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Immobilization of Fructofuranosidase from *Aureobasidium* sp. Onto TiO_2 and Its Encapsulation on Gellan Gum for FOS Production

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Abstract:
Fructofuranosidase (EC 3.2.1.26) from *Aureobasidium* sp. ATCC 20524, recovered from 5 L fermented medium, purified by two simple steps with a yield of 65 % and a purification factor of 16, was immobilized by adsorption onto titanium dioxide (FTIO). The enzyme was also covalently immobilized onto TiO_2 coated with polyethyleneimine (FTIOP) and encapsulated in gellan gum (FTIOPG). FTIO and FTIOP recorded an activity of 903 U g^{-1} and 9212 U g^{-1}, respectively. The immobilized enzyme showed high activity and stability at pH levels ranging from 4.0 to 8.0 and there were no changes in the temperature profile for either methodology when compared with free fructofuranosidase. The immobilized biocatalysts were reused 7 times for FOS production without significant activity loss, except FTIO at pH 5.0. Gellan gum was used for FTIOP encapsulation. FOS production was performed in a batch and a continuous reactor using FTIOPG as a biocatalyst. Batch conversion ($g_{FOS}/g_{initial\ sucrose}$) was around 60 % for initial sucrose concentrations of 100, 300 and 600 g L^{-1}, at a time of maximum conversion. Fixed-bed reactor operational stability was remarkable, providing a constant FOS production in the outlet of the column during 720 h.

Keywords: fructofuranosidase, fructooligosaccharide, titanium dioxide, immobilization

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1 Introduction

A prebiotic is a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth, activity (or both) of one or a limited number of bacteria in the colon, thereby improving the host's health (Gibson and Roberfroid 1995). Fructooligosaccharides (FOS), known and used as prebiotics, are renowned as natural food ingredients in most European countries, and have achieved GRAS status (Generally Regarded As Safe) in the U.S. and FOSHU status (Foods for Specified Health Use) in Japan (Antošová and Polakovič 2001; Hirayama 2002). Due to the broad range of their potential future applications there has been extensive research over the last three decades devoted to FOS production kinetics: physiological function, production source, and commercial application (Cáceres et al. 2004; Kurakake et al. 2010; Nemukula et al. 2009; Rastall and Maitin 2002; Sangeetha, Ramesh, and Prapulla 2005; Yun 1996).

FOS, type inulin, are composed of two to ten fructosyl units linked via β(2→1) glycosidic linkages with a D-glucose terminal head positioned at the non-reducing end (Hussein et al. 1998). Their general formula is GFn, where G is glucose and F is fructose, n = 2 is 1-kestose, n = 3 is nystose, and n = 4 is fructofuranosylnystose. When n > 10, the compounds are known as inulines, which are common in plants (Monsan and Ouarne 2009). Another kind of FOS are described as levan-type, with prebiotic activity, containing β(2 → 6) bonds between two fructose units or between a fructose and a glucose (Zambelli et al. 2014). FOS enzymatic production can be obtained by catalytic hydrolysis of inulin (Van Loo 2006) or synthesized from sucrose by the action of enzymes with transfructosylating activity (Jung et al. 1990; Hayashi et al. 1990; Duan et al. 1994; Lee, Chiang, and Tsai 1999; Antošová et al. 2008; Salinas and Perotti 2009; Alvarado-Huallanco and Maugeri-Filho 2010). FOS production with free enzyme is usually performed in batch process (Yoshikawa et al. 2008; Fernández et al. 2004; Shin

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et al. 2004) where enzyme is lost with the products. It is for this reason that much effort has also been put forth regarding the development of an enzyme immobilization method with the aim to obtain biocatalysts with good stability and minimal activity loss, thus paving the way for use in technology of said enzyme, where the main goal is finding a carrier.

Different methodologies and carriers have already been tested for the immobilization of enzymes with transfructosylating activity. Covalent immobilization of the enzyme has been performed on glass and silica activated with APTES, resulting in an activity yield of immobilization (YI) of 30.7 and 50.3 %, respectively (Hayashi et al. 1992a and 1992b), as well as on glutaraldehyde-activated chitosan with 82 % YI (Lorenzoni et al. 2014). Regarding physical adsorption, the enzyme has been immobilized on DEAE cellulose with 95 % YI (Hayashi et al. 1994), on niobium/graphite with 97 % YI (Aguiar-Oliveira and Maugeri 2010), and on nanoparticles of Fe_3O_4-chitosan with 65 % YI. Gel entrapment has also been carried out with alginate gel beads, resulting in 50.7 % YI (Ganaie et al. 2014; Fernandez-Arrojo et al. 2013). Therefore, one can conclude that the selection of the methodology and support used for enzyme immobilization is a process of trial and error.

In this paper we propose the use of titanium-based materials as an enzyme carrier of transfructofuranosidase immobilization. We used TiO_2, a low-cost, widely-used, thermally stable and non-flammable material which is non-toxic to humans and has been certified as a food additive by the Food and Drug Administration (FDA). It has been used in products like food coloring, water treatments, cosmetics and pharmaceuticals, etc. Since its introduction in 1923, as a commercial product, no health problems have been identified in relation to its exposure among the general population (Chen and Fayerweather 1988; Fryzek et al. 2003; Boffetta et al. 2004). Furthermore, TiO_2 has proven to be a practical enzyme carrier due to its mechanical characteristics and its renewed application as absorbent (Jesionowski, Zdarta, and Krajewska 2014). It presents high affinity with certain molecular groups, amino acids (Begonja et al. 2012), as well as carboxylic compounds (Araujo, Morando, and Blesa 2005; Roncaroli et al. 2010). Previous research has described protein immobilization by adsorption onto TiO_2 powder such as cysteine-peptidase (Llerena-Suster et al. 2009), human serum albumin (Oliva et al. 2003) and lipase B (Foresti et al. 2010). On the other hand, Bellino et al. (2010) immobilized polymerase onto mesoporous titania films.

TiO_2 has also been studied with polyethyleneimine (PEI) coating for use in medicine (Papa et al. 2013 and 2015) and electronics (Tang et al. 2006), among other applications. PEI is a cationic polymer that contains ionized primary, secondary and tertiary amino groups. Indeed, PEI is a very useful tool for enzyme immobilization. Enzyme immobilization on PEI-coated oxides such as aluminum or silica followed by cross-linking with a bifunctional reagent such as glutaraldehyde, is described in the bibliography (Oliveira et al. 2008; Piñuel, Mazzaferro, and Breccia 2011).

The aim of this work was to obtain an operational immobilized biocatalyst for FOS, type inulin, production. TiO_2 was used as the inorganic matrix and two strategies were evaluated for immobilization: adsorption and crosslinking with polyethylenimine. Additionally, immobilized FFase by crosslinking was encapsulated using gellan gum, and immobilized biocatalyst pellets were tested in batch and continuous reactors for FOS production.

2 Materials and methods

2.1 Materials

Gelrite® gellan gum, kestose, nystose, sucrose, fructose, glucose, polyethyleneimine and bovine serum albumine from Sigma Aldrich, and glutaraldehyde solution (50 % in water) from Merck were used in this research. Aeroxide® TiO_2 P25 was donated by Evonik Degussa Argentina S.A. and was used without further purification. Commercial glucose-oxidase kits from Wiener lab were also utilized. All other reagents were of analytical grade and used as received. All solutions were prepared with reverse osmosis water.

2.2 Microorganism and enzyme preparation

Aureobasidium sp. ATCC 20524 was used in this work. The strain, kept at −20 °C and in glycerol 20 %, was grown on a medium containing: sucrose 10 g L^{-1}, yeast extract 10 g L^{-1}, $NaNO_3$ 10 g L^{-1}, KH_2PO_4 5 g L^{-1}, and $MgSO_4.7H_2O$ 0.5 g L^{-1}. FFase production (E.C. 3.2.1.26) was conceptually similar to that previously described by Salinas and Perotti (2009). The two-step batch culture was carried out after adding 100 mL of inoculum culture to a 7 L stirred tank reactor (New Brunswick Series 100) containing 3.6 L of medium. When optical density achieved the value of 3 (λ = 620 nm), 1.3 L of 80 % sucrose was added to obtain a final concentration of 200 g

L^{-1} and an ending volume of 5 L. Temperature and pH were set at 30 ± 1 °C and 6.5 ± 0.1 respectively, and maintained throughout our experiments using automatic control. HCl was used for pH control and air flow, and agitation conditions were 2.5 vvm and 300 rpm, respectively. After cultivation, the cells were harvested by centrifugation at 10,000 rpm and 4 °C for 10 min. The supernatant was filtered on 0.22 µm membrane. The cell-free supernatant was diafiltrated using an Amicon® Ultra 100 K — NMWL filter and 100 mM citric acid/-NaOH pH 5.5 buffer as solvent. Then, two volumes of a chilled aqueous solution of ethanol were added to one volume of the diafiltered enzyme. The suspension was refrigerated at 4 °C for 1 h. The resulting precipitate was centrifuged at 5,000 rpm for 5 min. The enzyme pellet was collected and dissolved in 100 mM citric acid/NaOH buffer pH 5.5 and was used as the enzyme source. The total protein concentration was determined using the Lowry method (Lowry et al. 1951), using bovine serum albumin as standard.

2.3 Enzymatic activity

Transfructosylating activity was measured according to Jung et al. (1987). Sucrose 30 % (990 µL) prepared in a specific buffer (Citric acid/NaOH buffer (100 mM) for pH 3.0 to 6.0 and 100 mM Na_2HPO_4/NaH_2PO_4 buffer for pH 7.0 to 8.0) was mixed with free enzyme (10 µL), biocatalyst suspension (10 µL) or encapsulated biocatalyst (0.5 g). The reaction was performed at 40 °C (except for the experiments when temperature effect was studied) for 10 min. The reaction was stopped by heating at 100 °C for 5 min for free enzyme and, separated by filtration or centrifugation (5,000 rpm for 5 min) for heterogeneous biocatalyst. Samples were collected and used with commercial glucose-oxidase kits to quantify released glucose. One fructosyltransferase unit was defined as the amount of biocatalyst required to produce 1 µmol of glucose min^{-1} under the reaction conditions.

2.4 Enzyme immobilization

2.4.1 Preparation of FFase immobilized onto TiO_2 (FTIO)

FFase was immobilized by adsorption onto TiO_2 at six different pH levels, from 3 to 8. Immobilization was carried out by mixing 1 mL of a partially purified enzyme solution prepared in a corresponding buffer with 0.01 g TiO_2 for 1 h. Enzyme solutions of different concentrations were prepared in 100 mM buffer systems (described in Section 2.3.). The suspension was centrifuged after the immobilization period and the pellet (FTIO) was washed three times with buffer systems. The enzymatic activity of supernatant and pellet were measured at 40 °C, pH 5.5 and 30 % sucrose. The Lowry method was used to measure both the amount of protein offered to the support as well as proteins from buffers used for washing. The activity yield of immobilization was calculated.

2.4.2 Immobilization of FFase in TiO_2-PEI (FTIOP)

Polyethyleneimine was adsorbed onto TiO_2 support. TiO_2 (0.2 g) was suspended in 10 mL of PEI solution 10 % (w/v) and stirred at room temperature for 2 h. The suspension was centrifuged and the pellet (TiO_2-PEI) was washed several times with 100 mM Na_2HPO_4/NaH_2PO_4 buffer (pH 7). To confirm the PEI coating on the TiO_2 support, we tested its reaction in 1 % $CuSO_4$ solution and observed the blue complex formed with copper ions (Piñuel, Mazzaferro, and Breccia 2011). Immobilization was carried out by mixing 1 mL of enzyme solution prepared in the buffer at pH 7 with 0.02 g of TiO_2-PEI in 1 mL of buffer. Glutaraldehyde (GA) was added to reach a final concentration of 0.5 % and was stirred at room temperature for 2 h. The suspension was centrifuged and the pellets were washed several times and left in the buffer (pH 7) at 4 °C for conservation. A non-GA sample was used as control. The enzymatic activity of supernatant and pellet were measured at 40 °C, pH 5.5 and 30 % sucrose.

2.4.3 Activity yield of immobilization

For FTIO and FTIOP the activity yield of immobilization (YI) was calculated as:

$$YI = \frac{A_I}{A_{Tot} - A_{NI}} \times 100$$

where: A_I is the enzymatic activity of the immobilized biocatalyst, A_{NI} is the residual activity in supernatant and washings after the immobilization process, and A_{Tot} is the total enzymatic units offered to the support during the immobilization process. Immobilized protein was determined from the difference between the amount of protein offered for immobilization minus that recovered into the supernatant plus washings.

2.5 FTIOP encapsulated in gellan gum (FTIOPG)

FTIOPG was prepared using the cation-induced ionotropic gelation method (Agnihotri, Jawalkar, and Aminabhavi 2006). A 1.5 % gellan gum aqueous solution was prepared in water and 0.05 g FTIOP in 2.5 mL of water was added to 2.5 mL of this solution, stirring continuously, and then kept at 40 °C for 2 h to remove bubbles. The resulting gel was used to make the FTIOPG beads. The gel was dropped into 40 mL of gently stirred 100 mM $MgSO_4$ solution through a syringe needle (0.3 mm diameter) using a peristaltic pump (LKB 2132 Microperpex peristaltic pump). Beads were then left to harden in the $MgSO_4$ solution for 30 min at room temperature (Costas et al. 2012). The beads were filtered and washed three times with 100 mM Na_2HPO_4/NaH_2PO_4 buffer pH 7 and stored at 4 °C. Particle size distributions were determined from the FTIOPG picture using stereomicroscope and *Image J* software.

2.6 Operational stability of titania-based biocatalyst

Biocatalyst reuse was performed following the reaction in 30 % sucrose in buffer at 40 °C for 1 h per cycle. The reaction was restarted after the biocatalyst was separated from the reaction media and washed. Activity was measured in the supernatant. Biocatalyst reuse assays were carried out with FTIO at pH 4 and 5, FTIOP at pH 5.5 and FTIOPG at pH 5.5. The buffer was used as described in section 2.3.

2.7 Chromatographic sugar analysis

The separation of sugars and quantification were performed by Ion-Exclusion Chromatography, using HPLC (Knauer, Germany). A Rezex RSO-Oligosaccharide Ag^+ (4 %) column (200 mm x 10 mm i.d., particle size 8 µm, 4 % of cross-linking resin of Torrance, CA, USA) was used. The mobile phase consisted of water at a flow-rate of 0.2 mL min^{-1}, with a column temperature of 30 ± 1 °C (modification of Madlová et al. (2000)). Calibration curves for kestose, nystose, sucrose, fructose, and glucose were obtained using commercial standards.

2.8 FOS production

2.8.1 Batch production

FOS production was performed in a 20 mL sucrose solution with an initial sucrose concentration of 10, 30 and 60 % in 100 mM citric acid/NaOH buffer (pH 5.5, 50 rpm) at 40 °C and stirring continuously. FTIOPG was used as biocatalyst with an enzymatic activity of 0.2 U mL^{-1}. FOS production was followed by HPLC.

2.8.2 Continuous production

The reactor consisted of a glass column, filled with 930 units of FTIOPG beans. FOS production was evaluated in packed bed. The reactor was flow rate controlled with adjustable peristaltic pumps Masterflex L S. The packed bed column (height, 200 mm; inner diameter, 10 mm; volume, 15.7 mL) has an entrance at the bottom and an exit at the top, which was fitted with a polypropylene mesh to retain the particles within the column. FOS production was performed with an initial alimentary sucrose concentration of 25 % in 100 mM citric acid/NaOH buffer (pH 5.5) at 40 °C with an ascent rate from 0.165 cm min^{-1}. FOS production was followed by HPLC.

3 Results and discussion

3.1 Enzyme preparation

FFase production yielded 190 U mL^{-1} of final activity (specific activity of 170 U mg^{-1}), in a 7 L bioreactor after 96 hours under controlled conditions. A FFase final purification factor of 16.25 was obtained using diafiltration with a recovery of 95 % and by precipitation with ethanol up to a specific activity of 2765 U mg^{-1} with a recovery of 65 %. Likewise, small molecules can reduce the exposed surface for adsorption or covalent bonding of the enzyme to the matrix. SEM/EDS showed the presence of salt deposits on the inorganic matrix (Navntoft et al. 2007), and high ionic strength, over 200 mM of NaCl, which promotes the desorption of the enzyme in a agarose/PEI-glutaraldehyde system (Pessela et al. 2005); it is therefore important to diafiltrate the crude extract to reduce the salt content.

3.2 Enzyme immobilization

Two immobilization strategies were evaluated: physical adsorption onto TiO_2 powders (FTIO) and immobilization using a thin-layer of PEI coating onto TiO_2 powders (FTIOP).

Immobilization of FFase onto TiO_2 was carried out at different pH values (from 3 to 8), which is the activity range of the native enzyme. Figure 1 suggests that the behavior of the immobilization at different pH values is due to electrostatic interactions between TiO_2 and the enzyme, and the best results were obtained at a pH far from 7. There was weak evidence of interaction between enzyme and matrix surface at pH 6.5, around TiO_2 potential zero charge (PZC). In addition, a high residual enzymatic activity was obtained when FTIO was prepared at pH 3 and 4. The results of this study match those reported by Rajh, Ostafin, and Micic (1996) concerning a strong adsorption of cysteine onto TiO_2 at pH 4 due to the adsorption of the carboxylic groups of the molecule. The electrostatic nature of TiO_2 and its tendency to generate ligand-surface interactions between functional groups of enzymes, such as tiols, amines and carboxylic groups had previously been proposed by Oliva et al. (2003).

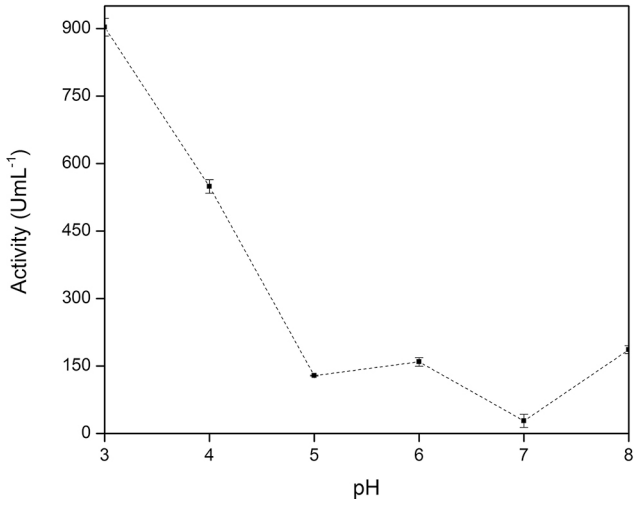

Figure 1: Influence of pH in immobilization process of FFase onto TiO_2 (-●-) FTIO. Dots represents the biocatalyst activity prepared at each pH, measured at pH 5.5 and T=40 °C.

On the contrary, the pH response of enzyme immobilization in FTIOP system could not be analyzed due to the glutaraldehyde stability restriction in the region of pH 7.

Table 1 shows the activity of the biocatalysts prepared by immobilization, the activity yield of immobilization (YI) and the immobilized protein. The FTIOP system was able to retain approximately 10 times more protein than FTIO and, accordingly, the activity per gram of FTIOP achieved a greater order of magnitude than FTIO. This is probably due to functional groups of PEI, with plenty of amine groups that interacted easily with the metal oxide surface and through the glutaraldehyde, thus facilitating the cross-linking with the amine group of the native enzyme. In the same context, Wasserman, Hultin, and Jacobson (1980) reported higher activity per gram of support when glucose-oxidase and catalase were immobilized by using PEI-functionalized glass beads.

Table 1: Immobilization of FFase by two different techniques.

	Activity U g^{-1} TiO_2	Activity Yield %	Immobilized protein mg of proteins g^{-1} TiO_2
FTIO	903	96	1.5
FTIOP	9212	92	17

YI reached a final value of over 90 % for both methods used. These values are better than those obtained by other authors such as Aguiar-Oliveira and Maugeri (2010) when FFase, parcially purified, was immobilized onto niobium with a final specific activity of 164 U g^{-1} (YI 97 %); Hayashi et al. (1992a) obtained 222 U g^{-1} (YI 44.4 %) when derivatized porous silica was used; and Lorenzoni et al. (2015) immobilized 2100 U g^{-1} of FFase (YI 42 %) onto derivatized chitosan with higher activity than that of FTIO.

3.3 Effect of pH and temperature on free FFase, FTIO and FTIOP activity and stability

Figure 2 assesses pH influence on the biocatalyst activity, comparing free enzyme with immobilized biocatalysts. Both methods for immobilization preserved FFase activity in a wide pH range. Furthermore, the FTIO system showed a slight increase of activity maintaining 60 % of its activity at a pH range of 4 to 6. FTIOP presented a higher increase of activity keeping 80 % of their maximum activity at a wide pH range of 4 to 7. Moreover, the pH profile of the immobilized enzyme was broader than that of the free enzyme, which could be related to a stability increase of the protein structure due to the immobilization by cross-linking (Lorenzoni et al. 2014). The expansion of the pH range where the biocatalyst maintains its activity could mean a technological advantage in FOS production (Pal and Khanum 2011).

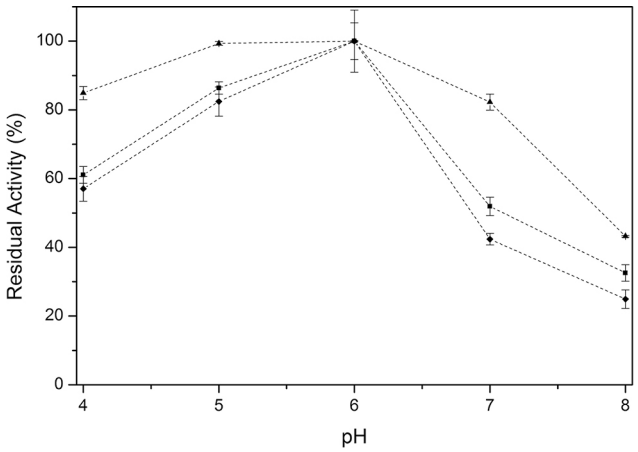

Figure 2: Effect of pH on biocatalysts activity. (- -) FFase, (-■-) FTIO, and (-▲-) FTIOP. Using 30% (w/v) sucrose, as substrate, prepared in 100 mM citric acid / NaOH buffer, for pH 3.0 to 6.0 and 100mM Na_2HPO_4 / NaH_2PO_4 buffer for pH 5.0 to 8.0. All measured activities were normalized setting the highest value at 100%.

The activity of both immobilized biocatalysts (FTIO and FTIOP) and the free enzyme at several temperatures showed similar behavior as shown in Figure 3. In fact, the highest activity for all the systems was obtained at 60 °C. It can be concluded that temperature does not change the enzyme behavior when these techniques are used, contrary to Hayashi et al. (1992a), who reported that below 55 °C the system FFase onto silica significantly lost activity when compared with the free enzyme. In the case of invertase onto sepabeads-PEI, Torres et al. (2002) described a maximum activity at 50 °C, but below this temperature, no changes were observed in the activity profile against temperature.

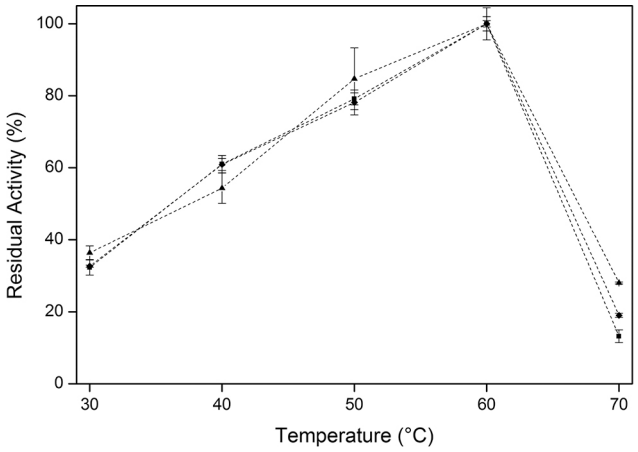

Figure 3: Effect of temperature on biocatalysts activity. (- -) FFase, (-■-) FTIO and (-▲-) FTIOP. Using 30% (w/v) sucrose, as substrate, prepared in 100 mM citric acid / NaOH buffer pH 5.5 at different temperatures. All measured activities were normalized setting the highest value at 100%.

In order to analyze their stability, the free enzyme and the immobilized derivatives, (FTIOP and FTIO) were incubated for 1 h at several pH and temperature ranges and residual activities were measured. The stability of free FFase, FTIO and FTIOP at several temperatures is shown in Figure 4. FFase and FTIOP were heat-labile over 50 °C and maintaining 85 % of their activity, while FTIOP preserved more than 95 % of the activity at 50 °C. The slight increase in the activity response of FTIOP at 50 °C could be attributed to intermolecular covalent bonds among the enzyme, PEI, and metal oxide. A small increase in immobilized FFase stability over 50 °C was also observed by other authors (Tanriseven and Aslan 2005; Hayashi et al. 1992a).

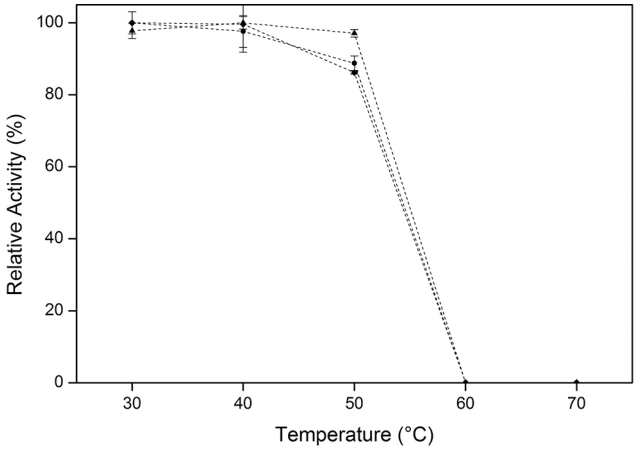

Figure 4: Effect of temperature on stability of (- -) FFase, (-●-) FTIO and (-▲-) FTIOP. Using 30% (w/v) sucrose, as substrate, prepared in 100 mM citric acid / NaOH buffer at 5.5 for 1 h. All measured activities were normalized setting the highest value at 100%. The activities were measured after 1 h incubation at each temperature.

FTIOP was stable in the pH range from 4 to 8, maintaining more than 90 % activity after 1 h incubation at each pH. However, FTIOP began to significantly lose its stability at pH under 4. This behavior is similar to that described by Hayashi et al. (1992a), who reported that the native enzyme maintained its stability at a pH range from 4 to 9 but an significant decrease occurred at pH 3 (50 %). FTIO stability was not evaluated at different pH levels because the enzyme immobilization onto TiO_2 is related to surface charges, thus pH could have released the adsorbed enzyme.

3.4 FTIOP encapsulation in gellan gum

It is necessary to take FTIOP or FTIO powder size into account in order to expand their use for industrial application. TiO_2 modal particle size is ca. 25 nm. This morphology implies the incorporation of a filter operation after the FOS reactor production. Thus, it might be necessary to develop larger particles of FTIOP and FTIO to avoid an additional recovery step. It is for this reason that the biocatalyst FTIOP was encapsulated in gellan gum, a linear polymer of a high molecular weight. Agnihotri, Jawalkar, and Aminabhavi (2006) demonstrated that the external morphology and porosity of gellan gum beads are significantly affected by pH. The gellan

gum beads prepared at pH 5.0 apparently created porous structures that reduced diffusional problems of substrates and products. The gellan gum is a promising encapsulating material with good porosity for biocatalyst activity at optimal pH conditions.

Moreover, gellan gum gelation is induced by different cations, such as group I cations (Na^+, K^+, Li^+); group II cations (Mg^{2+}, Ca^{2+}, Sr^{2+}, Ba^{2+}), and divalent transition metals (Zn^{2+}, Mb^{2+}) (Morris, Nishinari, and Rinaudo 2012). This condition allows choosing an appropriate cation in order to reduce the possibility of interaction among cations and reaction products, reaction raw materials, and enzyme activity modulation. Magnesium cation was used in this work as an ion-induced gelation, because it is not consumed during FOS production, therefore maintaining the stability of the gel structure.

Due to its specific activity, thermal stability and operational versatility FTIOP was selected as the biocatalyst for encapsulation in gellan gum to produce the biocatalyst FTIOPG. FTIOPG was tested at three shaking speeds in order to evaluate the enzyme leakage and there was no change in the activity, thus suggesting that diffusional effects were not significant. Encapsulation of FTIOP in gellan results in a strong structure for scaling up the biocatalyst for immobilized bed reactors. Blank test of FFase encapsulated in gellan gum was carried out to evaluate activity, but it was discharged because the enzyme was not encapsulated in the gel.

FTIOPG beads revealed spherical particles with a certain degree of polydispersity, with a mean bead size of 1826 ± 139 µm. This size therefore makes it possible to use the beads in fixed bed column reactors. Other authors have performed similar work in order to prepare biocatalysts suitable for reactors. Piñuel, Mazzaferro, and Breccia (2011) developed a biocatalyst of α-rhamnosyl-β-glucosidase immobilized on pectin and silica, reaching a mean bead size of 1670 ± 990 µm. In addition, Fernandez-Arrojo et al. (2013) immobilized FFase in alginate achieving a bead size between 1000 and 3000 µm and their application was shown on a fixed bed column to produce FOS.

3.5 Biocatalyst operational stability

When developing a biocatalyst for industrial use, one major challenge is to obtain a biocatalyst capable of being recycled; thus, it is necessary to study the ratio between best activity and high stability. In order to preserve the stability of the biocatalysts, the working temperature was fixed at 40 ºC. FTIO was tested at working pH 4 and 5. A working pH for FTIOP and FTIOPG was set at 5.5 because at a lower pH than 5.0 and higher than 6.0, the biocatalyst activity decreases sharply (see Figure 2).

Figure 5 shows that 95 % of relative activity of the biocatalysts FTIOP and FTIOPG was kept through seven reaction cycles. When FTIO was tested at pH 5.0 there was a significant performance loss (up to 40 % of residual activity). On the other hand, at pH 4.0, almost 80 % of enzymatic activity remained after the 7 cycles. This may be explained by the progressive leaching of the enzyme attached to the support due to the reduction of ionic interaction at a pH of 5. Fernandez-Arrojo et al. (2013) maintained more than 90 % activity after 13 recycles of FTase immobilized in DALGEEs (dried alginate entrapped enzyme).

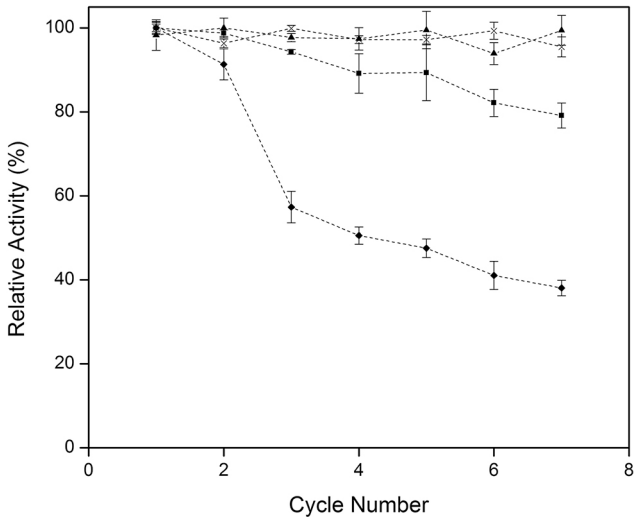

Figure 5: Immobilized biocatalyst reuse: (-■-) FTIO pH 4.0, (- -) FTIO pH 5.0, (-x-) FTIOP pH 5.5 and (-▲-) FTIOPG pH 5.5. Sucrose concentration 30% (w/v) at 40 °C. All measured activities were normalized setting the highest value at 100%.

3.6 FOS production

3.6.1 Batch production

FOS production was developed in three different concentrations of initial sucrose (100, 300, and 600 g L^{-1}). FOS conversion was measured as a percentage of $g_{FOS}/g_{initial\ sucrose}$, the time for maximum conversion (h) and the global FOS productivity (g_{FOS} h^{-1} L^{-1}), as shown in Table 2. In all three cases, global FOS productivity was kept constant at 2.5 g h^{-1}. Regardless, the time to achieve the maximum conversion increased considerably as initial sucrose concentration increased. Aguiar-Oliveira and Maugeri (2010) achieved 60 %, and Ganaie et al. (2014) achieved 67 % conversion for FOS production using FFase immobilized from several microorganisms. Other authors have produced FOS with free enzymes from *Aureobasidium* sp., recording yields of 53–62 %, even reaching values of 69 % by adding glucose isomerase (Yoshikawa et al. 2007 and 2008). With FTIOPG yields are similar to those achieved with free enzymes, but with considerable added advantages that immobilization provides, including easy separation of products and enzyme re-utilization, thus extending its operative lifetime, providing operational stability and opening up the possibility of using a continuous reactor.

Table 2: Maximum FOS conversion and productivity at different initial sucrose concentration.

Initial sucrose concentration [g L^{-1}]	$g_{FOS}/g_{initial\ sucrose}$ [%]	Time reaction for highest conversion [h]	Productivity gh^{-1} L^{-1}
100	60	24	2.5
300	59	72	2.5
600	62	143	2.6

Reaction products profiles are shown in Figure 6 (a, b and c). As reactions advanced, sucrose concentration fell considerably and was converted in FOS (kestose, nystose, fructosylnystose) and glucose. In the experiments with an initial sucrose concentration of 300 g L^{-1} and 600 g L^{-1}, residual fructose was not observed during the first 143 h. This behavior was also observed by Chiang et al. (1997). Vega and Zuniga-Hansen (2014) suggested no fructose production was related to high sucrose and kestose concentrations and low nystose concentration. Duan, Chen, and Sheu (1994) and Jung et al. (1989) showed similar FOS production profiles to those presented in this work.

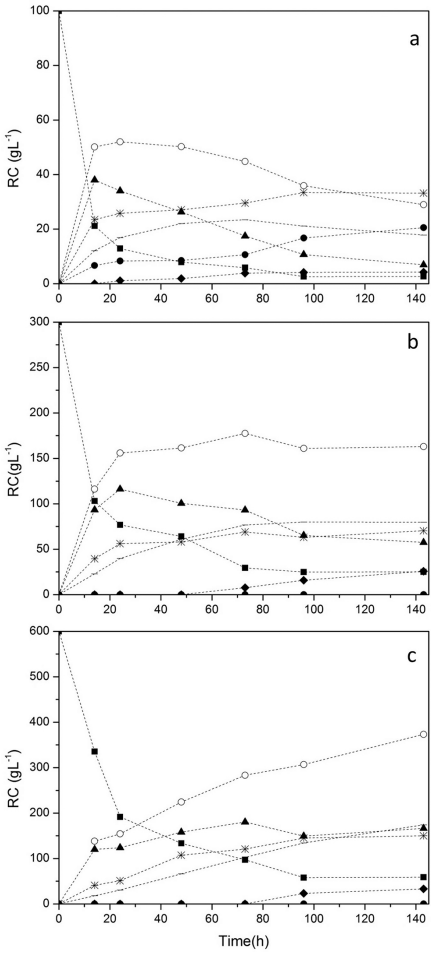

Figure 6: Influence of initial sucrose concentration ([S_0]) on FOS production using FTIOPG. a) [S_0] =100 g L^{-1}, b) [S_0] =300 g L^{-1}, c) [S_0] =600 g L^{-1}. (- -) fructosylnystose, (- - -) nystose, (-▲-) kestose, (-■-) sucrose, (- ▫ -) glucose, (-●-) fructose, (-O-) FOS (nystose + kestose + fructosylnystose). RC: reaction compounds.

3.6.2 Continuous production

Continuous FOS production was performed in a packed bed reactor (reactor volume 15.7 mL) at a flow rate of 0.13 mL min^{-1} equivalent to an ascent rate of 0.165 cm min^{-1}. Bioreactor operated continuously for 720 hours. The retention time was approximately 60 minutes with an average conversion of 35 %, and FOS concentration of 85 g L^{-1}.

Figure 7 shows the average composition during the production process. The stability of the biocatalyst under operational conditions remained satisfactory for a long time. The output of the column composition was: 31.51 ± 4.79 g L^{-1} glucose, 129.82 ± 19.39 g L^{-1} sucrose, 75.53 ± 7.69 g L^{-1} kestose and 9.26 ± 2.74 g L^{-1} nystose, fructose was no detected nor other oligosaccharides of higher degree of polymerization. Average system productivity of approximately 1011 g of FOS day^{-1} $L_{reactor}{}^{-1}$ was achieved. The scattered data could be associated to the variability of the process (pump pulse, temperature, flow, etc.). Also, sucrose was prepared periodically in order to avoid microbiology contamination.

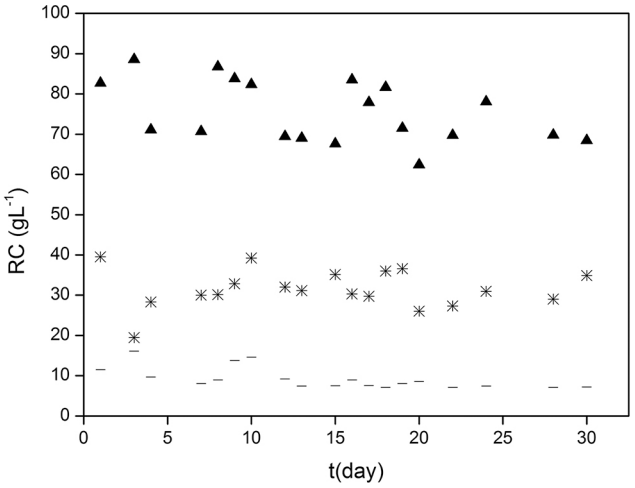

Figure 7: Continuous FOS production performed in a fixed bed reactor. [S_0] =250 g L^{-1}, (- - -) nystose, (-▲-) kestose, (-*-) glucose. RC: reaction compounds.

These results highlight the stability of the FTIOPG system to produce FOS in continuous regime. The effect of retention time, controlled by the feed flow as a critical parameter for the continuous reactors, was evaluated by Fernandez-Arrojo et al. (2013). Increasing of retention time may improve the conversion of sucrose to FOS and this could be done by decreasing the feed rate or increasing the height of the fixed-bed column whereby FOS production could be improved in the bioreactor design proposed in this work. Zambelli et al. (2016) developed a fixed bed system filled with a biocatalyst based on immobilized microorganisms with an important stability. This fixed-bed reactor was proposed in order to probe that FTIOPG, as a biocatalyst, achieved a satisfactory stability under operational conditions for a long time as first preliminary evidence. Further studies are being conducted on this matter.

4 Conclusion

TiO_2, a widely-available, low-cost material, proved to be a viable inorganic matrix for the immobilization of FFase from *Aureobasidium* sp. ATCC 20524, both by using it directly for enzyme adsorption or by functionalizing it and immobilizing the enzyme by covalent bonding. Enzyme immobilization on TiO_2 functionalized with PEI provided a biocatalyst with high activity per gram of support, with more than 95 % operational stability in optimal conditions. Gellan gum encapsulation was successful tool for size upload. In addition, a fixed-bed reactor was probed with FTIOPG as a biocatalyst achieving a satisfactory stability under operational conditions for a long time.

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Highlights

- **TiO_2 can successfully be used as inorganic matrix for FFase immobilization by adsorption**.
- **FFase can be immobilized onto TiO_2 functionalized with PEI and encapsulated in gellan gum**.
- **Operational stability of immobilized biocatalysts was remarkable, with continuous FOS production during 720 h**.

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