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High performance biocatalyst based on β-D-galactosidase immobilized on mesoporous silica/titania/chitosan material

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ABSTRACT

A new support for the immobilization of β-D-galactosidase from *Kluyveromyces lactis* was developed, consisting of mesoporous silica/titania with a chitosan coating. This support presents a high available surface area and adequate pore size for optimizing the immobilization efficiency of the enzyme and, furthermore, maintaining its activity. The obtained supported biocatalyst was applied in enzyme hydrolytic activity tests with *o*-NPG, showing high activity 1223 Ug^{-1}, excellent efficiency (74%), and activity recovery (54%). Tests of lactose hydrolysis in a continuous flow reactor showed that during 14 days operation, the biocatalyst maintained full enzymatic activity. In a batch system, after 15 cycles, it retained approximately 90% of its initial catalytic activity and attained full conversion of the lactose 100% (±12%). Additionally, with the use of the mesoporous silica/titania support, the biocatalyst presented no deformation and fragmentation, in both systems, demonstrating high operational stability and appropriate properties for applications in food manufacturing.

1. Introduction

The use of enzyme has become a powerful strategy to replace inorganic catalysts in the search for alternative methodologies in industrial processes. The advantages of using biocatalysts range from their high activity, specificity, and selectivity, to their general operation in milder temperature, pressure, and pH conditions, being considered eco-friendly alternatives for high value chemical synthesis (Chapman, Ismail, & Dinu, 2018; Madhavan, Sindhu, Binod, Sukumaran, & Pandey, 2017). Enzymes are, in general, applied in free conditions, which hinders their recuperation and reutilization. These drawbacks can be solved by the immobilization of the enzymes on solid supports, so improving the biocatalyst's stability under storage and operational conditions such as thermal and pH. The enzyme immobilization reduces costs, enabling the recovery of the biocatalyst (separation from the product) and its reutilization (Bilal et al., 2018; Sheldon & van Pelt, 2013). Therefore, the immobilization of enzymes has become a requirement for the use of biocatalysts in industrial operations since it improves the reaction control, avoids protein contamination of the product by easy separation, and enables the use of continuous flow biocatalysis reactors (Garcia-Galan, Berenguer-Murcia, Fernandez-Lafuente, & Rodrigues, 2011; Schöffer, Klein, Rodrigues, & Hertz, 2013). A better performance of the biocatalyst can be obtained by optimizing the immobilization process and combining the biochemical properties of the enzyme with the characteristics of the support (Bolivar, Consolati, Mayr, & Nidetzky, 2013).

β-D-galactosidase (E.C.3.2.1.23) is a glycoside hydrolase enzyme that catalyzes the hydrolysis of lactose by breaking glycosidic bonds to yield glucose and galactose. This enzyme holds great promise in food technology for the preparation of low-dosage lactose or lactose-free products, and the production of trans-galactosylation products or galactooligosaccharides (GOS) (Li et al., 2019; Xavier, Ramana, & Sharma, 2018). Several methods for enzyme immobilization have been proposed for β-D-galactosidase (Grosová, Rosenberg, & Rebroš, 2008), including enzyme cross-linking (Xu, Ji, Deng, & Agyei, 2020), characterized by the formation of covalent bonds between protein molecules by the use of bifunctional reagents. However, it has some drawbacks including limitations in mass transfer and loss of catalytic activity. Another method is the adsorption (Wolf & Paulino, 2019), which occurs by physical adsorption of the enzyme on a solid support but has a

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disadvantage in the low stability of the immobilized enzymes which leads to their leaching from the support. Finally, there is the covalent binding (Banjanac et al., 2016), which is formed by chemical bonds between amino acid side-chains of the enzymes to the functionalized surface of organic and inorganic supports. One of the main benefits achieved with covalent immobilization of enzymes is the potential to improve the catalyst's useful life due to the greater stability of the bond, and consequent decrease in the leaching of the enzyme from the support, leading to an increase in the operational stability of the immobilized enzyme (Grosová, Rosenberg, & Rebroš, 2008). This method was chosen to be applied in the present work.

Chitosan is a natural polysaccharide obtained by the partial deacetylation of chitin. Chitosan-based materials are applied as enzyme immobilization supports due to their polycationic, nontoxic, biocompatible, and biodegradable properties, and because they present reactive amino and hydroxyl groups that are available for chemical modifications (Krajewska, 2004). The use of chitosan as a support for enzyme immobilization has increased due to the possibility of obtaining it in the form of microspheres, membranes, coatings, and gels (Bilal & Iqbal, 2019). To enable the covalent attachment of the enzyme to the support, it is necessary to activate the matrix with a bifunctional reagent, such as glutaraldehyde, whose terminal functional groups (–CHO) simultaneously react with the binding sites of chitosan ($–NH_2$) and the terminal amino group of the enzyme. Urrutia, Bernal, Wilson and Illanes (2018) studied the immobilization of β-D-galactosidase in chitosan particles functionalized with glutaraldehyde and epichlorohydrin, and tested it in galacto-oligosaccharides synthesis. Other authors (de Albuquerque et al., 2018; Klein et al., 2012) reported the immobilization of β-D-galactosidase in chitosan spheres, showing great enzyme activity and high stability. However, supports based on chitosan suffer from low mechanical stability, which causes their deformation and disintegration when applied in reactors and continuous processes. Therefore, mechanical properties of chitosan supports have to be improved to hamper their deformation and enable their application in the food industry.

Inorganic oxides, such as silica, have interesting properties for the immobilization of enzymes due to their high thermal and mechanical resistance, posing no risk to health. Important textural properties, such as pore volume and pore size, can be designed by the production process and the surface of the oxides can be modified by the insertion of functional groups, which enable the immobilization of enzymes by several techniques such as adsorption and covalent binding. More recently, systems containing magnetic particles have been developed, so opening new perspectives (Rimola, Costa, Sodupe, Lambert, & Ugliengo, 2013; Xie & Zang, 2017; Zucca & Sanjust, 2014). Therefore, inorganic matrices with a planned texture, and coated with organic functional components, seem to be an excellent choice as supports for the immobilization of enzymes in enzymatic catalysis. This support will allow the combination of the properties of the inorganic and organic components, such as the textural, thermal, and mechanical stability from the inorganic component (oxide), and the biocompatibility and specific functional group reactivity from the organic fragment (chitosan) (El Kadib & Bousmina, 2012). A previous study (Ricardi et al., 2018) addressed the preparation of a silica chitosan composite as support for β-D-galactosidase covalent immobilization. However, even though the proposed system proved to be efficient in the hydrolysis of lactose, in this support the chitosan became distributed in micrometric domains where the immobilization of the enzyme in fact occurred. The direct immobilization of the enzyme on the silica moiety was not possible because it contained only hydroxyl groups, which are not adequate for the β-D-galactosidase immobilization. Therefore, the dispersion of chitosan at a molecular level, and to a greater extent in a porous support, is expected to increase the maintenance of the structural and functional properties of the enzyme.

Mixed silica/titania can be a more effective choice for the immobilization of biomolecules, due to the interaction of Lewis acid sites of the titania moiety with the basic nitrogen atom of the NH_2 group in biomolecules, allowing the adsorption of species (both chitosan and enzymes), without the need of functionalization (El Kadib, Molvinger, Guimon, Quignard, & Brunel, 2008). Magnetic silica/titania and silica/titania graphite composites modified with chitosan have been reported as supports for the direct immobilization of tyrosinase (Laranjo et al., 2019; Morawski et al., 2018). These materials were applied to the preparation of electrochemical biosensors. However, except for biosensor applications, to our knowledge there are no reports of mixed silica/titania modified with chitosan supports being applied to enzymatic catalysis.

In this work, silica/titania materials with adequate pore size for enzyme immobilization and improved activity were developed. Considering the affinity of the enzyme for the chitosan moiety, the porous silica/titania materials were modified with this biopolymer, using a method that seeks a thorough surface covering so enlarging the available area for enzyme immobilization. This composite material was used as support for β-D-galactosidase immobilization for the first time. For the sake of comparison, a porous silica/titania material without using cross linking agents was used as an enzyme support as well. Both prepared supported biocatalysts were applied in enzyme hydrolytic activity tests with *o*-NPG, in consecutive batch cycles, and in the lactose hydrolysis reactions in a continuous fixed bed reactor, and their immobilization parameters, temperature, pH, and operational stability were investigated afterwards.

2. Materials and methods

2.1. Materials

Tetraethyl orthosilicate (TEOS, 98%, Sigma-Aldrich, Germany), titanium(IV) isopropoxide (TIPOT, 97%, Sigma-Aldrich, USA), hydrochloric acid (HCl, 37%, Emsure Merck, Germany), hydrofluoric acid (HF, 40%, Sigma-Aldrich, USA), ethanol (99.9%, Emsure Merck, USA), chitosan low molecular weight (75–85% deacetylated, Sigma-Aldrich, USA), glutaraldehyde (25%, Sigma-Aldrich, USA) and sodium hydroxide (99.5%, Dinâmica, Brazil) were used in the synthesis of the support. *Kluyveromyces lactis* β-D-galactosidase (Maxilact LX-5000, Globalfood, Brazil) is the tested enzyme. *o*-nitrophenyl β-D-galactopyranoside (*o*-NPG, Sigma-Aldrich, Germany), potassium phosphate monobasic anhydrous (99.5%, Nuclear, Brazil), potassium phosphate dibasic anhydrous (99.5%, Nuclear, Brazil), sodium carbonate (99.5%, Neon, Brazil), sodium bicarbonate (99%, Neon, Brazil), sodium chloride (99.5%, Nuclear, Brazil), magnesium chloride (99.5%, Synth, Brazil), ethylene glycol (99.5%, Dinâmica, Brazil), D-(+)-lactose (Dinâmica, Brazil) and the D-glucose determination kit (In Vitro Diagnóstica) were used as a substrate for the enzyme performance evaluation. All reagents were of analytical grade without further purification and all solutions were prepared with distilled water.

2.2. Preparation of the silica/titania supports and immobilization of β-D-galactosidase

The supported biocatalysts were prepared according to the steps shown in Fig. S1, Supplementary Material. The first step was the preparation of the silica/titania material using sol–gel synthesis, with the purpose of obtaining 70% and 30% mol/mol of silica and titania similar to the material proposed by other authors (Laranjo et al., 2019). Firstly, the hydrolysis of the precursor was performed by adding TEOS (5 mL) to the solvent (ethanol, 7.0 mL). Then water (0.6 mL) and HCl 37% (0.2 mL) were added and the solution was stirred magnetically for 1 h. In sequence, TIPOT (2.0 mL) and a mixture of the catalysts, HF 40% (1.5 mL) and HCl 37% (1.0 mL) were added to this solution. The system was left at ambient conditions for 15 days for gelation and solvent evaporation and the obtained material was comminuted (Tyler standard sieve 80 mesh), washed to neutral pH and then vacuum-dried at 90 °C for 2 h. This porous powder is henceforth called SiTi.

Two supported biocatalysts were produced based on SiTi: i) SiTienz

was prepared by direct immobilization of the β-D-galactosidase (see left hand side of the diagram in Fig. S1); ii) SiTi-CHenz was prepared by a three step procedure (see right hand side of the diagram). Firstly, the support SiTi was covered with chitosan. The silica/titania surface was modified with chitosan by a grafting reaction, based on an already reported method (Laranjo et al., 2019). 150 mg of chitosan were dissolved in water (10 mL) and HF 40% (0.3 mL) under constant stirring until homogenization. NaOH (0.1 mol L^{-1}) was added to this solution until it reached pH 6, and finally 1.0 g of the SiTi was added to this mixture, which remained under magnetic stirring for 24 h. The resulting material was washed with portions of water and ethanol, and vacuum-dried for 2 h. This sample was designated SiTi-CH. In sequence the SiTi-CH was activated with glutaraldehyde. The material was incubated in a solution containing 0.1 mmol of glutaraldehyde per gram of material for three hours under agitation. The activated support was abundantly washed with water to remove the excess glutaraldehyde and then vacuum-dried for 2 h. In this intermediate stage, the support was denominated as SiTi-CHglut.

For the β-D-galactosidase immobilization, 1.0 mL of 0.1 mg mL^{-1} enzyme solution in immobilization buffer (0.1 mol L^{-1} potassium phosphate buffer, pH 7.0, containing 1.5 mmol L^{-1} $MgCl_2$) was added to 10 mg of the supports SiTi and SiTi-CHglut. The suspensions were mixed overnight (18 h) in an orbital shaker at room temperature (25 °C). To remove the unbound proteins, the materials were sequentially washed with an activity buffer, NaCl (1 mol L^{-1}) and ethylene glycol (30% v/v). These complete biocatalysts were denominated SiTienz and SiTi-CHenz, respectively.

2.3. Materials characterization

The micrographs were obtained using a field emission scanning electron microscope (FE-SEM) (FEI Inspect F50) operating at 20 kV, on powder samples dispersed on double-sided conductive tape and coated with a thin gold layer. Elemental analysis was accomplished with an energy dispersive X-ray spectrometer (Thermo-Fisher) coupled to a scanning electron microscope (JEOL LV5800). For this analysis the powder was compacted to pellets (4.6 ton cm^{-2}) and the SiO_2 and TiO_2 content was obtained with quantification procedures that used top-hat filter for background removal and φ(ρZ) matrix correction. The powder XRD patterns were obtained in the 5° to 90° 2θ range, using Cu-Kα radiation in a Siemens diffractometer (BRUKER AXS D-5000) operating at 40 kV/ 25 mA, with a graphite monochromator in the secondary beam. The samples were analyzed at 0.05° s^{-1}. Thermogravimetric analysis (TGA) was carried out using a Shimadzu equipment (TGA-50), under argon flow of 50 mL min^{-1} and a heating rate of 20 °C min^{-1}, from room temperature up to 850 °C. The nitrogen adsorption/desorption isotherms were acquired at liquid N_2 boiling point (77 K), in a Micromeritics instrument (Tristar II 3020 with Krypton option). 100.0 mg of each sample were previously degassed at 60 °C for 8 h. The specific surface area was calculated by the Brunauer, Emmett and Teller method (BET), and the pore-size distribution curves were obtained by using the Barret, Joyner and Halenda method (BJH) (Gregg & Sing, 1982; Webb & Orr, 1997). Fourier transform infrared spectroscopy (FT-IR) spectra were performed between 500 and 4000 cm^{-1}, using 68 scans, with a Perkin Elmer spectrophotometer (Spectrum 1000 FT-IR), using the KBr technique.

2.4. Enzymatic activity assays

Free and immobilized β-D-galactosidase hydrolytic activities were assayed using *o*-NPG (30 mmol L^{-1}) as the substrate at 37 °C in a thermostatic bath. The released *o*-nitrophenol was measured in a spectrophotometer at 415 nm. Per definition one β-D-galactosidase activity unit (U) is the quantity of enzyme required to convert 1 μmol of the substrate per minute under the reaction conditions.

Free enzyme hydrolytic activity was measured by incubating the diluted enzyme solution (50 μL) in the immobilization buffer (270 μL) and *o*-NPG (180 μL) for 2 min. The reaction was halted with the addition of sodium carbonate buffer (1.5 mL, 0.1 mol L^{-1}, pH 10). For immobilized β-D-galactosidase, 10 mg of the dried biocatalyst was incubated with an immobilization buffer (1.62 mL) and *o*-NPG (1.08 mL) for 30 s under continuous stirring. The reaction was halted with the addition of sodium carbonate buffer (9 mL, 0.1 mol L^{-1}, pH 10). All experiments were conducted in triplicate.

The immobilization parameters were determined according to the following equations (Sheldon & van Pelt, 2013):

$$Immobilization\ yield\ (\%) = \frac{Immobilized\ activity}{Starting\ activity} \times 100$$

$$Efficiency\ (\%) = \frac{Observed\ activity}{Immobilized\ activity} \times 100$$

$$Activity\ recovery\ (\%) = \frac{Observed\ activity}{Starting\ activity} \times 100$$

2.5. Study of the effect of pH on enzyme activity and study of thermal and storage stability of the biocatalysts

The optimal pH of free and immobilized β-D-galactosidase was acquired by measuring enzyme activity in different pH values of the phosphate buffer using pH values of 5.7, 6.0, 6.5, 7.0, 7.5 and 8.0 at 37 °C. Tests were done in triplicate.

The thermal stability of free and immobilized β-D-galactosidase was assessed by incubating the biocatalyst in a thermostatic bath (40, 50, 60 and 70 °C) in tubes with a phosphate buffer (pH 6.5). After preset exposure times, the remaining enzyme activities were measured as described above in 2.4. Assays were made in triplicate.

In order to investigate the storage stability of the biocatalysts, they were stored in a phosphate buffer (pH 6.5) in the fridge at 4 °C for 30 days. Following the storage period, the biocatalysts were employed in the hydrolysis of *o*-NPG to determine remaining enzyme activities, as described in 2.4. Tests were done in duplicate every seven days.

2.6. Determination of kinetic parameters

The kinetic parameters, Michaelis-Menten constant (K_M) and the maximum rate of reaction (V_{max}) of free and immobilized enzymes were determined by a Lineweaver–Burk plot under optimal conditions, using increasing concentrations of *o*-NPG in solution, from 5 mmol L^{-1} to 50 mmol L^{-1} in the phosphate buffer (pH 6.5). The K_M and V_{max} values were obtained from a $\frac{1}{V} x \frac{1}{[S]}$ graph where V is the rate of reaction and [S] is the concentration of *o*-NPG. Using the linear regression of the Lineweaver-Burk plot $\frac{1}{V} = \frac{1}{V_{max}} + \frac{K_m}{V_{max}} \frac{1}{[S]}$, which presents a slope of $\frac{K_m}{V_{max}}$, intersecting the axis at $\frac{1}{V_{max}}$.

2.7. Operational stability

The operational stabilities of the biocatalysts were evaluated by lactose hydrolysis in batch cycles. The experiment was carried out by incubating 10 mg of each biocatalyst (SiTienz or SiTi-CHenz) in 1.0 mL aliquot of lactose solution (50 g L^{-1}) in phosphate buffer (pH 6.5). Each cycle was carried out at 37 °C for 60 min under stirring. At the end of each cycle, the biocatalysts were filtered, washed with the activity buffer and added to a fresh lactose solution. Samples of the hydrolyzed product were collected and analyzed enzymatically for glucose formation by the colorimetric method, without deproteinization (GOD-PAP), by enzymatic oxidation in the presence of glucose oxidase. Each collected aliquot of the hydrolyzed product was diluted 20 times in NaCl solution (0.01 mol L^{-1}). The test was performed by mixing 10.0 μL of the diluted product with 1.0 mL of the reagent, for 15 min at 37 °C. Afterwards, the absorbance was measured at 505 nm. This experiment was performed in

duplicate.

The operational stability was also evaluated with the use of a fixed bed reactor. This type of reactor consists of a glass cylindrical tube (diameter = 1 cm; height = 5 cm) filled with an immobile stack of biocatalyst particles (1 g of SiTi-CHenz), through which a substrate solution is pumped and converted into products, under temperature control (37 °C). The substrate was a 50 g L^{-1} lactose solution diluted in phosphate buffer (pH 6.5). Flow rate was maintained at 0.3 mL min^{-1} using a peristaltic pump Watson Marlow 120U. Samples of the hydrolyzed product were collected in triplicate after preset times and analyzed enzymatically for glucose formation as described above.

2.8. Statistical analysis

Experiments were carried out in triplicate, using three independent samples. Statistical analysis was conducted using Past statistical software (freeware from Oyvind Hammer v. 4.03). The results were expressed as "mean ± standard deviation" (as "X ± SD") of three determinations. The analysis of variance (ANOVA) and distinction between the mean values of the different treatments was evaluated using Tukey's test ($\rho \leq 0.05$).

3. Results and discussion

3.1. Structural and textural characterization

The microanalysis results obtained on several areas of the pressed powder pellets found 75.9 wt% SiO_2 (±1.4 wt%) and 24.1 wt% TiO_2 (±1.4 wt%), compatible with the planned three to one ratio of the oxides. A SEM image of one of the analysis regions and a representative X-ray spectrum showing the characteristic peaks of Ti, Si, and O are shown in Fig. S2, Supplementary Material. The organic content of the samples was determined by TGA, which is more appropriate for the determination of the organic fraction in hybrid materials, and the results are presented in Fig. S3, Supplementary Material. The weight loss from room temperature to 150 °C was attributed to water desorption. In the range from 150 °C to 650 °C, the weight loss can be related to the dehydroxylation reactions of silica/titania and organic moiety decomposition. Based on the weight loss values, the organic content incorporated after successive modifications was estimated and it is shown in Table 1. Comparing the relevant temperature range between 150 °C and 650 °C in the TGA curves, it can be seen that the weight loss increased after the incorporation of chitosan: for SiTi the loss was of 2.22% and for SiTi-CH of 7.34%, allowing the conclusion that the 5% difference was due to the loss of incorporated chitosan. After further modifications of SiTi-CH, an additional weight loss of 0.87% showed the loss of incorporated glutaraldehyde and the weight loss of 2.32% indicated the loss of the successfully incorporated enzyme (Table 1).

Fig. 1a and 1b show the high-resolution SEM images of the SiTi and SiTi-CH supports. Fig. 1a shows a representative image of SiTi obtained by the sol–gel method, where the xerogel is formed by agglomerates of nanometric primary particles. Compared to SiTi in Fig. 1a, no significant morphological differences were observed in SiTi-CH, after chitosan coating (Fig. 1b).

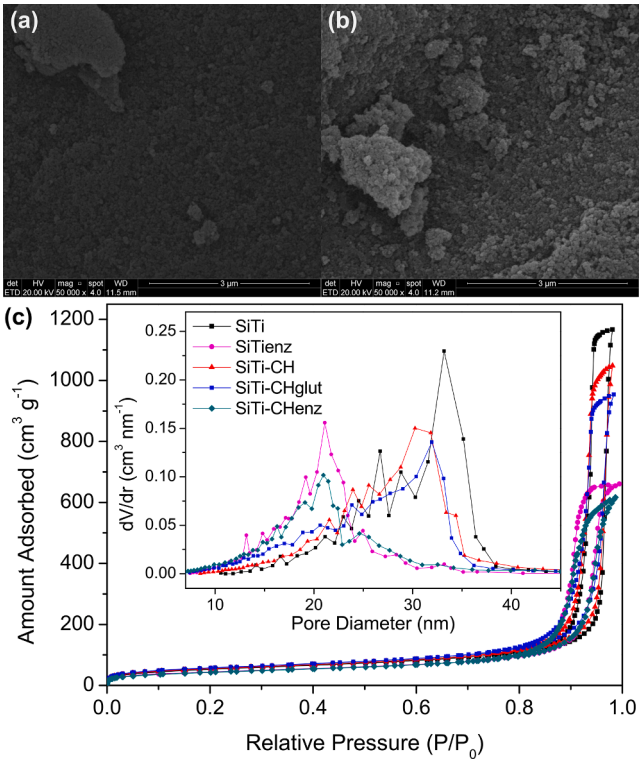

Fig. 1. (a) SEM image of the support SiTi, (b) SEM image of the support SiTi-CH and (c) N_2 adsorption–desorption isotherms and pore-size distribution curves (Inset Fig. 1(c)): SiTi, SiTienz, SiTi-CH, SiTi-CHglut and SiTi-CHenz samples.

Fig. 1c shows the N_2 adsorption/desorption isotherms of SiTi, SiTienz, SiTi-CH, SiTi-CHglut and SiTi-CHenz. All are type IV isotherms (Gregg & Sing, 1982), characteristic of mesoporous materials. After surface modifications and enzyme immobilization the isotherms showed a decrease of the amount of adsorbed nitrogen. Pore size distribution curves are presented as an inset of Fig. 1c, showing a maximum pore size of approximately 35 nm for the support. After the addition of chitosan and glutaraldehyde, a reduction of maximum pore size to 30 nm was observed, evidencing that the organics covered the inner pore surface. The pore size presented by the support, with and without modifications, is compatible with the enzyme tetramer size, estimated as 15.1 nm × 17.1 nm × 10.7 nm (Pavel et al., 2017), thereby allowing the immobilization, after which the pore size maximum shifts to approximately 20 nm. In both biocatalysts, SiTi-CHenz and SiTienz, the direct enzyme immobilization occurred in these same pore regions and resulted in a similar curve, showing that both systems promote a very good dispersion of the enzyme on the surface of the supports.

Table 2 shows details of the textural properties of the materials. The modification of SiTi support with chitosan, glutaraldehyde, and enzyme immobilization, caused a decrease in the amount of N_2 adsorbed and, as a result, there was a decrease of pore volume from 1.79 cm^3 g^{-1} for SiTi,

Table 1
Weight losses obtained by TGA in different ranges of temperature and estimated organic content incorporated in each step.

Samples	0 – 150 °C (%)	150 – 650 °C (%)	Organic content (%)	
			Total	For each step
SiTi	1.24 (±0.01)	2.22 (±0.02)	–	–
SiTi-CH	2.51 (±0.02)	7.34 (±0.05)	5.12 (±0.05)	5.12 (±0.05)
SiTi-CHglut	1.63 (±0.01)	8.21 (±0.05)	5.99 (±0.05)	0.87 (±0.01)
SiTi-CHenz	3.54 (±0.03)	10.53 (±0.05)	8.31 (±0.05)	2.32 (±0.05)

Table 2
BET surface area and pore volume results.

Sample	S_{BET} (m^2 g^{-1})	Pore Volume (cm^3 g^{-1})
SiTi	190 (±8)	1.79 (±0.05)
SiTienz	155 (±6)	1.02 (±0.04)
SiTi-CH	195 (±8)	1.61 (±0.05)
SiTi-CHglut	204 (±8)	1.46 (±0.04)
SiTi-CHenz	153 (±6)	0.94 (±0.04)

to 0.94 $cm^3\ g^{-1}$ for SiTi-CHenz, and to 1.02 $cm^3\ g^{-1}$ for SiTienz. The specific surface area remained constant (within experimental error) after modifications with chitosan and glutaraldehyde (around 190 $m^2\ g^{-1}$) and decreased after the enzyme immobilization to 155 $m^2\ g^{-1}$ for SiTienz and to 153 $m^2\ g^{-1}$ for SiTi-CHenz. This allows us to infer that chitosan and glutaraldehyde cover the surface of the pores as a nanometric coating, leaving the pore volume almost unchanged. This is very important for the immobilization of the enzyme inside the pores, because it promotes the availability of active sites and consequent enzyme stability. The marked textural changes occurred only after the addition of the enzyme, indicating that the enzymes were in fact immobilized inside the mesopores. Additionally, the pore size of 20 nm and pore volumes of 1.02 $cm^3\ g^{-1}$ (SiTienz) and 0.94 $cm^3\ g^{-1}$ (SiTi-CHenz), which are maintained after enzyme immobilization, are very suitable for substrate access and enhanced enzyme activity (Sigurdardóttir et al., 2018).

Fig. 2a shows the XRD patterns of the SiTi and the SiTi-CH supports. In the XRD pattern of SiTi, a typical halo of amorphous material is observed near 20°, and an incipient crystallization of titania in anatase structure can be seen. These features were not modified by the chitosan coating, as expected, considering that the coating is very thin and all surface modifications were performed at ambient temperature.

Fig. 2b shows the infrared spectra of SiTi, SiTi-CH, SiTi-CHglut and SiTi-CHenz samples. The wide band at 3300 cm^{-1} was ascribed to the O-H stretching mode of silanol groups (Si-OH). The band at 2950 cm^{-1} appeared after the incorporation of chitosan in the material and was attributed to the C-H stretch. The bands corresponding to silica tetrahedral harmonic vibration are located at 1995 and 1880 cm^{-1} (Costa, Gallas, Benvenutti, & Jornada, 1997). The band at 1633 cm^{-1} was assigned to the angular deformation of adsorbed water. In the spectrum of the SiTi-CHenz sample, this band appears larger indicating the presence of C═O stretching of the enzyme. The band at 1550 cm^{-1} is due to the symmetric angular deformation of $–NH_2$ of the chitosan, confirming the incorporation of chitosan in the material. This band suffered a slight decrease in intensity after the incorporation of glutaraldehyde due to the interaction of the amino with the aldehyde groups. The band with a maximum at 1100 cm^{-1} was assigned to the combination of silica network vibrations (Si-O-Si). The band at 930 cm^{-1} in the SiTi sample was attributed to the Si-O stretch of silicon not bridged to another silicon, i.e., Si-OH and Si-O stretching vibrations of Si-O-Ti bonds so demonstrating the connection of the silica and titania phases. The shoulder at 970 cm^{-1} is due to the Ti-O stretching of TiOH. The relative change of these bands, after the incorporation of glutaraldehyde and enzyme immobilization, can indicate an interaction of the Ti-OH groups with the aldehyde groups or CO groups of the enzyme, and H interaction of Si-OH groups with NH_2 enzyme. The 800 cm^{-1} band corresponds to vibrations of the silica tetrahedra. Other bands that could prove the bond Ti-O-C and the presence of glycosidic rings are in the region between 1000 and 1150 cm^{-1} and are possibly masked by the silica bands, considering that more than 70% of the sample consists of silica (Anaya-Esparza et al., 2020).

3.2. Immobilization parameters

The prepared materials were used as supports for β-D-galactosidase immobilization and the results of enzyme activity, immobilization yield, efficiency, and activity recovery, are shown on Table 3. The SiTi-CHenz biocatalyst showed higher (73 ± 1)% values of immobilization yield if compared to the SiTienz biocatalyst (40 ± 1)% ($\rho < 0.05$), thus indicating that a large part of the enzyme offered to the SiTi matrix did not remain attached to the support. Even so, the immobilization parameters presented by this biocatalyst were quite appreciable since the enzyme can link directly to the support via the acidic sites in titania without the need of support surface functionalization. The amount of protein immobilized on the support, determined by the Lowry method, was (9 ± 1) μg mg^{-1}, for SiTi-CHenz and (5 ± 1) μg mg^{-1}, for SiTienz. The biocatalyst containing chitosan (SiTi-CHenz) showed the highest observed activity per gram of support (1223 ± 45) U g^{-1}, efficiency (74 ± 1)%, and activity recovery (54 ± 2)% ($\rho < 0.05$). The obtained process parameters indicated that the chitosan coating increased the enzyme molecules retention capacity (immobilization yield) and assisted in the maintenance of the enzymatic activity of the immobilized protein (immobilization efficiency), offering a favorable microenvironment to maintain the enzyme catalytic capacity and, consequently, increasing the recovered activity and contributing to a greater observed activity per gram of support.

Improvements in the stability of enzymes, when attached to chitosan supports, have been observed in previous works (de Albuquerque et al., 2018; Duarte et al., 2017; Flores et al., 2019; Klein et al., 2012; Urrutia et al., 2018). In general, the authors agree that the protection provided by the 3D structure formed between the enzyme and chitosan is responsible for the increase in stability of the immobilized enzyme in comparison with the free enzyme. At the pH conditions used in this work, it is generally assumed that the enzyme binds to the support at a single point, which is the amino terminal of the protein (Barbosa et al., 2014). However, in addition to weak multipoint interactions that could be established between chitosan and the enzyme, some authors speculate that there might be multipoint covalent bonds, even at low pH values (Barbosa et al., 2014; Duarte et al., 2017; Flores et al., 2019; Schöffer et al., 2017). These suggestions are based on the evidence that the pKa of amino acids are strongly influenced by the microenvironment. Therefore, the pKa of an amino acid such as Lysine can vary from

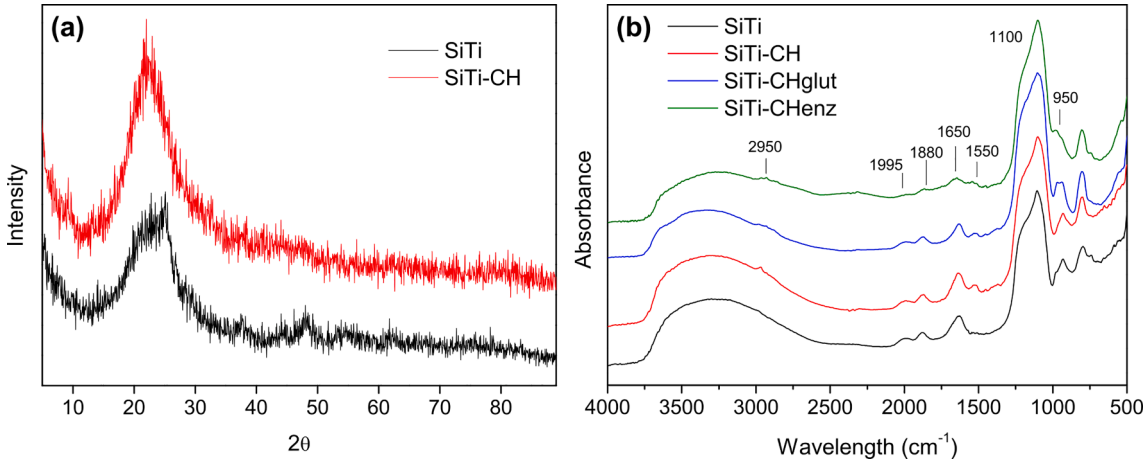

Fig. 2. (a) XRD of the supports SiTi and SiTi-CH. (b) Infrared spectra obtained at room temperature for SiTi, SiTi-CH, SiTi-CHglut and SiTi-CHenz.

Table 3
Immobilization process parameters of SiTienz and SiTi-CHenz samples.

Sample	Protein concentration (μg mg^{-1} of support)	Observed Activity (U g^{-1})	Immobilization Yield (%)	Efficiency (%)	Activity Recovery (%)
SiTienz	5 (± 1)	711 (± 24)[a]	40 (± 1)[a]	53 (± 1)[a]	21 (± 2)[a]
SiTi-CHenz	9 (± 1)	1223 (± 45)[b]	73 (± 1)[b]	74 (± 1)[b]	54 (± 2)[b]

Any two means in the same column followed by the same letter are not significantly ($\rho \leq 0.05$) different according to Tukey test.

5.3 to 10.4 according to the region of the residue (Isom, Castañed, Cannon, & García-Moreno, 2011). Thus, even if a small percentage of deprotonated amines (protein surface lysins) is present at a lower pH, this would be sufficient to allow the reaction with glutaraldehyde previously bound to chitosan, forming multipoint connections (Migneault, Dartiguenave, Bertrand, & Waldron, 2004), increasing the rigidity of the protein structure and strengthening the connection between the enzyme molecule and the support. From a practical point of view, this is important because it allows the use of biocatalysts in batch repetitions or in continuous flow reactors without the leaching of enzyme molecules during the operation.

3.3. Study of the effect of pH on enzyme activity and study of thermal and storage stability of the biocatalysts

The influence of pH on relative activities of both free and immobilized enzymes was analyzed in the range of 5.7 and 8.0 (Fig. S4, Supplementary Material). The biocatalysts showed the maximum value of relative activity at pH 6.5. With respect to pH values above 6.5, the SiTi-CHenz biocatalyst showed slightly higher activity than those of the free enzyme and SiTienz, keeping the value of (45 ± 1)% at pH 8.0, while the free form and the SiTienz were near (30 ± 3)%.

The thermal stabilities of free and immobilized enzymes are presented in Fig. S5, Supplementary Material. In experiments performed at 40 °C (Fig. S5a, Supplementary Material), SiTienz and SiTi-CHenz biocatalysts showed lower decay of relative activity over time when compared with the free enzyme. After 24 h of 40 °C incubation, SiTienz retained (40 ± 4)% of the initial activity, while SiTi-CHenz maintained (30 ± 2)% and the free enzyme (20 ± 5)%. After 24 h of incubations at 50 °C, 60 °C, and 70 °C (Figs. 5b, 5c and 5d, Supplementary Material) both free and immobilized enzymes tended to a total loss of relative activity.

Although the relative activity of the immobilized enzyme does not show marked improvements in more acidic and alkaline pH, nor does it present a better thermal stability; the immobilization process maintains the basic properties of the enzyme, with similar behavior as the free enzyme. This consistency of properties in relation to optimum pH and thermal stability of the immobilized *β*-D-galactosidase was found by Ricardi et al. (2018), when the enzyme was immobilized in a silica/chitosan composite.

The Fig. S6, Supplementary Material, shows the enzymatic activity of the biocatalysts, measured every seven days, aiming to evaluate the storage stability of free and immobilized enzymes. The enzymatic activity was maintained near 90% for five weeks under storage.

3.4. Determination of kinetic parameters

The kinetic parameters, Michaelis-Menten constant (K_M) and the maximum rate of reaction (V_{max}) of free and immobilized enzyme were obtained varying the substrate concentration of *o*-NPG in solution. Fig. S7, Supplementary Material, shows the Lineweaver-Burk plot and linear regression line of free and immobilized enzyme. The linear regression equation is $\frac{1}{V} = 0.0004\frac{1}{[S]} + 0.0535$ for free enzyme, and $\frac{1}{V} = 9.10^{-6}\frac{1}{[S]} + 0.0011$ for SiTi-CHenz. The values obtained for K_M were 7.48 μmol mL^{-1} for free enzyme and 8.18 μmol mL^{-1} for SiTi-CHenz. For V_{max}, the values obtained were 149.52 μmol min^{-1} mg^{-1} of protein for free enzyme and 99.63 μmol min^{-1} mg^{-1} of protein for SiTi-CHenz. The V_{max} value slightly decreased when the enzyme was immobilized and the K_M value showed a small increase. This result is important because the SiTiCHenz biocatalyst presented an enzyme/substrate affinity very similar to the free enzyme.

3.5. Operational stability

The biocatalysts were evaluated for their operational stability using batch and fixed bed reactors, as previously described. Results are shown in Fig. 3.

The operational stability of immobilized enzymes (SiTienz and SiTi-CHenz) was evaluated under repeated batch reactions for the hydrolysis of lactose. After the first cycle, shown in Fig. 3a, the SiTienz biocatalyst achieved (85 ± 5)% conversion of lactose hydrolysis, while SiTi-CHenz reached (100 ± 12)%. The highest relative activity loss after the first hydrolysis cycle observed for SiTienz can be attributed to the existence of covalent as well as weak enzyme-silica/titania surface interactions, which lead to partial enzyme leaching. This leaching suggests the existence of non-covalent type interactions, such as weak physical enzyme adsorption, that can be favored by the enzyme entrapment into the pores (Ghannadi, Abdizadeh, Miroliaei, & Saboury, 2019; Prazeres et al., 2019). These conversion values were considered as 100% of the relative activity. As seen in Fig. 3a, the relative activity of SiTienz decreased to (70 ± 5)% after the second cycle, retaining only (23 ± 6)% of the relative activity after 15 cycles, showing lower operational stability. In contrast, the relative hydrolytic activity of the biocatalyst containing chitosan (SiTi-CHenz) remained close to (100 ± 10)% after 15 cycles of lactose hydrolysis. In the SiTi-CHenz biocatalyst, the enzyme remained attached to the support without changing its active sites throughout the 15 hydrolysis cycles, and without a considerable loss of relative activity.

The operational stability of the biocatalyst SiTi-CHenz was also evaluated in a fixed bed reactor for the lactose hydrolysis reaction. In Fig. 3b, it can be seen that the biocatalyst SiTi-CHenz maintained its hydrolytic activity close to (100 ± 10)% after 14 days of continuous operation of the reactor. The hydrolytic conversion rate of lactose, forming glucose and galactose, was (85 ± 4)%. This conversion percentage can probably be increased by optimizing the reactor conditions, such as increasing the reactor column volume, increasing the concentration of immobilized enzyme, and enlarging the substrate residence time of the lactose in the reactor. However, our reactor experiment demonstrated the feasibility of using our biocatalyst in continuous processes.

4. Conclusion

Silica/titania-based mesoporous powders, modified with chitosan and glutaraldehyde, were prepared and applied for the first time to the immobilization of *β*-D-galactosidase from *Kluyveromyces lactis*. The organic components chitosan and glutaraldehyde were gradually incorporated on the surface, inside of the pores of the SiTi matrix, in a nanometric dispersion level. The enzyme was immobilized in these coated pores, and the obtained biocatalyst showed a very suitable texture for enzymatic catalysis, presenting a pore size distribution maximum around 20 nm and a surface area of 153 m^2 g^{-1}. These characteristics allowed its high performance as a biocatalyst, resulting in high activity (1223 U g^{-1}) and excellent operational stability. Particularly the SiTi-CHenz biocatalyst showed excellent stability without significant loss of enzymatic activity after 15 batch cycles of lactose

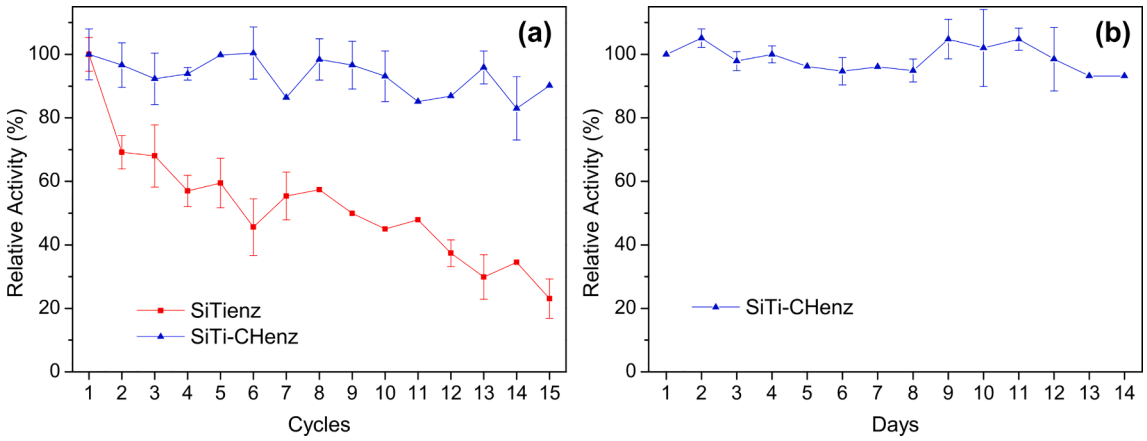

Fig. 3. Operational stability of the immobilized enzyme, (a) in a batch reactor and (b) in a fixed bed reactor, at 37 °C and pH 6.5.

hydrolysis and after 14-day operation in a continuous flow reactor.

In conclusion, the incorporation of chitosan to a large extent in a porous mixed oxide support was determinant in improving the physicochemical properties of the biocatalyst. The design of composition and pore texture of the inorganic matrix, as well as the synthesis of the final composite support, were fundamental steps in the development of this biocatalyst, promoting catalytic activity and operational stability of the enzyme. The porous silica/titania/chitosan hybrid material proposed in this work has the potential to be applied as support for other enzymes, in the development of other hybrid biocatalysts for the food industry.

CRediT authorship contribution statement

Natália Carminatti Ricardi: Conceptualization, Investigation, Validation, Formal analysis, Writing - original draft, Writing - review & editing, Visualization. **Leliz Ticona Arenas:** Conceptualization, Writing - original draft, Writing - review & editing, Visualization, Resources, Methodology. **Edilson Valmir Benvenutti:** Investigation, Resources, Writing - review & editing, Visualization, Funding acquisition, Methodology. **Ruth Hinrichs:** Investigation, Methodology, Validation, Resources. **Elí Emanuel Esparza Flores:** Investigation, Validation, Formal analysis. **Plinho Francisco Hertz:** Conceptualization, Resources, Writing - review & editing, Visualization, Funding acquisition, Methodology, Project administration. **Tania Maria Haas Costa:** Conceptualization, Writing - original draft, Writing - review & editing, Resources, Project administration, Supervision, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2021.129890.

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