REVISTA DESAFIOS
ISSN: 2359-3652
v. 11 n. 3 (2024): Dossiê Temático: Programa Institucional de Bolsas de Iniciação Científica da Universidade Federal do Tocantins. *DOI:*

http://dx.doi.org/10.20873/2024_v3_17

ARTIGO RECEBIDO: 14/12/2023 – APROVADO: 26/03/2024 - PUBLICADO: 22/04/2024

IMMOBILIZATION OF A MICROBIAL FRUCTOSYLTRANSFERASE ON BIOCHAR AND BIOCHAR FUNCTIONALIZED WITH GLUTARALDEHYDE AIMING AT FRUCTOOLIGOSSACHARIDES PRODUCTION

IMOBILIZAÇÃO DE FRUTOSILTRANSFERASE MICROBIANA EM BIOCARVÃO E BIOCARVÃO FUNCIONALIZADO COM GLUTARALDEÍDO OBJETIVANDO A PRODUÇÃO DE FRUTOOLIGOSSACARÍDEO

INMOVILIZACIÓN DE FRUCTOSILTRANSFERASA MICROBIANA SOBRE BIOCARBONO Y BIOCARBONO FUNCIONALIZADO CON GLUTARALDEHÍDO ORIENTADO A LA PRODUCCIÓN DE FRUCTOOLIGOSACÁRIDO

Thais Feitosa da Silva,[1]; Kauã Marques de Oliveira[1], Rafaela Rocha Pinto[1], Larissa Santos Saraiva[1], Raquel Marchesan[2], Rafael Firmani Perna[3], Sergio Andres Villalba Morales[1], Michelle da Cunha Abreu Xavier*[*1]

[1] Federal University of Tocantins, Graduate Program in Food Science and Technology, Palmas, Tocantins, Brazil

[2] Federal University of Tocantins, Graduate Program in Forest Engineering, Gurupi, Tocantins, Brazil.

[3] Federal University of Alfenas, Graduate Program in Chemical Engineering, Institute of Science and Technology, Poços de Caldas Campus, Minas Gerais, Brazil.

[*] Correspondence: michellecax@mail.uft.edu.br

RESUMO

Os frutooligossacarídeos (FOS) são um grupo de carboidratos que possuem unidades de frutose, com uma molécula de glicose no final. São estruturas que podem ser encontradas em uma grande variedade de vegetais, sendo também produzidas quimicamente pela reação enzimática de transfrutosilação da sacarose. O presente trabalho teve como objetivo a imobilização da enzima FTase extracelular de Aspergillus oryzae-IPT-301 em biocarvão de Eucalyptus e biocarvão de Eucalyptus funcionalizado com glutaraldeído, para a conversão enzimática de sacarose em FOS. Foram realizados testes de imobilização e reutilização e calculado o rendimento de imobilização e atividade recuperada. O rendimento de imobilização (RI) e a atividade recuperada (AR) da FTase imobilizada em biocarvão foram de 10,92% e 77,67%, respectivamente. RI e AR da FTase imobilizada no biocarvão funcionalizado foram 22,44% e 96,51%, respectivamente. A enzima imobilizada em biocarvão funcionalizado demonstrou atividade de transfrutosilação por mais ciclos que a enzima imobilizada em biocarvão. O uso de glutaraldeído permitiu 48% de atividade enzimática inicial no terceiro ciclo. As análises foram conduzidas até o sexto ciclo, no entanto, apenas 7% de atividade enzimática inicial foi

alcançado no ciclo final. Estes resultados sugerem que o biocarvão funcionalizado se destaca como material de suporte para imobilização de FTase visando a produção de FOS catalisada em biocatalisadores heterogêneos.
Palavras-chave: Frutooligossacarídeos. Frutosiltransferase. Biocarvão

ABSTRACT
Fructooligosaccharides (FOS) are a group of carbohydrates that have fructose units, with a glucose molecule at the end. These are structures that can be found in a wide variety of vegetables, being also produced chemically by the enzymatic transfructosylation reaction of sucrose. The present work aimed at the immobilization of the extracellular FTase enzyme from *Aspergillus oryzae*-IPT-301 on Eucalyptus biochar and Eucalyptus biochar functionalized with glutaraldehyde, for the enzymatic conversion of sucrose into FOS. Immobilization and reuse tests were carried out and immobilization yield and recovered activity calculated. The immobilization yield (IY) and recovered activity (RA) of the FTase immobilized on biochar were, 10.92% and 77.67%, respectively. The IY and RA of the FTase immobilized on the functionalized biochar were, 22.44% and 96.51%, respectively. The enzyme immobilized on functionalized biochar demonstrated transfructosylation activity for more cycles than the enzyme immobilized on biochar. The use of glutaraldehyde allowed 48% of initial enzymatic activity in the third cycle. Analyzes were conducted up to the sixth cycle, however, only 7% of initial enzymatic activity was achieved in the final cycle. These results suggest that functionalized biochar stands out as a support material for FTase immobilization aiming at FOS production catalyzed on heterogeneous biocatalysts.
Keywords*:* Fructooligosaccharides. Fructosyltransferase. Biochar.

RESUMEN
Los fructooligosacáridos (FOS) son un grupo de carbohidratos que tienen unidades de fructosa, con una molécula de glucosa al final. Estas son estructuras que se pueden encontrar en una amplia variedad de vegetales, y también se producen químicamente mediante la ocurrencia enzimática de la transfructosilación de sacarosa. El presente trabajo tuvo como objetivo inmovilizar la enzima FTasa extracelular de Aspergillus oryzae-IPT-301 sobre biocarbón de eucalipto y biocarbón de eucalipto funcionalizado con glutaraldehído, para la conversión enzimática de sacarosa en FOS. Se realizaron pruebas de inmovilización y reutilización y se calculó el rendimiento de inmovilización y la actividad recuperada. El rendimiento de inmovilización (RI) y la actividad recuperada (AR) de FTasa inmovilizada en biocarbón fueron 10,92% y 77,67%, respectivamente. RI y AR de FTasa inmovilizada en el biocarbón funcionalizado fueron 22,44% y 96,51%, respectivamente. La enzima inmovilizada en biocarbón funcionalizado demostró actividad de transfructosilación durante más ciclos que la enzima inmovilizada en biocarbón. El uso de glutaraldehído permitió 48% de actividad enzimática inicial en el tercer ciclo. Los análisis se realizaron hasta el sexto ciclo, sin embargo, en el ciclo final solo se logró el 7% de la actividad enzimática inicial. Estos resultados sugieren que el biocarbón funcionalizado se destaca como material de soporte para la inmovilización de FTasa con el objetivo de la producción de FOS catalizado en biocatalizadores heterogéneos.
Descriptores*:* Fructooligosacáridos. Fructosiltransferasa. Biocarbón.

INTRODUÇÃO

Nowadays, people are looking for a healthier lifestyle, which leads them to look for foods that have beneficial effects on their health. Therefore, the food industry aims at providing differentiated products, with natural and preserved sensory properties, and whenever possible, functional foods, among which fructooligosaccharides (FOS) stand out (Macedo *et al.*, 2020).

FOS have two to ten saccharide monomers, known as oligosaccharides, belonging to the fructan group. Fructooligosaccharides can be found in more than 36,000 plant species, including artichokes, garlic, asparagus, bananas, beets, onions, chicory, tomatoes, wheat and yacon (Cunha, *et al*., 2019; Macedo, 2020; Passos e Park, 2003). It is known that FOS improve the sensorial quality of foods, provide nutritional benefits to the animal organism and have a low caloric potential. It is also known that they are compounds highly soluble in water and have low crystallization and precipitation characteristics. They are used to change the freezing temperature of foods and can be applied to products to increase their humidity and reduce their water activity. In addition, they help in the treatment of diseases such as anemia, osteoporosis, hypertension, diabetes, lactose intolerance and kidney failure (Araújo *et al.,* 2023; Faria *et al*., 2021; Macedo *et al*., 2020).

Enzymatic hydrolysis and the enzymatic sucrose transfructosylation reaction are commercial ways to obtain FOS. One way to strengthen sugar production by the synthesis of FOS is to use biotechnological processes, employing the enzyme Fructosyltransferase (FTase, EC 2.4.1.9) immobilized on supports. The immobilization technique has the principle of confining the enzyme on a support. When this happens, the enzyme is protected by the support and its structure does not suffer from possible adverse environmental conditions, that is, the enzyme does not suffer damage and, therefore, it does not destabilize as easily as the enzyme that is soluble in the medium. Strong interactions between enzyme and support allow the reuse of enzymes, thus reducing operational costs and, consequently, reducing the value passed on to the consumer (Garcia *et al*., 2021).

In addition to allowing the reuse of the enzyme, the use of the immobilization technique promotes its high tolerance to changes in the operational conditions of the bioprocess, such as variations in solvents and salts in the medium and chemical (pH) and physical conditions

(temperature and agitation). Unlike immobilized enzymes, free enzymes are expensive to produce and purify and have an unstable three-dimensional structure, resulting in a short shelf life (Faria *et al*., 2021).

Biochar is a material similar to coal, being produced from plant materials such as forest residues that are decomposed at high temperatures, often during renewable energy production. This by-product has been used as an enzyme immobilizer support, since it presents interesting properties for this purpose, considering that it provides advantages in technical aspects, such as large surface area and abundance of pores, and advantages in environmental aspects, since it is a forest residue from the Amazon biome, which is abundant (Basiri *et al*., 2019; Pandey *et al*., 2000). Furthermore, the immobilization of enzymes on biochar can be maximized by the functionalization process, which increases the reactivity of its surface with the enzyme (Bijoy *et al.,* 2022).

Glutaraldehyde is a compound capable of reacting with different types of supports and enzymes, being considered a good functionalizing agent. The enzyme is covalently immobilized on the support by a reaction with its amino groups which, in turn, bind to the aldehyde groups of the functionalized support (Souza *et al*., 2017).

There are several microorganisms that produce the enzyme responsible for the transfructosylation activity (FTase, EC 2.4.1.9), especially the fungi *Aureobasidium pullulans, Aspergillus niger, Aspergillus oryzae, Rhodoturola diarensis* and *Cryptococcus* sp. (Cunha *et al*., 2019; Macedo *et al.*, 2020). Thus, the objective of the present work was the development of a heterogeneous biocatalyst by the immobilization of an extracellular FTase from *Aspergillus oryzae* - IPT-301 in biochar from agro-industrial or forestry waste, aiming at the production of fructooligosaccharides.

MATERIAL AND METHODS

Raw material

Eucalyptus biomass was collected in the rural region of the Federal University of Tocantins (UFT), Gurupi Campus – TO. The trees were transformed into logs and later into wood shavings. These shavings were dried under ambient conditions, exposed directly to the sun. The particle size separation process was performed using 16, 40 and 60 mesh sieves, and the material retained in the 40 mesh was used to prepare the biochar.

Biochar preparation

The granulometrically separated Eucalyptus particles were subjected to the process of pyrolysis with an initial temperature of 150 °C and a final temperature of 500 °C, and a heating rate of 5 °C/min, in a muffle furnace (Edutec).

Determination of the moisture content

To determine the moisture content, approximately 1g of biochar was dried in an oven (New Ethics, model 402/5N) at 70°C until constant weight was obtained. The calculation of the moisture content was carried out according to Eq. 1. The experiments were conducted in triplicate and all reagents used were of Sigma and Vetec analytical grade.

$$Moisture\ (\%) = \frac{A.100}{B} \quad (1)$$

In Equation 1, "A" means the mass of water lost during drying (g) and "B", the initial mass of the sample (g).

Support Functionality

To functionalize the support, the biochar particles were dried in an oven (New Ethics, model 402/5N) at 60 °C for 24 hours, and then functionalized in a 25% glutaraldehyde solution ($v.v^{-1}$) and distilled water. The solution was kept stirring for 18 hours, at 25 °C and 50 rpm, in a Shaker-type orbital shaker. The subsequent step was to separate the support from the solution, using vacuum filtration, and to reserve it in a desiccator with dry silica, of blue color, for later use.

Microorganism

The fungal strain *Aspergillus oryzae*-IPT-301 was provided by the Industrial Biotechnology Laboratory of the Technological Research Institute of the State of São Paulo (LBI/IPT-SP).

Inoculum preparation

To prepare the inoculum, the methodology of (Cunha, *et al*., 2019) was followed. The pellet containing lyophilized spores of the fungus *Aspergillus oryzae*-IPT-301 was resuspended in 10 mL of sterile distilled water, and aliquots of 70 µL were inoculated on solid PDA medium composed of 2.0% ($m.v^{-1}$) of potato dextrose agar, 2.5% ($m.v^{-1}$) of glycerin, 0.5% ($m.v^{-1}$) of yeast extract and 2.5% ($m.v^{-1}$) of glucose. The pH of the medium was adjusted to 5.5 and the material was incubated at 30 °C

for 7 days in a B.O.D. incubator. The PDA medium was sterilized in an autoclave at 121 °C for 15 minutes. Subsequently, the spores produced were collected by scraping with a Drigalsky loop, using 10 mL of a solution containing 0.95% NaCl ($m.v^{-1}$) and 0.1% Tween-80 ($v.v^{-1}$). Then, the suspension containing the spores was filtered using glass wool, and then 20% glycerin solution ($m.v^{-1}$) was added and, using a Neubauer chamber, spore concentration was adjusted to approximately 1.0 x 10^7 spores.mL^{-1}. The suspension was frozen and stored in a freezer.

Submerged cultivation

For the submerged cultivation, the methodology of Faria *et al*. (2021) was employed. The spore suspension with a concentration of 1.0 x 10^7 spores.mL^{-1} was inoculated in liquid medium containing 150 g/L ($m.v^{-1}$) of sucrose, 5.0 g/L ($m.v^{-1}$) of yeast extract, 5.0 g/L ($m.v^{-1}$) of $NaNO_3$, 2.0 g/L ($m.v^{-1}$) of KH_2PO_4, 0.5 g/L ($m.v^{-1}$) of $Mg_2SO_4.7H_2O$, 0.3 g/L ($m.v^{-1}$) of $MnCl_2.4H_2O$ and 0.01 g/L ($m.v^{-1}$) of $FeSO_4.7H_2O$. The culture was incubated for 64 hours at 30 °C and 200 rpm in a Shaker-type orbital shaker. The pH of the culture medium was adjusted to 5.5 using 0.1 M NaOH ($m.v^{-1}$) or 0.1 M HCl ($m.v^{-1}$), and the medium was sterilized in an autoclave at 121°C for 15 min. After cultivation, the medium was vacuum-filtered and the permeate, containing the extracellular enzyme (enzyme broth), was stored at refrigeration temperature. The experiments were performed in triplicate.

Scanning electron microscopy (SEM)

The morphology of the biochar particles was observed by scanning electron microscopy (SEM) using an electron microscope (Zeiss EVO MA-10, Germany), with an acceleration voltage of 15 kV and magnifications of 120, 250 and 500 times. The support material samples were deposited on a carbon tape in a sample holder (stub) and were covered by a thin film of gold-palladium alloy by the sputtering process, using a mini Sputter Coater model POLARON EMITECH SC7620. The purpose of the process was to be able to observe quantitative aspects of porosity of the support material in a non-functionalized condition. The analyses were performed at the Industrial Biotechnology Laboratory at IPT-SP.

Immobilization of the microbial extracellular enzyme – Kinetic assay

For the immobilization of the extracellular enzyme, approximately 1.0 g of biochar was added to 20 mL of the enzyme broth, and the solution was kept under stirring in a Dubnoff bath for 6 hours, at 35 °C and 175 rpm. Within the total analysis time (6 hours), every 1 hour, 0.1 mL aliquots of the supernatant were removed, and these data were used to assemble the adsorption kinetic curve.

After the end of the estimated time for immobilization, the medium was retained (cake) in vacuum and the support containing the immobilized enzymes and the aliquots of the kinetic points was stored in an 2.0 mL Eppendorf tube under refrigeration for subsequent tests of enzymatic activity and operational stability. The immobilization yield (IY) and recovered activity (RA) were calculated according to Ramos *et al.* (2015), who suggest Eqs. (2) and (3):

$$IY(\%) = \frac{A_{ti} - A_{tf}}{A_{ti}}. 100 \quad (2)$$

$$RA(\%) = \frac{A_{td}}{AD}. 100 \quad (3)$$

being A_{ti} and A_{tf} the transfructosylation activities (U) of the supernatant before and after immobilization, respectively; A_{td}, the transfructosylation activity (U) of the enzyme-support derivative and AD, the activity lost in the immobilization process.

Enzymatic activity

FTases, produced during fermentation, present transfructosylation activity (TA) characterized by the transfer of fructosyl groups between sucrose molecules, and this activity can be quantified. Experiments to determine the activity of the extracellular and immobilized FTases were conducted by the addition, in a test tube, of 0.1 mL of the enzymatic solution (assay for the soluble FTase) or 1 g of the support (assay for the immobilized FTase) in a reaction medium consisting of 3.7 mL of a sucrose solution (63.6%, $m.v^{-1}$) and 1.2 mL of 0.2 $mol.L^{-1}$ tris-acetate buffer, at pH 5.5 (Fernandez *et al*., 2007). The reactions were carried out for 60 minutes in a Dubnoff bath with stirring at 190 rpm and a temperature of 50 ºC. Once this stage was finished, the glassware containing the reagents was immersed for 10 minutes in a bath with boiling water, followed by cooling in an ice bath. After cooling, the solution was used for the quantitative analysis of glucose and total reducing sugars (TRS).

The transfructosylation activities (TA) were obtained using Equations 4, 5 and 6. One unit of transfructosylation activity is defined as the amount of enzyme that produces one micromol (1μmol) of transfructosylated fructose per minute, under experimental conditions (Cunha, *et al*., 2019; Ottoni *et al.,* 2012; Fernandez *et al*., 2007). Thus, FOS concentration consists in 1-kestosis, nystose and fructofuranosylnystosis (Fernandez *et al*., 2007; Ottoni *et al*., 2012). A_T activity was calculated according to Eq. (4).

$$A_t = \frac{[F_t].V_R}{t.W} \quad (4)$$

where [F_t] is the molar concentration of transfructosylated fructose (μmol.L^{-1}), V_R is reaction volume (L), t is reaction time (min) and W is the mass of biocatalyst (g) or volume of the fermented broth used in the enzymatic reaction (mL).

The values for fructose and transfructosylated fructose were calculated using Eqs. (5) and (6), respectively.

$$[F] = [ART] - [G] \quad (5)$$

$$[F_t] = [G] - [F] = [2G] - [ART] \quad (6)$$

being [ART], [F] and [G] the concentrations (μmol.L^{-1}) of total reducing sugars, fructose and glucose, respectively.

Quantification of the total reducing sugars (ART)

The quantification of the total reducing sugars present in the reaction medium was performed using the method adapted from Miller (1959), as described by Vasconcelos *et al.* (2013), using 3,5-dinitrosalicylic acid (DNS). Initially, 0.5 mL of the sample obtained from the enzymatic activity reaction was collected and inserted into a test tube. Then, in this same test tube, 0.5 mL of a DNS solution was added. This tube was then placed in a water bath with water at boiling temperature, for 15 minutes. After this time, the reaction was stopped by subjecting the test tube to cooling in an ice bath for 5 minutes, at which stage 4.0 mL of distilled water was added to dilute the sample. Aliquots from the test tube, after cooling, were taken and analyzed in a spectrophotometer (Bioespectro, model SP-22) stabilized at the wavelength of 540 nm. This procedure aimed to obtain absorbance values, used to determine ART concentrations based on an analytical calibration curve previously obtained for the DNS method.

Glucose quantification

The quantification of the glucose present in the reaction medium was determined by the method GOD-PAP® - commercial glucose oxidase enzyme kit (Cunha, *et al.,* 2019; Ganaie *et al.*, 2014). For this analysis, 35 µL of the sample obtained from the enzymatic activity reaction were added to 3.5 mL of a reagent solution (enzymatic kit) in a test tube. The reaction was performed in a Dubnoff bath (model NI 1232 from Bunker®) at 37 ºC for 5 minutes. After the reaction period, the absorbance at the wavelength of 505 nm was verified using a spectrophotometer (Bioespectro, model SP-22). This procedure aimed to obtain the absorbance values, used to determine the glucose

concentrations based on an analytic calibration curve previously obtained for the GOD-PAP® method - glucose-oxidase commercial enzyme kit.

Operational stability of the immobilized FTases

The operational stability of the immobilized enzymes was evaluated during cycles of sequential batch reactions, according to Faria *et al.,* (2021), where 1 g of the support containing the immobilized enzyme (biocatalyst) was added to a reaction medium containing 3.7 mL of a 47% commercial sucrose solution ($m.v^{-1}$) and 1.2 mL of 0.2 mol/L tris-acetate buffer, pH 5.5. After each reaction cycle, the biocatalyst was separated from the reaction medium by vacuum filtration and used in a new cycle, ending when a low transfructosylation activity was observed.

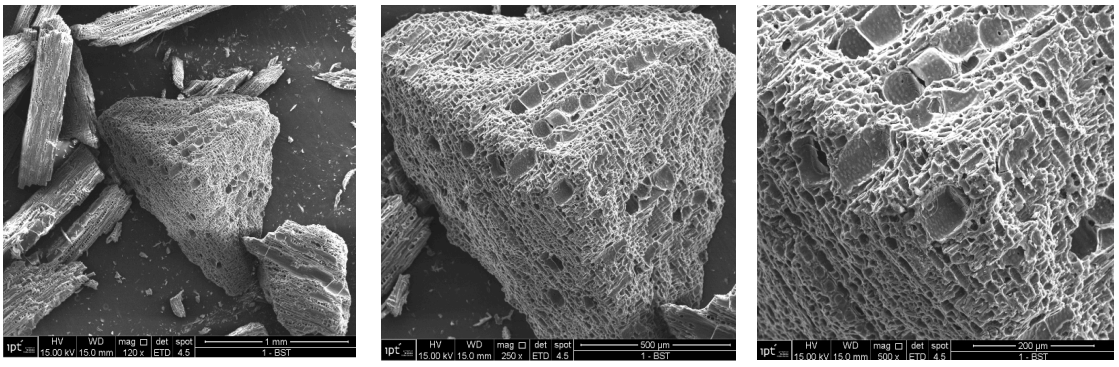
RESULTS AND DISCUSSION

Scanning electron microscopy (SEM)

The SEM images referring to the morphology of the non-functionalized biochar particle samples before the immobilization process are shown in Figure 1. The sample presented a surface with aspects of roughness and porosity, in a heterogeneous character, along its entire length. The heterogeneous porosity aspect occurs because the sample presents porosity with depths and regions that vary, with pores with smaller dimensions and depths prevailing. Figure 1-A presents a 120 times approximation, Figure 1-B presents a 250 times approximation and Figure 1-C presents a 500 times approximation.

Figure 1 - Electron micrographs of the non-functionalized biochar particle samples. A: 120 times approximation; B: 250 times approximation; C: 500 times approximation

A B C

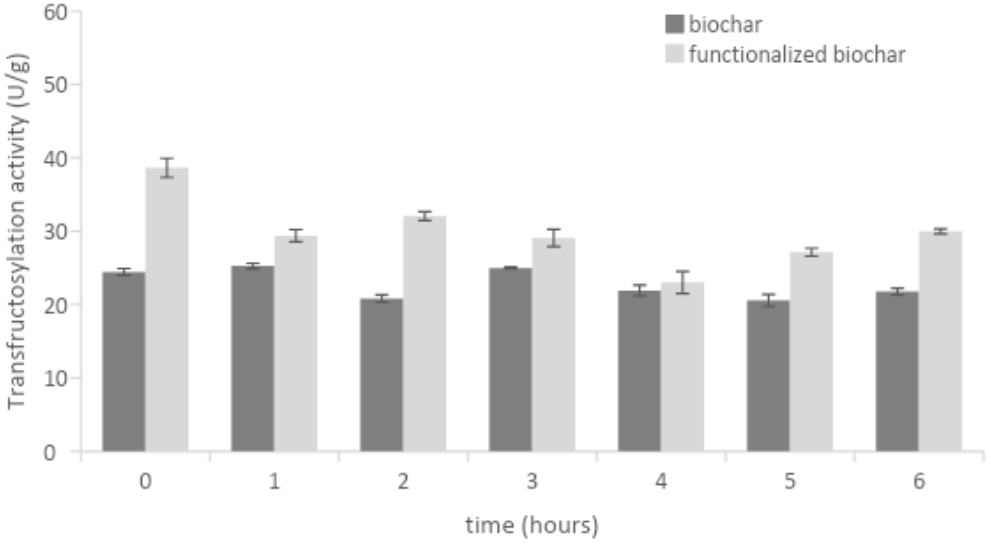


Source: Author's own elaboration

Kinetic profile of enzyme immobilization

The kinetic profile assays were performed aiming at mapping the kinetic transfructosylation behavior of the extracellular FTase immobilized on biochar and functionalized biochar. Figure 2 shows the results of the enzyme immobilization kinetic profiles for 6 hours at 35 °C.

Figure 2 - Kinetic profile of the extracellular FTase immobilized under two support conditions



Source: Author's own elaboration

Observing Figure 2, it can be noted that there was an interaction between the enzyme and both support conditions, since a reduction in enzymatic activity was observed over the 6 hours analyzed. The transfructosylation activity of the crude extract in the presence of biochar at time 0 hour was 24.44% (±0.092) and, after 6 hours of immobilization, the activity decreased to 21.77% (±0.050). The transfructosylation activity in the presence of the biochar functionalized with glutaraldehyde was 38.64% (±0.075) at the beginning of the immobilization and 29.97% (±0.021) at the end.

Araújo *et al.*, (2023) studied the immobilization process of the extracellular microbial FTase enzyme, using polyhydroxybutyrate (PHB) as support, in the pure form and functionalized with glutaraldehyde, aiming at FOS production. The authors report an enzyme adsorption behavior on the pure support relatively similar to that of the enzyme covalently immobilized on the functionalized support, showing that support functionalization will not always guarantee advantages in enzyme immobilization. Nonetheless, for the biochar support, the functionalization of this material was essential to obtain better FTase immobilization results. The performance of these kinetic tests made it

possible to determine parameters of immobilization performance and recovered activity, which are presented in Table 1.

Table 1 - Immobilization parameters for the pure and functionalized biochar after 6 hours of immobilization

Support	Immobilization yield (IY) (%)	Recovered activity (RA) (%)
Biochar	10.92	77.67
Functionalized biochar	22.44	96.51

Source: Author's own elaboration

Observing Table 1, after 6 hours of immobilization, there was a gain in the immobilization yield and recovered activity of the FTase immobilized on the functionalized biochar compared to the condition of just biochar. The immobilization yield for the functionalized support was around 11.52% higher than the immobilization yield for the support without functionalization, and the activity recovered on the functionalized support was 18.84% higher than the activity recovered on the support without functionalization.

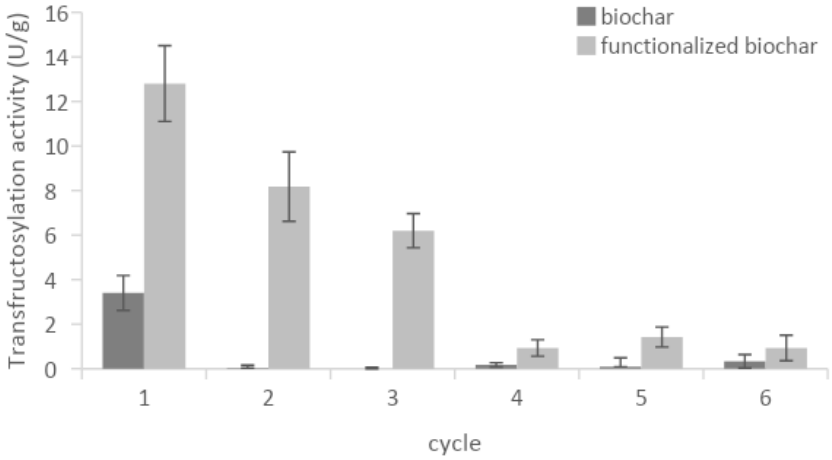
Araújo *et al.*, (2023) used the technique of support functionalization (PHB with glutaraldehyde) and found a better immobilization performance when in a functionalized condition. Nevertheless, the recovered activity was slightly higher for the FTase adsorbed to the biopolymer without glutaraldehyde. The authors comment, and are corroborated by Garcia-Galan *et al.*, (2011), that there are some effects on the immobilized enzymes, directly linked to the immobilization method applied, capable of altering the catalytic activity and selectivity. These include the distortion of the enzyme, if multiple interactions occur between the enzyme and the support, blockage of the active site, or diffusion problems.

Having a high recovered activity is important for the production of FOS for several reasons, such as: process efficiency, cost reduction, sustainability and market competitiveness. When an enzyme maintains a high recovered activity, this means that it is more stable and can be reused in multiple stages of the FOS production process, reducing the need for frequent replacement of the enzyme, saving time, resources and decreasing the production of waste generated, leading to efficiency in the production process and competitiveness in the market.

Operational stability of the enzyme

The operational stability during the recycling of the FTase immobilized on biochar and functionalized biochar was evaluated, and the results are shown in Figure 3.

Figure 3 - Operational stability of *Aspergillus oryzae*-IPT-301 FTase immobilized on biochar and functionalized biochar



Source: Author's own elaboration

In Figure 3, it is noted that, for all cycles evaluated, the values of TA on biochar without glutaraldehyde were lower than those of the enzyme immobilized on the functionalized biochar, which showed a greater activity retention. The difference between the values is already significant from cycle 1 to cycle 2, in the biochar condition only. The operational stability obtained under the condition of immobilization on the functionalized biochar decreased 36% from the first to the second cycle, and 93% from the first to the sixth cycle. The operational activity obtained when immobilized on the biochar not functionalized in glutaraldehyde dropped completely from the first to the second cycle, that is, 100% at the beginning. The operational stability behavior without the use of glutaraldehyde as a functionalizing agent demonstrated zero performance. This demonstrates the importance of functionalizing the immobilizing agent.

The fact that the functionalized biochar presents greater operational stability can be justified by the fact that the covalent bond, used in the adsorption of enzymes on activated supports, promotes the rigidification of their three-dimensional structure, resulting in a difficulty in enzyme desorption from the support (Mendes *et al.,* 2011). Operational stability is important for FOS production because it ensures that production processes are consistent and predictable, reducing the likelihood of unplanned failures. This parameter also minimizes the costs associated with unexpected stops, corrective

maintenance and waste of raw materials, contributing to the efficiency and sustainability of FOS production, and also ensuring compliance with standards and avoiding fines and legal problems.

Binhayeeding *et al.,* (2020) reported high operational stability, with activity retention greater than 50% after fourteen cycles for the lipase of *Candida rugosa* immobilized on PHB by a combination of immobilization techniques, physical adsorption and cross-linking with glutaraldehyde. Miranda *et al.*, (2014) reported that the lipase from *Thermomyces lanuginosus* was immobilized on mesoporous PHB particles and the biocatalyst retained approximately 70% of the initial activity after five consecutive cycles of esterification reactions.

CONCLUSION

Given the results presented, the immobilization parameters showed that the enzyme immobilized on the functionalized biochar presented a higher immobilization yield and recovered activity compared to the biochar without functionalization. Regarding operational stability, the FTase immobilized on the functionalized biochar showed a greater stability compared to the non-functionalized biochar. These results make clear a potential application of the covalent bond immobilization technique for the synthesis of extracellular FTase biocatalysts from *Aspergillus oryzae* IPT-301 applicable in FOS production.

ACKNOWLEDGMENTS

The authors are grateful for the financial support provided by CNPq and Capes and the technological support provided by IPT/SP.

Todos os autores declararam não haver qualquer potencial conflito de interesses referente a este artigo.

REFERÊNCIAS

ARAÚJO, I. M., BECALETTE, P. C., DA SILVA, E. S., DE SOUZA DIAS, G., XAVIER, M. C. A., DE ALMEIDA, A. F., MAIORANO, A. E., MORALES, S. A. V., PERNA, R. F. Enhancement of fructosyltransferase stability by immobilization on polyhydroxybutyrate and glutaraldehyde-activated polyhydroxybutyrate for fructooligosaccharides production. **Journal of Chemical Technology & Biotechnology**, 98(2), 419–430, 2023.

BASIRI, N., CLARA, S., WATER, V., FULCHER, A. F., WALKER, F., JAHROMI, N. B., FULCHER, A. (2019). **What Is Biochar and How Different Biochars Can Improve Your Crops**. Disponível em https://www.researchgate.net/publication/336124619. Acesso em 16/11/2023.

BIJOY, G., RAJEEV, R., BENNY, L., JOSE, S., VARGHESE, A. Enzyme immobilization on biomass-derived carbon materials as a sustainable approach towards environmental applications. **Chemosphere**, 307, 135759, 2022.

BINHAYEEDING, N., YUNU, T., PICHID, N., KLOMKLAO, S., SANGKHARAK, K. Immobilisation of Candida rugosa lipase on polyhydroxybutyrate via a combination of adsorption and cross-linking agents to enhance acylglycerol production. **Process Biochemistry**, 95, 174–185, 2020.

CUNHA, J. S., OTTONI, C. A., MORALES, S. A. V., SILVA, E. S., MAIORANO, A. E., PERNA, R. F. Synthesis and characterization of fructosyltransferase from aspergillus oryzae ipt-301 for high fructooligosaccharides production. **Brazilian Journal of Chemical Engineering**, 36(2), 657–668, 2019.

FARIA, L. L., MORALES, S. A. V., PRADO, J. P. Z., DIAS, G. de S., DE ALEMIDA, A. F., XAVIER, M. DA C. A., DA SILVA, E. S., MAIORANO, A. E., PERNA, R. F. Biochemical characterization of extracellular fructosyltransferase from Aspergillus oryzae IPT-301 immobilized on silica gel for the production of fructooligosaccharides. **Biotechnology Letters**, 43(1), 43–59, 2021.

FERNANDEZ, R. C., OTTONI, C. A., DA SILVA, E. S., MATSUBARA, R. M. S., CARTER, J. M., MAGOSSI, L. R., WADA, M. A. A., DE ANDRADE RODRIGUES, M. F., MARESMA, B. G., MAIORANO, A. E. Screening of β-fructofuranosidase-producing microorganisms and effect of pH and temperature on enzymatic rate. **Applied Microbiology and Biotechnology**, 75(1), 87–93, 2007.

GANAIE, M. A., LATEEF, A., GUPTA, U. S. Enzymatic trends of fructooligosaccharides production by microorganisms. **Applied Biochemistry and Biotechnology**, 172(4), 2143–2159, 2014.

GARCIA, R. L., DIAS, G. S., MORALES, S. A. V., XAVIER, M. C. A., SILVA, E. S., MAIORANO, A. E., TARDIOLI, P. W., PERNA, R. F. Glutaraldehyde-crosslinked cells from Aspergillus oryzae IPT-301 for high transfructosylation activity: optimization of the immobilization variables,

characterization and operational stability. **Brazilian Journal of Chemical Engineering**, 38(2), 273–285, 2021.

GARCIA-GALAN, C., Berenguer-Murcia, Á., Fernandez-Lafuente, R., & Rodrigues, R. C. Potential of Different Enzyme Immobilization Strategies to Improve Enzyme Performance. **Advanced Synthesis & Catalysis**, 353(16), 2885–2904, 2011.

MACEDO, L. L., VIMERCATI, W. C., DA SILVA ARAÚJO, C. Fructooligosaccharides: Nutritional, technological and sensory aspects. **Brazilian Journal of Food Technology**, 23, 2020.

MENDES, A. A., DE CASTRO, H. F., DE S. RODRIGUES, D., ADRIANO, W. S., TARDIOLI, P. W., MAMMARELLA, E. J., DE C. GIORDANO, R., DA L. C. GIORDANO, R. Multipoint covalent immobilization of lipase on chitosan hybrid hydrogels: Influence of the polyelectrolyte complex type and chemical modification on the catalytic properties of the biocatalysts. **Journal of Industrial Microbiology and Biotechnology**, 38(8), 1055–1066, 2011.

MILLER, G. L. Use of Dinitrosalicylic Acid Reagent for Determination of Reducing Sugar. **Analytical Chemistry**, 31(3), 426–428, 1959

MIRANDA, J. S., SILVA, N. C. A., BASSI, J. J., CORRADINI, M. C. C., LAGE, F. A. P., HIRATA, D. B., MENDES, A. A. Immobilization of Thermomyces lanuginosus lipase on mesoporous poly-hydroxybutyrate particles and application in alkyl esters synthesis: Isotherm, thermodynamic and mass transfer studies. **Chemical Engineering Journal**, 251, 392–403, 2014.

OTTONI, C. A., CUERVO-FERNÁNDEZ, R., PICCOLI, R. M., MOREIRA, R., GUILARTE-MARESMA, B., SABINO DA SILVA, E., RODRIGUES, M. F. A., MAIORANO, A. E. MEDIA OPTIMIZATION FOR β-FRUCTOFURANOSIDASE PRODUCTION BY Aspergillus oryzae. **Brazilian Journal of Chemical Engineeing.** V.29(01), 49–59, 2012.

PANDEY, A., SOCCOL, C. R., MITCHELL, D. New developments in solid state fermentation: I-bioprocesses and products. **Process Biochemistry**, Vol. 35, 2000.

PASSOS, L. M. L., PARK, Y. K. Frutooligossacarídeos: implicações na saúde humana e utilização em alimentos. **Ciência Rural**. V.33(2), 385–390. 2003

RAMOS, E. Z., JÚNIOR, R. H. M., DE CASTRO, P. F., TARDIOLI, P. W., MENDES, A. A., FERNANDÉZ-LAFUENTE, R., HIRATA, D. B. Production and immobilization of Geotrichum candidum lipase via physical adsorption on eco-friendly support: Characterization of the catalytic properties in hydrolysis and esterification reactions. **Journal of Molecular Catalysis B: Enzymatic**, 118, 43–51. 2015.

SOUZA, L. T. de A., VERÍSSIMO, L. A. A., PESSELA, J. B. C., SANTORO, M. M., RESENDE, R. R., & MENDES, A. A. IMOBILIZAÇÃO ENZIMÁTICA: PRINCÍPIOS FUNDAMENTAIS E TIPOS DE SUPORTE. **Biotecnologia Aplicada à Agro&Indústria**, v. 4, p. 529-568, 2017.

VASCONCELOS, N. M. DE ADOLFO, G., PINTO, S., ANTÔNIO, F., ARAGÃO, S. **Empresa Brasileira de Pesquisa Agropecuária** - Embrapa Agroindústria Tropical. Boletim de Pesquisa e Desenvolvimento no 88. Determinação de Açúcares Redutores pelo Ácido 3,5-Dinitrosalicílico. 2013.