* min under the chosen experimental conditions. The concentrations of reducing sugars and glucose were quantified by the enzymatic Glucose kit (GOD-PAP®) and by the 3,5-dinitrosalicylic acid (DNS)

method, respectively.[24]

Statistical analysis

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

Results and Discussion

Properties of the support

Table 2 shows the results of the specific surface area and average pore diameter obtained for CCP and TCCP. The experimental results showed that the values of the specific surface area and average pore diameter of TCCP were lower than those of CCP. It is known that pretreatment induces an alteration in the morpho-chemical structure of corncob due to hydrolysis, which promotes an effective lignin removal by the saponification of the ester bonds between lignin and the hemicelluloses, causing a structural change in the cellulose fibers.[36,37] Pretreatment can produce a change in the total degree of crystallinity of the lignocellulosic material because of the partial removal of amorphous regions (lignin and hemicellulose). Pretreatment, by changing the degree of crystallinity and morphology of the material as a whole, can change the values of specific surface area and average pore diameter.[38-40] This information, observed in Table 2, showed that the treatment promoted a decrease in the specific surface area and pore diameter, which negatively affects the immobilization of the enzyme on the support.

Table 2. Experimental results of specific surface area, and average pore diameter of the support

Sample	Specific surface area / (m^2 g^{-1})	Average pore diameter / Å
CCP	1.92 ± 0.02	111.89
TCCP	0.34 ± 0.03	37.74

CCP: corncob particles; TCCP: alkali-treated corncob particles.

Figure 1 presents the FTIR spectra of TCCP before and after FTase adsorption. In both materials, there are peaks in the regions of 3300, 1650 and 1000 cm^{-1}. In this case, the peak regions of 3300 cm^{-1} refer to the O–H stretching vibration of hydroxyl groups. The peak 1650 cm^{-1} is associated with the C=C stretching of aromatic groups. The peak in the region of 1000 cm^{-1} can be attributed to ether and ester groups (C–O stretching).[41-43]

For the corncob particles before the immobilization process, Ojedokun and Bello[41] obtained peaks in the regions of 3301, 1643 and 1029 cm^{-1}. Padilla *et al.*[42] obtained peaks in the regions of 3459, 1659 and 1044 cm^{-1}. Piña-Barrera *et al.*[43] observed peaks in the regions of 3296, 1650 and 950 cm^{-1}. The similar results observed in various studies[5,37,41-43] indicated the material is, indeed, corncob.

For the support with the immobilized FTase, it is noted a significant increase in the peaks in the regions of 3300 and 1650 cm^{-1}. The wide peak at 3300 cm^{-1} possibly corresponds to amino group stretching (N–H), probably with overlap peaks related to O–H stretching and C=C stretching, respectively. On the other hand, the increase in the peak at 1000 cm^{-1} is probably related to the amide groups (C–O and C–N stretching).[44,45] These results confirm that there was a significant increase in the organic groups on the surface, possibly indicating that FTase was immobilized on the corncob particles.

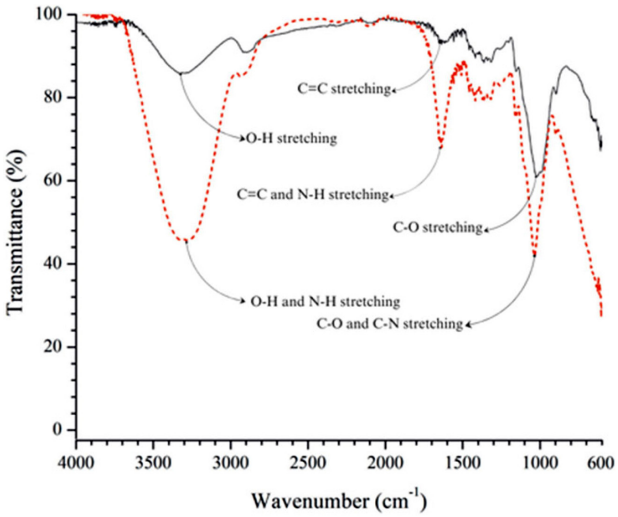

Figure 1. FTIR (KBr) spectra of TCCP before and after immobilization for the values above 600 cm^{-1}. Continuous line: support without FTase; dashed line: support with the immobilized FTase.

To visualize the interference of the chemical treatment on the corncob microstructure, Figure 2 presents the SEM micrographs of the non-treated (Figure 2a) and alkali treated (Figure 2b) corncob particles at 150× magnification. In Figure 2c, alkali-treated corncob particles at 1000× magnification. It was possible to observe the effect of the chemical treatment on the corncob surface, causing morphological and structural changes (Figures 2a and 2b). Similar results were obtained by Fatmawati *et al.*,[46] using a biological pretreatment of corncob.

Figure 2a shows a larger specific surface area and mean pore diameter for the untreated samples in relation to Figure 2b (with treatment), which corroborates the results already presented in Table 2, regarding the data obtained

by BET. The increase in specific surface area and mean pore diameter interferes with the access of the enzyme to the structure, causing an increase in immobilization yield. Figure 2c highlights the high number of pores and irregular voids of the pore morphology obtained after treatment in the corncob structure and the exposure of the surface area that facilitates enzyme immobilization.

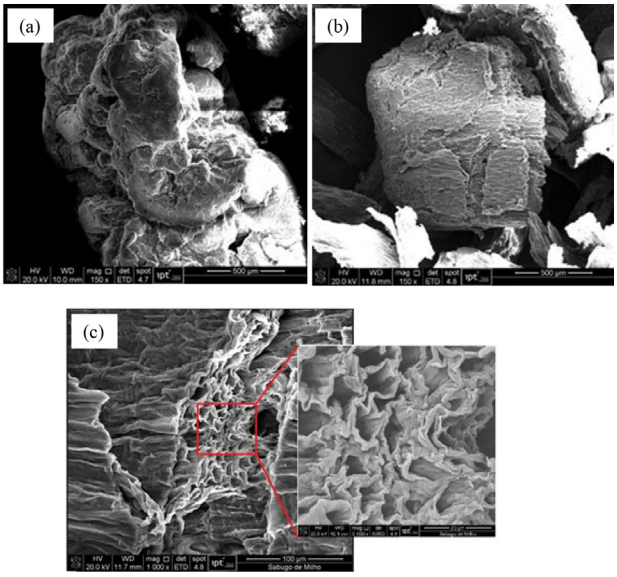

Figure 2. Micrographs of the non-treated (a) and alkali-treated (b) corncob particles at 150× magnification, and (c) alkali-treated corncob particles at 1000× magnification. FTase immobilization in corncob and alkali-treated corncob.

The extracellular FTase from *A. oryzae* IPT-301 was immobilized on CCP and TCCP. After 6 h of immobilization, the enzymatic activity in the supernatant was about 18.62 and 19.86% of its initial value (12.52 ± 2.02 U mL^{-1}) in the presence of CCP and TCCP, respectively. These results suggest the FTase was spontaneously immobilized on the organic support by adsorption, wherein the enzyme was transferred from the liquid phase to the solid surface and attached to it by weak intermolecular forces, such as van der Waals attractive forces, ionic interactions, hydrophobic bonding, or a combination of these interactions.[27,47] Although the physical adsorption mechanisms showed low linking energy between enzyme and support, in comparison to immobilization techniques such as covalent bonding or crosslinking, this technique is useful, simple, low cost and one of the most used techniques in the production of heterogeneous biocatalysts, as reported in the works of Garcia *et al.*[28] and Brígida *et al.*[48]

The greatest immobilization yield was obtained using CCP as support (IY of 74.3 ± 0.2%), while the immobilization yield of the FTase on TCCP was 64.6 ± 3.0%. Immobilization yield may be limited by the presence of metabolites and residues in the culture broth, including amino acids, low-weight polypeptides, traces of mineral salts and residual sucrose, all of which can be adsorbed on the support surface or interact with the enzyme, hindering its immobilization.[27,28] Additionally, it was shown that FTase immobilization yield on TCCP decreased about 1.2-fold compared to the enzyme immobilized on CCP. One of the reasons that may have led to the decrease in this parameter derived from the reduction in the specific surface area and average pore diameter of the material promoted by the alkali treatment of the corncob particles (Table 2).

On the other hand, the FTase immobilized on TCCP showed a recovery activity 4-fold higher than that shown by the FTase immobilized on CCP (RA of 9.1 ± 0.6% and 2.3 ± 0.2%, respectively). The pretreatment provided a significant increase in the organic groups on the surface, that is, a greater number of components on the surface of the support to interact with the immobilized biocatalyst. The increase in these organic groups has a positive impact on the recovered activity, since it allows an enzyme-support conformation which facilitates the interaction with the substrate in a more effective way than in the absence of pretreatment, minimizing diffusional limitations.[49,50]

This increase is probably related to the higher accessibility to the reactive hydroxyl groups (OH) at the positions of the carbon atoms C-2, C-3, and C-6 of the glucose units of the cellulose present on TCCP, because of the partial lignin and hemicellulose solubilization that might also have promoted the transformation of the cellulose polymorph from I to II, which is thermodynamically more favorable.[2,51] Nevertheless, this greater number of intermolecular bonds promoted by TCCP and the FTase could be the reason for the lower enzymatic activity observed after immobilization. This higher cellulose reactivity could have led to problems such as the distortion of the enzyme due to its multiple interactions with the support, which allows possible conformational changes in the three-dimensional structure of the biocatalyst, the blocking of the active site of the enzyme promoted by stereochemical effects, diffusional limitations derived from the access of substrate molecules to the active site of the FTase, as well as desorption of the enzyme because of its weak attachment to the support.[27,28,32]

This is the first work that specifically reports FTase immobilization on corncob particles. Nevertheless, there are studies that show the potential of corncob powder as a support material for the immobilization of other enzymes. A commercial glucoamylase immobilized on corncob powder by single-point chemical bonding was studied[5] and presented IY and RA values of 95.9 ± 1.2% and 8.4 ± 0.8%, respectively, after 24 h of immobilization at 145 rpm and 25 °C. Furthermore, a commercial trypsin

was immobilized on corncob powder activated with glyoxyl groups, glutaraldehyde and iminodiacetic acid-glyoxyl for 24 h at 25 °C, and the derivatives presented immobilization yields higher than 83% and retention of the catalytic activity higher than 74%.[37] In turn, Galárraga *et al.*[52] immobilized a peroxidase on a highly activated corncob powder for 3 h at 25 °C, achieving IY and RA equal to 74.3 ± 2.2% and 57.7 ± 1.1%, respectively.

Optimization of the temperature and pH of the reaction medium for the enzymatic activity of the immobilized FTase

The matrix of the experimental design, with the factors and levels employed, is shown in Table 3. It was observed that the highest values of the transfructosylation activity were achieved for the central points (50 °C and pH 5.5), with the average value of 4.98 ± 0.34 U g^{-1}. The central points presented low variation, indicating the good reproducibility of the process.

The optimum temperature and pH of the reaction medium obtained for this work are in accordance with results reported in the literature involving immobilization studies with the extracellular FTase from *A. oryzae* IPT-301. Faria *et al.*[32] immobilized the FTase on silica-gel and the studies on the experimental design presented maximum A_t at 50 °C and pH 5.5 for the biocatalyst obtained. Higher A_t values were also reported by Cunha *et al.*[26] for the same temperature and pH of the reaction medium when evaluating the biochemical properties of the soluble FTase. The results indicated that immobilization on different supports (corncob and silica-gel) did not affect the pH and temperature of the reaction medium. On the other hand, Aguiar-Oliveira and Maugeri[53] immobilized a FTase from *Rhodotorula* sp. by adsorption onto niobium ore. The immobilized enzyme exhibited maximum A_t at pH 4.5 and optimum temperature at about 62 °C. Yun and Song[54] immobilized a FTase from *Aureobasidium pullulans* KFCC 10524 on the ion exchange resin Diaion HPA25® and reported maximum A_t values at 55 °C and pH 5.5.

Table 4 shows the data on the estimated effects, standard error and *p*-value (statistical test to estimate the confidence interval of the model) of the effects of temperature and pH on the transfructosylation activity of the immobilized enzyme. The results of the *p*-values showed that only the quadratic pH had a significant influence at a 90% confidence level. According to Rodrigues and Iemma,[55] the bioprocess involving enzymes and microorganisms presents a great variability and, therefore, it is recommended to apply higher significance levels. These results suggest that the enzymatic activity depends significantly on pH and there is less influence of temperature on the FTase immobilized on chemically treated corncob particles. The influence of the pH in the reaction medium is one of the reasons for the preservation of the protein structure and enzymatic activity.[56,57] Enzymes have ionic groups at their active sites that should be in a suitable form (acidic or basic) to be functional. The enzymatic activity can be altered depending on changes in pH, that is, in the ionic form of the active site, which can change the three-dimensional shape of the biocatalyst, corroborating to the activity of enzymes in a pH range or value.[57]

Table 3. Experimental design matrix with values of the response variable

Run	Level (factor)		Transfructosylation activity (A_t) / (U g^{-1})	
	Temperature / °C	pH	Experimental values	Predicted values
1	–1 (43)	–1 (4.8)	2.82	3.40
2	–1 (43)	+1 (6.2)	3.41	3.40
3	+1 (57)	–1 (4.8)	1.17	3.40
4	+1 (57)	+1 (6.2)	3.20	3.40
5	–1.41 (40)	0 (5.5)	3.64	4.98
6	+1.41 (60)	0 (5.5)	4.52	4.98
7	0 (50)	–1.41 (4.5)	1.91	1.84
8	0 (50)	+1.41 (6.5)	2.13	1.84
9	0 (50)	0 (5.5)	4.70	4.98
10	0 (50)	0 (5.5)	5.35	4.98
11	0 (50)	0 (5.5)	4.88	4.98

Table 4. Estimated effects, standard error, and *p*-value for the evaluation of the effects of temperature and pH on the enzymatic activity (significance level of 10%, *p*-value ≤ 0.10)

Variable	Estimated effect	Standard error	*p*-value
Mean	4.977	0.401	0.000
T	–0.077	0.246	0.767
pH	0.366	0.246	0.196
T^2	–0.548	0.293	0.120
pH^2	–1.578	0.293	0.003
(T × pH)	0.360	0.348	0.348

T: temperature (°C).

The analysis of variance (ANOVA) was presented for the quadratic model with interactions applied to the transfructosylation activity of the immobilized enzyme (Table 5). The adjustment of the model for the experimental results was evaluated by the coefficient of determination of error (R^2), *p*-value, and the *F*-test. The statistical model (equation 2) explained 86.70% of the variability of the observed responses.

$$A_t\ (U\ g^{-1}) = 4.977 - 1.578(pH^2) \quad (2)$$

Table 5. Results of the analysis of variance (ANOVA) for the quadratic model with interaction for the evaluation of the effects of temperature and pH on the enzymatic activity

Source	Sum of square	Degree of freedom	Mean square	*F*-test	*p*-value
Model	15.75	5	3.15	6.52	0.030
Residues	2.42	5	0.48		
Lack of fit	2.19	3			
Pure error	0.22	2			
Total	18.17	10			
R^2 / %	86.70				
$F_{5;5;0.05}$	5.05				

R^2: coefficient of determination of error.

According to the ANOVA results, both *p*-value and *F*-test indicated that the statistical quadratic model was adequate for the prediction of A_t, represented by the response surface (Figure 3a) and contour curves (Figure 3b). The high values of A_t were obtained for an optimum zone in the temperature range from 42 to 58 °C at pH 5.5 for the biocatalyst.

Thermal stability of the soluble and immobilized enzymes

Figure 4 shows the inactivation rate of the soluble (Figure 4a) and immobilized (Figure 4b) FTase of *A. oryzae* IPT-301 at different incubation temperatures. After thermal pretreatments, the residual activity of the soluble and immobilized enzymes were 13.68% (1.27 ± 0.67 U mL^{-1}) and 19.89% (1.07 ± 0.42 U g^{-1}) at 30 °C, respectively. Furthermore, the decrease in residual activity was dependent on incubation temperature (40, 50 and 60 °C) and time, suggesting a thermal denaturation of the biocatalysts. It was observed that after 24 h, the immobilized FTase showed activity retentions of about 18, 15 and 5% at 40, 50 and

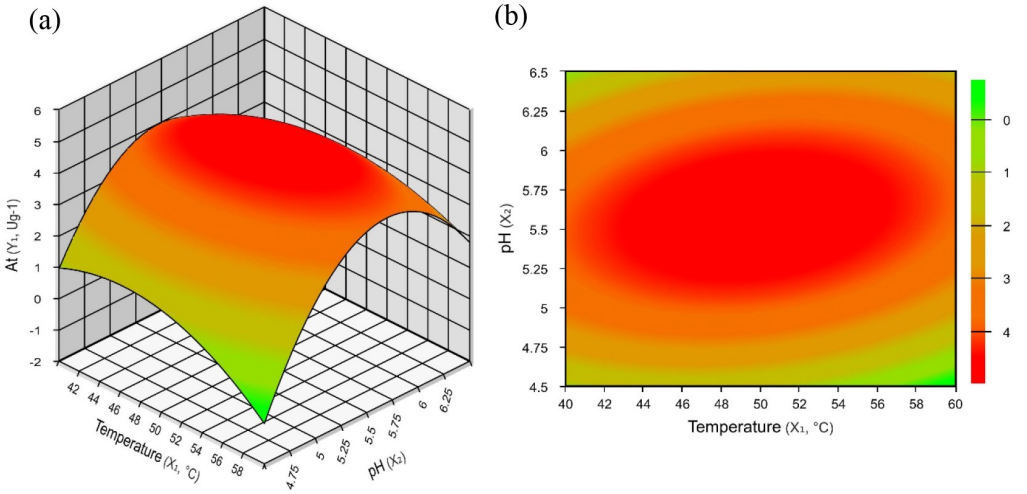

Figure 3. Response surface (a) and contour curves (b) for the transfructosylation activity of the biocatalyst as a function of the pH and temperature of the reaction medium.

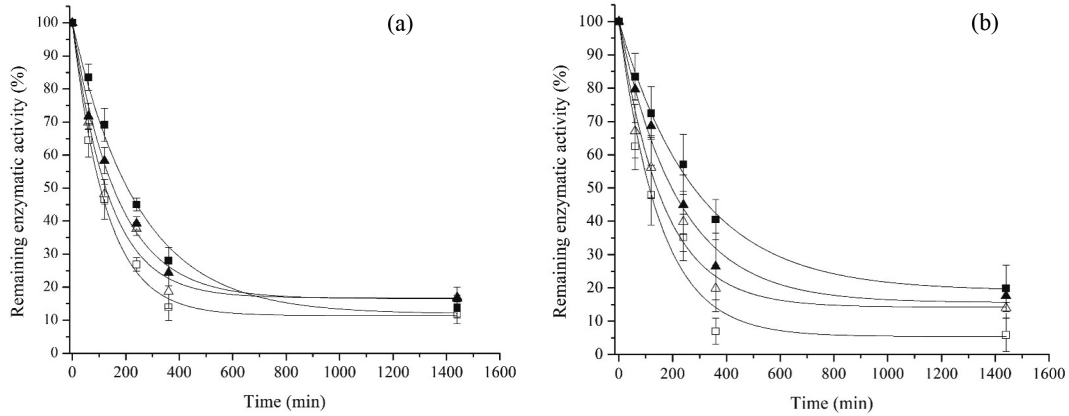

Figure 4. Thermal stability of the soluble FTase (a) and the FTase immobilized on alkali-treated corncob particles (TCCP) (b) over 24 h of incubation at different temperatures: 30 °C (■), 40 °C (▲), 50 °C (Δ) and 60 °C (□). Continuous line: thermal inactivation model according to Sadana and Henley[35] fitted to the experimental data. The maximum activity for soluble (9.29 ± 0.99 U mL^{-1}) and immobilized FTase (5.36 ± 0.48 U g^{-1}) was defined as 100% of relative activity.

60 °C, respectively, whereas the soluble enzyme presented, for all these incubation temperatures, activity retentions of around 15%. Usually, the exposure of enzymes to high temperatures results in an irreversible loss of their catalytic properties, since there is a cleavage of non-covalent interactions and conformational changes.[5,58]

Based on the binding of the enzyme to the support, several studies have associated the effect of immobilization with the thermal stability of the process. Onderková *et al.*[59] studied the immobilization of an FTase from *Aureobasidium pullulans* CCY 27-1-94 on an acrylic carrier. The immobilized biocatalysts showed thermostability from 20 to 50 °C, with around 100% of retention of the transfructosylation activity after 1 h of incubation. Faria *et al.*[32] immobilized an FTase from *A. oryzae* on silica gel by adsorption and obtained activity retention from 90 to 40% after 17 h of incubation at temperatures from 30 to 50 °C, respectively. Another recent study[27] evaluated the thermal stability of an FTase from *A. oryzae* IPT-301 immobilized by physical adsorption on polyhydroxybutyrate (PHB) and by covalent bonding on glutaraldehyde-activated PHB. The authors reported activity retentions from 70 to 20% for the FTase immobilized on PHB and from 75 to 30% for the enzyme immobilized on the activated support after 24 h of incubation at temperatures from 30 to 60 °C, respectively.

The thermodynamic parameters of the soluble and immobilized FTase are shown in Table 6. The half-life ($t_{1/2}$) of the soluble and immobilized enzymes decreased progressively and the first-order thermal denaturation constant (k_D) increased progressively with the increase in incubation temperature. The immobilized FTase showed a $t_{1/2}$ value about 1.3 and 1.1-fold higher than that of the soluble biocatalyst at 30-40 and 50-60 °C, respectively, indicating its greater thermostability when adsorbed on the organic support. The half-life is the time required for the enzymatic activity to decrease to 50% of its initial activity at a given temperature.[32,60]

Therefore, the higher $t_{1/2}$ values for the immobilized enzyme compared to those of the soluble enzyme indicate higher reaction rates for a longer period, allowing its application in industrial processes.[27,61,62] In a previous study,[27] an FTase from *A. oryzae* IPT-301 immobilized on PHB and glutaraldehyde-activated PHB showed $t_{1/2}$ values about 1.8 and 2.6-fold higher than the soluble enzyme at 30 °C, respectively. Furthermore, the same FTase immobilized on silica gel showed a stability factor about 2.5-fold higher than the soluble biocatalyst at 30 °C.[32] On the order hand, it was reported[53] that the half-life of a FTase from *Rhodotorula* sp. immobilized onto niobium ore increased 4-fold compared to the soluble enzyme at 50 °C.

Table 6 also shows the values of the activation energy of denaturation (E_D) for the soluble and immobilized FTase. This parameter expresses how much energy is needed to

Table 6. Thermodynamic parameters of the soluble FTase and the FTase immobilized on TCCP, incubated at different temperatures. The soluble and immobilized FTases were incubated at pH 5.5 (0.2 mol L^{-1} tris-acetate buffer) in the absence of the substrate at 30, 40, 50, and 60 °C

Parameter	FTase	Temperature / °C			
		30	40	50	60
R^2	soluble	99.33	99.55	98.64	99.71
	immobilized	99.70	98.96	98.18	96.83
k_D / min^{-1}	soluble	4.07×10^{-3}	5.95×10^{-3}	7.35×10^{-3}	7.92×10^{-3}
	immobilized	3.46×10^{-3}	4.57×10^{-3}	6.19×10^{-3}	6.62×10^{-3}
$t_{1/2}$ / min	soluble	205.91	153.51	124.90	104.87
	immobilized	278.43	196.34	141.23	113.59
SF		1.35	1.28	1.13	1.08
E_D / (kJ mol^{-1})	soluble	18.67			
	immobilized	18.97			
ΔH_D / (kJ mol^{-1})	soluble	16.15	16.07	15.99	15.91
	immobilized	16.45	16.37	16.29	16.20
ΔG_D / (kJ mol^{-1})	soluble	98.43	100.78	103.52	106.60
	immobilized	98.84	101.46	103.98	107.09
ΔS_D / (kJ mol^{-1} K^{-1})	soluble	0.272	–0.271	–0.271	–0.272
	immobilized	–0.272	–0.272	–0.271	–0.273

TCCP: alkali-treated corncob particles; R^2: correlation coefficient for k_D values; k_D: first-order thermal denaturation constant; $t_{1/2}$: biocatalyst half-life; SF: stability factor; E_D: activation energy of denaturation; ΔH_D: enthalpy of activation of denaturation; ΔG_D: Gibbs energy of activation of denaturation; ΔS_D: entropy of activation of denaturation; FTase: enzyme fructosyltransferase.

promote biocatalyst denaturation under the conditions assessed.[27,32,53] High E_D values indicate high enzyme thermostability.[27,60,62] The E_D values obtained show that the soluble (18.67 kJ mol^{-1}) and immobilized (18.97 kJ mol^{-1}) FTase presented similar energy values, suggesting enzyme thermostability did not change when it was immobilized by adsorption. The thermal stability studies of the FTase from *A. oryzae* IPT-301 showed E_D values around 56.8 and 41.6 kJ mol^{-1} for the biocatalyst immobilized by adsorption on silica gel[32] and PHB,[27] respectively.

In turn, E_D is related to the enthalpy of activation of denaturation (ΔH_D), which is an important thermodynamic parameter associated to the total amount of energy necessary to denature the enzyme.[25,63,64] Table 6 shows high and positive ΔH_D values for the whole range of incubation temperatures investigated, thus indicating that the soluble and immobilized FTase presented a thermostable behavior. Faria *et al*.[32] obtained ΔH_D values around 54 kJ mol^{-1} for the FTase immobilized on silica gel, while Araújo *et al*.[27] reported, for this parameter, values of 39 kJ mol^{-1} for the same enzyme immobilized on PHB.

Although high ΔH_D values suggest the biocatalyst is thermostable, it is also important to analyze the Gibbs energy of activation of denaturation (ΔG_D) and entropy of activation of denaturation (ΔS_D). ΔG_D is the most precise and reliable thermodynamic parameter to evaluate enzyme thermostability, since it includes the enthalpic and entropic contributions.[60,64] Thus, higher ΔG_D values indicate higher enzyme thermostability, as shown in Table 6. In addition, positive ΔG_D values were obtained for the soluble and immobilized FTase, suggesting a greater amount of enzyme in the native state than in the denatured state at equilibrium conditions.[60-62] Therefore, these values indicate that the thermal denaturation of the soluble and immobilized enzyme is a non-spontaneity process.[27,62,65]

On the other hand, negative ΔS_D values were obtained for the soluble and immobilized FTases at all temperatures tested. ΔS_D expresses the amount of energy *per* degree involved in the transition from a native to a denatured state.[60,63,64] The results shown in Table 6 indicate enzyme transition to a more ordered state, since enzyme resistance to unfolding because of stronger hydrophobic interactions overcomes the enzyme tendency to fall apart due to weakened polar interactions at high temperatures.[66,67]

Influence of sucrose concentration on the enzymatic activity and kinetic parameters

The influence of sucrose concentration on relative A_t is shown in Figure 5. The highest A_t (5.35 ± 0.70 U g^{-1}) was obtained at the sucrose concentration of 470 g L^{-1}. Similarly,

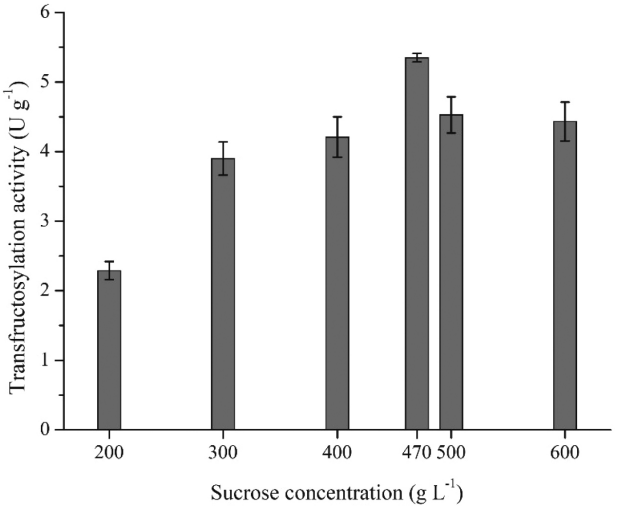

Figure 5. Effect of sucrose concentration on the transfructosylation activity of the FTase from *A. oryzae* IPT-301 immobilized on alkali-treated corncob particles (TCCP). Reaction conditions: 20, 30, 40, 47, 50 and 60% (m v^{-1}) sucrose solution and 0.2 mol L^{-1} of tris-acetate buffer (pH 5.5), 190 rpm at 50 °C for 60 min. The maximum activity for the FTase immobilized on TCCP (5.35 ± 0.70 U g^{-1}) was defined as 100% of relative activity.

Cunha *et al*.[26] and Faria *et al*.[32] have reported that the soluble FTase and the FTase immobilized on silica gel also showed the highest A_t at 470 g L^{-1}, suggesting the influence of sucrose concentration on FTase A_t was not modified by its immobilization on the functionalized corncob.

Figure 6 shows the fit of the Hill model to the data on the A_t of the biocatalyst as a function of sucrose concentration. The coefficient of determination (R^2) was 0.86 and the parameters v_{max}, $K_{0.5}$ and n were 4.80 ± 0.46 U g^{-1}, 205.34 ± 21.28 g L^{-1} and 3.99, respectively. The low v_{max} could be attributed to limitations in the transport of the sucrose molecules to the active sites of the enzyme.[27]

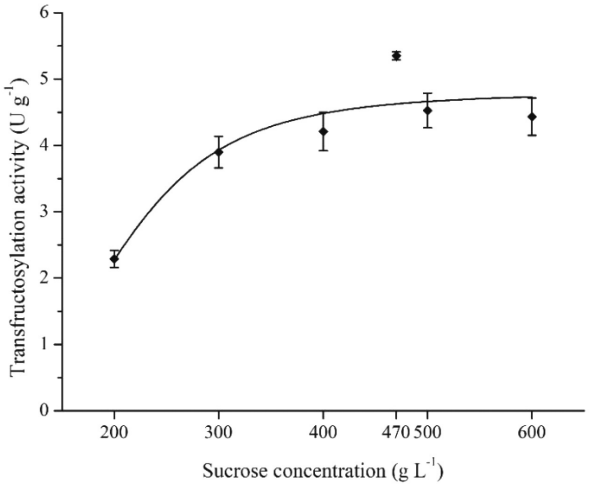

Figure 6. Data on the transfructosylation activity and its fit to the Hill model for the determination of the kinetic parameters of the FTase from *A. oryzae* IPT-301 immobilized on alkali-treated corncob particles (TCCP).

The positive Hill coefficient (n) value suggests there is a positive cooperative behavior between the multiple active sites of the enzyme and the substrate molecules.[68,69] This is in accordance with Aguiar-Oliveira *et al*.,[53] who reported that most of the FTases are found in their dimeric form, which means they have at least two active sites. On the other hand, Cunha *et al*.[26] reported that the soluble FTase from *A. oryzae* IPT-301 was successfully adjusted to the Michaelis-Menten model and showed R^2, v_{max} and K_M of 0.991, 16.23 U g^{-1} and 50.41 g L^{-1}, respectively. This suggests FTase immobilization on TCCP changed the kinetics behavior of the transfructosylation reaction. This alteration in kinetics was also observed for the FTase immobilized on silica gel.[32]

Effect of pH on the stability of the immobilized enzyme

Figure 7 shows the influence of pH on the enzymatic activity of the biocatalyst. The highest A_t (5.36 ± 0.48 U g^{-1}) was obtained at pH 5.5 and was considered as 100% of relative A_t. Considerably lower relative A_t values (lower than 30%) were obtained at pH values below 4.5. The relative A_t values at the pH range from 5.5 to 7.0 were higher than 75%. A similar behavior was observed for the FTase immobilized on silica gel.[32] Nevertheless, the soluble biocatalyst showed the highest relative activity at pH 6.0.[26] Additionally, the soluble FTase showed a lower A_t at higher pH values than those obtained in this work, suggesting the immobilization of the FTase on TCCP improved enzyme stability at the pH range from 5.5 to 7.0.

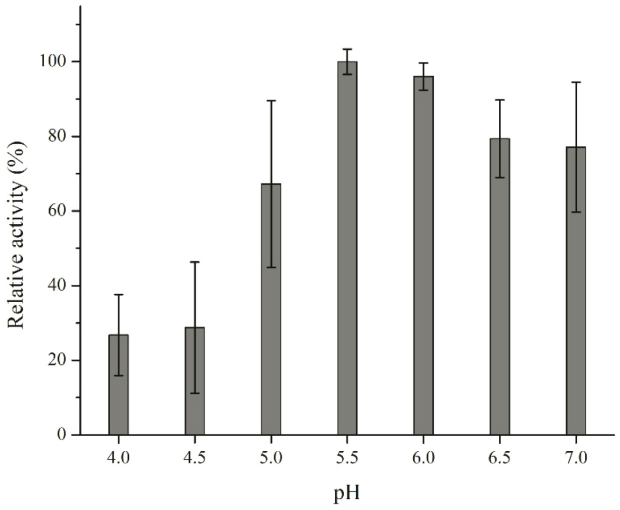

Figure 7. Stability of the FTase immobilized on alkali-treated corncob particles (TCCP) for 24 h of incubation at 4 °C in tris-acetate buffer solutions at different pH values. Reaction conditions: 47% (m v^{-1}) of sucrose solution and 0.2 mol L^{-1} of tris-acetate buffer (pH 5.5), 190 rpm at 50 °C for 60 min. The maximum activity for the FTase immobilized on TCCP (5.36 ± 0.48 U g^{-1}) was defined as 100% of relative activity.

Operational stability

The operational stability of the biocatalyst is shown in Figure 8. The highest A_t was 5.86 ± 0.62 U g^{-1} (100% of relative A_t). It can be observed that the A_t of the enzyme decreased along the reaction cycles and was about 3.8% at the sixth cycle, which could be attributed to enzyme dragging or denaturation during the transfructosylation reaction. Nevertheless, it is worth mentioning that in the second reaction cycle, the enzymatic activity still showed relative A_t of about 53%, suggesting an important potential of being reused during FOS production. This means FTase immobilization on TCCP allowed the synthesis of a biocatalyst with important operational advantages in comparison with enzymatic processes based on soluble enzymes that cannot be reused. Recently, a FTase immobilized on silica gel showed relative A_t of 100% during the two first reaction cycles, indicating higher operational stability.[32] Nonetheless, it must be considered that silica gel is an inorganic, widely used material for enzyme immobilization, whereas corncob is an organic, renewable, and shortly explored material, which can be abundantly obtained from agro-industrial waste. Furthermore, the applicability of other functionalization and treatment methods in corncob could improve the operational stability of the enzyme.

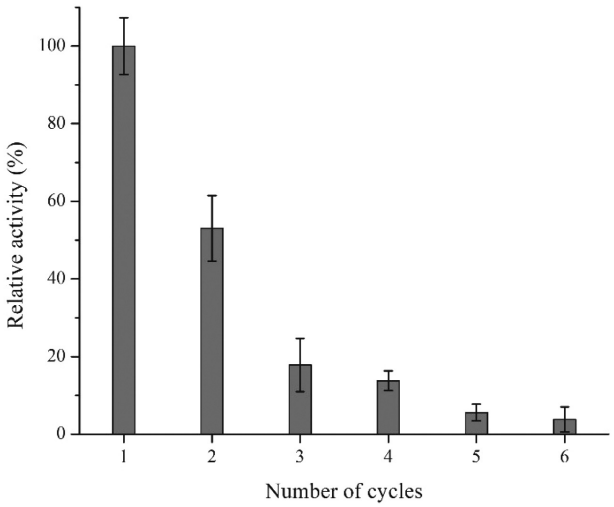

Figure 8. Operational stability of the FTase from *A. oryzae* IPT-301 immobilized on alkali-treated corncob particles (TCCP) during six consecutive reaction batches. Reaction conditions: 47% (m v^{-1}) of sucrose solution and 0.2 mol L^{-1} of tris-acetate buffer (pH 5.5), 190 rpm at 50 °C for 60 min. The maximum activity of the FTase immobilized on TCCP (5.86 ± 0.62 U g^{-1}) was defined as 100% of relative activity.

Conclusions

Alkali-treated corncob particles (TCCP) proved to be a potential material for the immobilization of

an extracellular FTase from *A. oryzae* IPT-301. The presence of the immobilized enzyme in the corncob was confirmed by FTIR analysis. The immobilized FTase presented greater thermal and pH stability, confirming the quality of the bond between support and enzyme for the immobilization. Enzyme immobilization changed the kinetic behaviour of the reaction of sucrose transfructosylation; nonetheless, the Hill model described in this work indicated a favorable performance of the interaction between enzyme and substrate. The operational stability achieved by the biocatalyst demonstrated a relevant operational advantage using the immobilized enzyme in the reaction of sucrose transfructosylation. Additionally, further studies about the functionalization of corncob particles (CCP) can improve its performance as a support material for FTase immobilization, bringing new opportunities for future research. This work is a breakthrough for fructooligosaccharides (FOS) production via new heterogeneous enzymatic catalysts, in which the immobilization process was improved by the use of a low cost, abundant and sustainable industrial residue.

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Author Contributions

Richard S. Pereira was responsible for conceptualization, investigation, methodology, validation, formal analysis, writing original draft; Elda S. Silva and Alfredo E. Maiorano for formal analysis, resources, writing (review and editing); Ana C. Vieira, Sylma C. Maestrelli, Paula C. Leite, Michelle C. A. Xavier, Melina S. Lopes, Ariela V. de Paula and Sergio A. V. Morales for writing (review and editing); Rafael F. Perna for writing (review and editing), project administration, supervision, resources, visualization.

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