Application of cellulosic materials as supports for single-step purification and immobilization of a recombinant β-galactosidase via cellulose-binding domain

Adriano Gennari [a,b], Renate Simon [a], Nathalia Denise de Moura Sperotto [c], Cristiano Valim Bizarro [c,d], Luiz Augusto Basso [c,d,e], Pablo Machado [c,d,e], Edilson Valmir Benvenutti [f], Gaby Renard [c], Jocelei Maria Chies [g], Giandra Volpato [h], Claucia Fernanda Volken de Souza [a,b,*]

[a] *Laboratório de Biotecnologia de Alimentos, Brazil*
[b] *Universidade do Vale do Taquari - Univates, Lajeado, RS, Brazil*
[c] *Instituto Nacional de Ciência e Tecnologia em Tuberculose, Centro de Pesquisas em Biologia Molecular e Funcional, Brazil*
[d] *Programa de Pós-Graduação em Biologia Celular e Molecular, Brazil*
[e] *Pontifícia Universidade Católica do Rio Grande do Sul, Porto Alegre, RS, Brazil*
[f] *Instituto de Química, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil*
[g] *Quatro G Pesquisa & Desenvolvimento Ltda, Porto Alegre, RS, Brazil*
[h] *Instituto Federal de Educação, Ciência e Tecnologia do Rio Grande do Sul - IFRS, Campus Porto Alegre, Porto Alegre, RS, Brazil*

ARTICLE INFO

ABSTRACT

This study aimed to develop single-step purification and immobilization processes on cellulosic supports of β-galactosidase from *Kluyveromyces* sp. combined with the Cellulose-Binding Domain (CBD) tag. After 15 min of immobilization, with an enzymatic load of 150 U/g$_{support}$, expressed activity values reached 106.88 (micro-crystalline cellulose), 115.03 (alkaline nanocellulose), and 108.47 IU/g (acid nanocellulose). The derivatives produced were less sensitive to the presence of galactose in comparison with the soluble purified enzyme. Among the cations assessed (Na$^+$, K$^+$, Mg^{2+}, and Ca^{2+}), magnesium provided the highest increase in the enzymatic activity of β-galactosidases immobilized on cellulosic supports. Supports and derivatives showed no cytotoxic effect on the investigated cell cultures (HepG2 and Vero). Derivatives showed high operational stability in the hydrolysis of milk lactose and retained from 53 to 64% of their hydrolysis capacity after 40 reuse cycles. This study obtained biocatalyzers with promising characteristics for application in the food industry. Biocatalyzers were obtained through a low-cost one-step sustainable bioprocess of purification and immobilization of a β-galactosidase on cellulose via CBD.

1. Introduction

β-D-galactosidase is a glycosidic hydrolase that catalyzes the conversion of lactose into glucose and galactose. This enzyme is widely used in dairy industries to hydrolyze the lactose of milk and milk-derived products to meet the needs of consumers intolerant to this sugar [1–3]. For large-scale use, microbial β-galactosidases are preferred [4], although this enzyme is also present in other organisms, like plants and animals [5]. Enzyme purification and stabilization are the main limitations of applying β-galactosidase in industrial bioprocesses since they can affect the biocatalyzer performance and the production cost [6].

The heterologous production of this enzyme using recombinant DNA technology is a biotechnological alternative to overcome these limitations. That way, it is possible to manipulate genes to obtain more stable metabolites with specific binding characteristics [7]. Also, protein engineering enables us to include in a specific matrix binders or tags that codify amino acid sequences with high binding affinity [8]. This strategy

---

results in recombinant proteins with high yield and purity [9]. Some binders used for this purpose are His-tag, Calmodulin Binding Proteins, Maltose Binding Proteins, Cellulose Binding Domain (CBD), and Chitin Binding Proteins [10].

The CBD tag has been used as a biomarker due to its specific binding affinity with cellulose and capacity to bind or immobilize biomolecules in matrices formed by this biopolymer [11,12]. The immobilization of enzymes on solid supports like cellulose develops biocatalyzers with higher operational and thermal stability and reusability [13–15]. In addition, pore size and volume (Barrett-Joyner-Halenda – BJH) and the surface area (Brunauer, Emmett & Teller – BET) of the cellulose support structure may favor the oriented binding of high enzyme concentrations [16]. The CBD tag also increases the affinity of the active site of the immobilized enzyme with its substrate [17,18]. By including a CBD sequence in a β-galactosidase codifying gene, enzyme immobilization and purification processes can be done in one step by binding the enzyme to a cellulosic support [19]. This is a sustainable low-cost bioprocess that enables the reuse of β-galactosidase in reactions of lactose hydrolysis [20].

Previous works have reported the cloning and expression of β-galactosidase from *Kluyveromyces* sp. with the CBD tag (β-galactosidases-CBD) [20]. Therefore, this study aimed to immobilize β-galactosidases-CBD by binding them to cellulosic supports through simultaneous enzyme purification and immobilization processes. The immobilization processes were carried out via combination with CBD by using four different supports: microcrystalline cellulose, nanocellulose obtained by alkaline hydrolysis, nanocellulose obtained by acid route, and solvent-treated cellulose. The enzymatic load and the immobilization time were analyzed to determine immobilization conditions. Textural and morphological characteristics of the supports and biocatalyzers obtained were assessed. The soluble purified enzyme and the biocatalyzers obtained were characterized regarding pH and temperature reaction conditions, presence of ions and galactose, kinetic parameters, thermal and storage stability, and cytotoxic effect on cell cultures. In addition, the immobilized enzyme's operational stability was assessed during the hydrolysis of skim milk lactose through a batch process.

2. Materials and methods

2.1. Materials

Immobilization experiments were carried out by using crude enzymatic extract with β-galactosidase-CBD [20]. Ortho-nitrophenyl-β-D-galactopyranoside (ONPG), activated factor X (Xa) from bovine plasma, *N,N*-Dimethylacetamide (DMAc), and microcrystalline cellulose (MCC) were acquired from Sigma-Aldrich® (Missouri, US). The glucose quantification kit was acquired from Labtest® (Minas Gerais, Brazil). Powdered skim milk was purchased from Brazil Foods S.A. (Rio Grande do Sul, Brazil). Analytical-grade reagents were acquired from Sigma-Aldrich® (Missouri, US).

2.2. Methods

2.2.1. Synthesis of cellulosic supports

Supports for immobilizing β-galactosidase-CBD were prepared using three different treatments of microcrystalline cellulose (MCC): alkaline, acid, and solvent. In the first treatment, nanocellulose was obtained by

alkaline hydrolysis (ALNC) using NaOH 7% *w/v* and urea 12% w/v following the methodology of Gennari et al. [21]. Nanocellulose was obtained by acid route (ACNC) using HCl 6 M following Gennari et al. [21]. The nanocelluloses produced (ALNC and ACNC) were reconstituted at the concentration of 50 mg of cellulose per mL of ultrapure water and stored at 4 °C.

For the solvent-treated cellulose (SC), the solution solidification method proposed by Carrick et al. [22] was used with adaptations. A solution with 100 mL of LiCl 5% *w/v* in DMAc was prepared and heated in a drying oven (Ethik Technology®, 403-3*D*, Brazil) at 105 °C for 30 min. The solution was cooled in an ice bath until it reached room temperature (25 °C), and 10.5 g of MCC was added to it. Afterward, the solution was saturated with N_2, and ethanol 99.5% *v/v* was added to it by continuing dripping, resulting in a nonsolvent solution. The solidified cellulose was separated by centrifugation (Hettich®, Universal 320R, Germany) (2790 $\times g$, 4 °C, 2 min), washed twice with ultrapure water, and then dried in a drying oven (Ethik Technology®, 403-3D, Brazil) at 50 °C for 2 h.

2.2.2. Immobilization of recombinant β-galactosidase on cellulose by CBD

The oriented immobilization processes of β-galactosidase-CBD (Gal) were conducted by incubating 6 mL of the immobilization solution (100 to 300 $U_{enzyme}/g_{support}$ in 50 mM sodium phosphate buffer solution with 3 mM of $MgCl_2$, pH 7.0) with 100 mg of support (MCC, ALNC, ACNC, or SC). The solutions were maintained under rocking and rolling motion (Didática SP®, Roller Mixer MRII, Brazil) at 100 rpm and 25 °C for 15 min. During this process, supernatant samples were collected periodically to determine the immobilization yields (Eqs. (1) and (2)) [23,24]. In the end, β-galactosidase immobilized on cellulose was separated by centrifugation (Hettich®, Universal 320R, Germany) (2790 $\times g$, 4 °C, 2 min), and the derivatives (MCC-Gal, ALNC-Gal, ACNC-Gal, and SC-Gal) were washed 5 x with 6 mL of Tris-HCl buffer (50 mM, pH 7.5) and analyzed to determine efficiency and expressed activity following Eqs. (3) and (4), respectively [23].

$$Yield\ (\%) = \frac{Initial\ activity - Supernatant\ activity}{Initial\ activity} \times 100 \tag{1}$$

$$Protein\ Yield\ (\%) = \frac{Initial\ protein\ concentration - Supernatant\ protein\ concentration}{Initial\ protein\ concentration} \times 100 \tag{2}$$

$$Efficiency\ (\%) = \frac{Suspension\ Activity}{Initial\ activity - Supernatant\ activity} \times 100 \tag{3}$$

$$Expressed\ Activity\ (IU/g) = \frac{Suspension\ Activity}{Initial\ activity} \times Activity\ load \tag{4}$$

All experiments were carried out in triplicate. Statistical verification of the immobilization parameters was performed using a one-way analysis of variance (ANOVA). The significance of the model was determined using Fisher's F-test. In the significant models, means were compared using the Tukey test with a significance level of 0.05 (*p*-value <0.05). The SPSS® Statistics 26.0 software was used for the statistical analysis.

A cleavage sequence was used in the construction of the recombinant protein to obtain β-galactosidase in its free form [20]. The enzyme immobilized on cellulose was incubated in a drying oven (Ethik Technology®, 403-3D, Brazil) in the proportion of 100:1 with activated factor X for 2.5 h at 37 °C. In the end, the mixture was centrifuged (Hettich®, Universal 320R, Germany) (2790 $\times g$, 4 °C, 5 min), and the free enzyme (free Gal) of the solution supernatant was separated for

later characterization.

2.2.3. Enzyme activity and protein determination

The enzymatic activity of β-galactosidase-CBD was determined by using the substrate ortho-nitrophenyl-β-D-galactopyranoside (ONPG) following the methods described by Rech et al. [25] with some modifications. For this purpose, 100 μL of enzymatic samples were added to 1 mL of 13 mM ONPG (prepared in a 50 mM sodium phosphate buffer solution with 3 mM of $MgCl_2$, pH 7.5) at 41 °C. The reaction was interrupted after 1 min by adding 200 μL of 1 M sodium carbonate, and the absorbance was measured using a spectrophotometer (Shimadzu®, UV-2600, Japan) at 405 nm. One unit (U) or international unit (IU) of activity was defined as the amount of enzyme that catalyzes the release of 1 μmol of ortho-nitrophenol (ONP) (ε_{ONP} = 3.1 mL/μmol.cm) per minute under assay conditions.

Protein content was determined using the method proposed by Bradford [26]. The protein profile of the crude enzymatic extract containing β-galactosidase-CBD of the derivatives MCC-Gal, ALNC-Gal, and ACNC-Gal, and free Gal was assessed with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE 12% *w/v*) by using the molecular weight marker Page Ruler™ (Thermo Scientific®, US) [25]. The gel was stained with Coomassie Brilliant Blue R-250 (0.1% w/v). The derivatives obtained were heated for 15 min for protein desorption and visualization.

2.2.4. Textural and morphological assessment of the cellulosic supports and the derivatives produced

Textural characterization of the cellulosic supports (MCC, ALNC, and ACNC) and derivatives obtained (MCC-Gal, ALNC-Gal, and ACNC-Gal) was performed by using N_2 adsorption-desorption isotherms at liquid N_2 boiling temperature using a Micromeritics device (Micromeritics®, Tristar II Kr 3020, USA). Samples had been previously degassed at 100 °C under vacuum for 8 h. The Brunauer-Emmett-Teller (BET) multipoint technique was used to determine the specific surface area. Pore size distribution was obtained by using the Barret-Joyner-Halenda (BJH) method [26].

The structure morphology of samples (MCC, MCC-Gal, ALNC, ALNC-Gal, ACNC, and ACNC-Gal) was analyzed using Scanning Electronic Microscope with Field Emission (SEM-FE) (FEI®, Inspect F50, Japan) with an acceleration voltage of 20 kV and magnification of 10.000 ×. Before the analysis, samples had been dried in a drying oven (Ethik Technology®, 403-3D, Brazil) at 60 °C for 12 h and dispersed in iso-propyl alcohol. The material was then mounted on carbon tapes and coated with gold. The elemental composition of the samples was determined by Energy Dispersive Spectroscopy (EDS) (FEI®, Inspect F50, Japan), with an acceleration voltage of 20 kV.

2.2.5. Characterization of catalytic properties of free and immobilized β-galactosidase

2.2.5.1. Determination of reaction conditions of pH and temperature and kinetic parameters.
Optimal pH and temperature values were determined for the activity of free and immobilized β-galactosidases by using the Central Composite Design (CCD) method following Gennari et al. [20]. The catalytic activities of the free enzyme (free Gal) and their derivatives (MCC-Gal, ALNC-Gal, and ACNC-Gal) were analyzed at temperatures ranging from 25 to 75 °C and solutions with pH ranging from 5 to 9. Table S1 shows the complete matrix of the factorial design 2^2 with the real and codified levels of the different combinations of conditions assessed.

Experimental designs were developed and analyzed using the software Statistica 13.1 (Dell Statistica®, EUA). Statistical verification of the models was performed using ANOVA. The significance of the regression coefficients (p-value <0.05) of the variables assessed (pH and temperature) and associated probabilities (p(t)) were determined by the

Student *t*-test. The Fisher's F-test was used to assess the significance of models of second-order equations. The variance explained by the model is given by the coefficient of multiple determination, R^2. The quadratic models of β-galactosidases were represented by contour surface (2D) for the variables assessed. All experiments were carried out in triplicate.

The kinetic parameters of free (Free Gal) and immobilized (MCC-Gal, ALNC-Gal, and ACNC-Gal) β-galactosidases were determined by the concentration variation of three different substrate solutions: ONPG (6.5 to 52.0 mM), lactose (9.13 to 584 mM), and skim milk (9.13 to 584 mM of lactose) [27,28]. The reactions with ONPG were conducted for 1 min at 41 °C, as described in item 2.2.3. The hydrolysis of the solutions with lactose was conducted for 5 min at 41 °C, with subsequent determination of glucose concentration using a glucose oxidase kit (Labtest®, Minas Gerais, Brazil). All substrate solutions were prepared in a sodium phosphate buffer solution (50 mM with 3 mM of $MgCl_2$, pH 7.5).

Michaelis constant values (K_M) and maximal reaction velocity (V_{max}) were determined using the Michaelis-Menten model and the Lineweaver-Burk linearization. Catalytic specificity constants (k_{cat}) of free (free Gal) and immobilized (MCC-Gal, ALNC-Gal, and ACNC-Gal) β-galactosidases were determined by the relation between the maximal velocity and the Michaelis constant. Kinetic parameters were analyzed using a one-way ANOVA, and the significance of the model was determined by the Fisher's F-test. In the significant models, means were compared using the Tukey test with a significance level of 0.05 (*p*-value <0.05). The SPSS® Statistics 26.0 software was used for the statistical analysis.

2.2.5.2. Determination of the effect of the presence of galactose and ions.
The inhibitory action of galactose in the enzymatic activity was determined by the incubation of free (Free Gal) and immobilized (MCC-Gal, ALNC-Gal, and ACNC-Gal) (50 U) β-galactosidases in the presence of different concentrations of galactose (1 to 5% m/v). The commercial β-galactosidase from *Kluyveromyces lactis* (50 U) (Prozyn®, Lactomax Pure, Brazil) was also subjected to the same conditions to assess the effect of this monosaccharide on the activity of enzymes used in the industry. Enzymatic reactions were conducted using ONPG as substrate, as described in Item 2.2.3. The activity of β-galactosidase in the absence of galactose was used as control (100%) to determine the relative activity of the enzyme in the presence of the monosaccharide.

The effect of the presence of ions in the activity of free (free Gal) and immobilized (MCC-Gal, ALNC-Gal, and ACNC-Gal) β-galactosidases was assessed as follows: (i) for 1 min in the reaction conditions of determination of the enzymatic activity, and (ii) for 30 min in the Tris-HCl buffer solution (50 mM, pH 7.5) [19,29].

In (i), the test was conducted using 10 to 20 U of β-galactosidases with the substrate ONPG (41 °C for 1 min), as described in item 2.2.3. ONPG was prepared in a sodium phosphate buffer solution (50 mM, pH 7.5) with different cation concentrations (chloride salts): Na^+ (20 and 40 mM), K^+ (40 and 80 mM), Ca^{2+} (30 and 60 mM), and Mg^{2+} (4 and 8 mM). For the preparation, different anion concentrations (magnesium salts) were used: Cl^- (8 and 16 mM) and $(SO_4)^{2-}$ (8 and 16 mM). The activity of β-galactosidase in the absence of ions was used as control (100%) to determine the relative activity of the enzyme in the presence of ions.

In (ii), the test was conducted with 10 and 20 U of the β-galactosidases incubated for 30 min at 25 °C in a Tris-HCl buffer solution (50 mM, pH 7.5). The solutions contained the different ion concentrations described above. After that, the enzymatic activity of each solution was determined following the methods described in item 2.2.3. The activity of β-galactosidase in the absence of ions was used as control (100%) to determine the relative activity of the enzyme in the presence of ions.

2.2.5.3. Determination of thermal and storage stabilities.
The thermal stability of free and immobilized β-galactosidases was determined by incubating the samples (50 U) in thermostatized bath (Marconi®, MA

156, Brazil) at 55, 60, 65, and 70 °C. Aliquots were collected periodically to determine the residual activity of the enzymes. The kinetic inactivation constant (k), half-life time ($t_{1/2}$), and stabilization factor (SF) of the β-galactosidases (Free Gal, MCC-Gal, ALNC-Gal, and ACNC-Gal) were determined following Gennari et al. [27].

Storage stability was determined by incubating the free (free Gal) and immobilized (MCC-Gal, ALNC-Gal, e ACNC-Gal) β-galactosidase in Tris-HCl buffer (50 mM, pH 7.5) at 4 °C. Samples were collected periodically for 90 days to determine the enzymatic activity, as described in Item 2.2.3. The activity of each β-galactosidase in the first days of storage was used as control (100%) to calculate the residual activity.

2.2.6. Cytotoxicity determination of the supports and β-galactosidase preparations

Cellular viability determination after incubation with the enzyme samples was performed using two different methods: the Methyl Thiazol Tetrazolium (MTT) and neutral red (NR) uptake assays [30,31]. HepG2 and Vero cells were grown in Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen, US) supplemented with 10% inactivated fetal bovine serum, 1% antibiotic (penicillin-streptomycin), and 0.01% antifungal (amphotericin B). Cells were seeded at 5×10^3 (HepG2) or 2×10^3 cells/well (Vero) in a 96-well microtiter plate and incubated overnight. The supports (MCC, ALNC, and ACNC) and enzyme samples (Free Gal, MCC-Gal, ALNC-Gal, and ACNC-Gal) (10 and 20 U) were solubilized in 6 M H_2SO_4, and a 12 M NaOH solution was added dropwise until the mixtures reached pH 7.0. Then, samples were incubated with the cell lines for 72 h at 37 °C under 5% CO_2. For the MTT assay, cultures were subjected to the same conditions with MTT reagent (2 mg/mL) for 4 h, and absorbance was measured at 570 nm using an EZ Read 400 microplate reader (Biochrom®, US). Precipitated purple formazan crystals were directly proportional to the number of live cells with active mitochondria. The mean absorbance of vehicle control wells was set as 100% viability, and the values of treated cells were calculated as the percentage of cell viability. For the NR assay, after 72 h of incubation with the samples, cells were washed with Phosphate-Buffered Saline (PBS) before the addition of 200 μL of NR dye solution (25 μg/mL) prepared in serum-free medium, and the plate was incubated for an additional 3 h at 37 °C under 5% CO_2. Cells were washed with PBS, followed by incubation with 100 μL of a desorb solution (ethanol/acetic acid/water, 50:1:49 v/v/v) for 30 min, with gentle shaking to extract NR dye from viable cells. Absorbance was measured at 562 nm using an EZ Read 400 microplate reader (Biochrom®, US). Cell viability was expressed as percentage, considering the vehicle control cell as 100% cell viability. Statistical analysis was performed using one-way ANOVA followed by Dunnett's Multiple Comparison post-test using GraphPad Prism 9 (San Diego, US).

2.2.7. Determination of the reusability of immobilized β-galactosidase

The reusability of immobilized β-galactosidases in a batch process was assessed by means of the hydrolysis reaction of skim milk lactose (prepared to 5% w/v lactose). For this purpose, 100 mg of the derivatives (MCC-Gal, ALNC-Gal, and ACNC-Gal) were incubated at 25 °C for 30 min with 10 mL of milk under rocking and rolling motion (Didática SP®, Roller Mixer MRII, Brazil) at 150 rpm. After each reuse cycle, the immobilized enzyme was separated from milk by centrifugation (Hettich®, Universal 320R, Germany) (1260 ×g, 1 min, 25 °C), and the supernatant fluid was collected to establish the degree of hydrolysis using the colorimetric enzyme kit of oxidase-peroxidase (Labtest®, Glucose Liquiform, Brazil) to form glucose. The derivative was washed with 2 mL of Tris-HCl buffer (50 mM, pH 7.5). A new solution of skim milk was added, starting a new reuse cycle. The degree of hydrolysis of each derivative after the first reuse cycle was defined as 100%.

3. Results and discussion

3.1. Oriented immobilization of recombinant β-galactosidase on cellulose

The recombinant β-galactosidase was immobilized by using enzymatic loads ranging from 100 to 300 U/$g_{support}$ on different cellulosic bases (MCC, ALNC, ACNC, and SC). Table 1 shows the results for yield (activity and protein), efficiency, and expressed activity.

The presence of the CBD tag bound to β-galactosidase enabled a rapid immobilization (15 min) on the four supports assessed (Table 1). Activity and protein yield and expressed activity did not significantly differ ($p < 0.05$) for enzymatic loads up to 150 U/$g_{support}$. The derivatives MCC-Gal, ALNC-Gal, and ACNC-Gal showed efficiency values higher than 95% regardless of the enzymatic load used. The efficiency value of the derivative SC-Gal was approximately 80%. This difference between the derivatives can be attributed to different bonds between β-galactosidase-CBD and chemical groups formed in each cellulose preparation, which can affect the tridimensional structure of the enzyme and consequently its enzymatic activity. In the alkaline treatment (ALNC), the cellulose glycosidic bond was broken, and a double conjugated bond was formed due to electron transfer [32]. The acid nanocellulose-based support (ACNC) was produced by occasional depolymerization caused by the acid medium and the temperature used, resulting in the hydrolysis of cellulose α (1 → 4) bonds [33]. The use of DMAc in SC enables the solubilization of MCC by destroying its crystallinity and hydrogen

Table 1
Immobilization parameters of β-galactosidase-CBD on the prepared cellulosic supports using different activity loads.

Derivative	Immobilization Parameter	Activity Load (U/$g_{support}$)				
		100	150	200	250	300
MCC-Gal	Yield (%)	75.75 ± 3.42 [abc]	69.79 ± 3.13 [bcdef]	60.95 ± 3.17 [fgh]	48.43 ± 2.32 [ij]	35.68 ± 2.45 [k]
	Protein Yield (%)	73.77 ± 2.88 [abc]	65.09 ± 1.80 [bcde]	57.05 ± 3.62 [fgh]	48.66 ± 3.56 [ij]	35.03 ± 2.16 [k]
	Efficiency (%)	103.71 ± 2.20 [ab]	102.13 ± 4.61 [ab]	100.28 ± 2.97 [a]	98.64 ± 2.75 [ab]	100.82 ± 1.46 [ab]
	Expressed activity (IU/g)	78.51 ± 5.84 [hi]	106.88 ± 4.57 [cbde]	118.98 ± 5.89 [abc]	119.70 ± 2.38 [abc]	107.40 ± 7.39 [bcde]
ALNC-Gal	Yield (%)	83.81 ± 3.01 [a]	75.42 ± 2.62 [abcd]	65.53 ± 2.40 [cdef]	52.42 ± 2.28 [ghi]	42.99 ± 1.96 [ij]
	Protein Yield (%)	79.78 ± 3.73 [a]	70.63 ± 2.56 [bcd]	63.35 ± 2.29 [cde]	49.48 ± 2.32 [ghi]	40.34 ± 2.87 [ij]
	Efficiency (%)	101.17 ± 2.62 [ab]	101.62 ± 1.94 [ab]	100.26 ± 3.07 [ab]	101.36 ± 3.13 [ab]	101.37 ± 2.21 [ab]
	Expressed activity (IU/g)	84.74 ± 5.58 [fgh]	115.03 ± 7.36 [abcd]	131.49 ± 8.46 [a]	132.81 ± 5.37 [a]	130.78 ± 7.31 [a]
ACNC-Gal	Yield (%)	78.42 ± 2.99 [ab]	72.61 ± 2.17 [bcde]	60.04 ± 2.88 [efg]	50.87 ± 3.44 [hij]	41.28 ± 4.40 [ijk]
	Protein Yield (%)	75.79 ± 3.64 [ab]	69.49 ± 2.49 [bcd]	58.34 ± 2.79 [efg]	46.92 ± 3.76 [ij]	38.21 ± 3.20 [ijk]
	Efficiency (%)	101.36 ± 2.23 [ab]	99.55 ± 3.66 [ab]	103.50 ± 3.48 [ab]	96.58 ± 1.89 [ab]	99.90 ± 3.53 [ab]
	Expressed activity (IU/g)	79.44 ± 1.30 [ghi]	108.47 ± 6.37 [bcde]	124.33 ± 6.15 [ab]	122.86 ± 5.90 [ab]	123.79 ± 8.15 [ab]
SC-Gal	Yield (%)	73.24 ± 4.36 [bcd]	73.38 ± 4.30 [bcd]	64.42 ± 4.48 [def]	49.64 ± 4.13 [ij]	40.78 ± 5.16 [jk]
	Protein Yield (%)	71.97 ± 1.47 [bcd]	67.64 ± 0.87 [bcd]	62.60 ± 1.43 [def]	45.48 ± 2.76 [ij]	36.39 ± 1.51 [jk]
	Efficiency (%)	83.68 ± 3.44 [c]	80.93 ± 4.03 [c]	78.37 ± 4.56 [c]	82.52 ± 1.96 [c]	80.95 ± 3.60 [c]
	Expressed activity (IU/g)	61.38 ± 5.93 [i]	89.08 ± 6.94 [efgh]	100.81 ± 6.21 [cdef]	102.40 ± 6.67 [cdef]	98.69 ± 6.20 [defg]

Different letters in the same immobilization parameter between the different activity loads and supports represent a significant difference ($p < 0.05$). Each value represents the mean of three experiments conducted in duplicate and the standard deviation.

bond network. Then, polymeric aggregates are produced when the mixture is dripped in a nonsolvent solution (water, ethanol, acetone, or methanol) [34].

Immobilization efficiency values were higher in this study than those observed by Lu et al. [35]. Lu et al. [35] produced β-galactosidase from *Lactobacillus bulgaricus* L3 bound to CBD to synthesize galactooligosaccharides. They observed the immobilization of 97.6 U/g_{MCC} and 61% of efficiency after 20 min of contact using crude enzymatic extract. Wang et al. [36] also produced a β-galactosidase bound to a CBD. The recombinant β-galactosidase was purified using a commercial column with affinity to His-tag and then immobilized using cellulosic filter paper. Wang et al. [36] reported a yield of 100% and efficiencies higher than 100% after 2 h of immobilization. Similar results were obtained in this study for the immobilization parameters. Immobilization parameters were obtained after only 15 min of contact between the recombinant enzyme and the cellulosic support, and β-galactosidase-CBD was purified in one step.

The values of expressed activity (106.88, 115.03, and 108.47 IU/g) of the derivatives MCC-Gal, ALNC-Gal, and ACNC-Gal, respectively, did not significantly differ ($p < 0.05$) for enzymatic loads higher than 150 U/$g_{support}$. Based on that, they were selected for the characterization experiments with a load of 150 U/$g_{support}$.

Fig. 1 shows the gel electrophoresis of the crude enzymatic extract (30 μg of protein), supernatant after the immobilization process (10 μg of protein), proteins immobilized on the cellulosic supports (10 μg of protein), and free β-galactosidase (10 μg of protein).

Fig. 1 shows the band corresponding to β-galactosidase-CBD (~120 kDa) in the crude enzymatic extract (I). The recombinant enzyme was not present in the supernatant of the solutions (II, III, and IV). This result may be attributed both to the binding of the largest part of the enzyme to cellulosic supports and to the low amount of proteins in the supernatant. There was also a strong presence of β-galactosidase-CBD in the derivatives MCC-Gal, ALNC-Gal, and ACNC-Gal (V, VI, and VII). This result proves the functionality and high specificity of the CBD tag in the one-step purification and immobilization processes of the recombinant enzyme on cellulosic supports. The enzyme bound to the cellulose was released by cleaving the protein sequence using the Factor Xa. Therefore, the purified recombinant β-galactosidase in its soluble form (VIII) can be obtained by immobilization on cellulose.
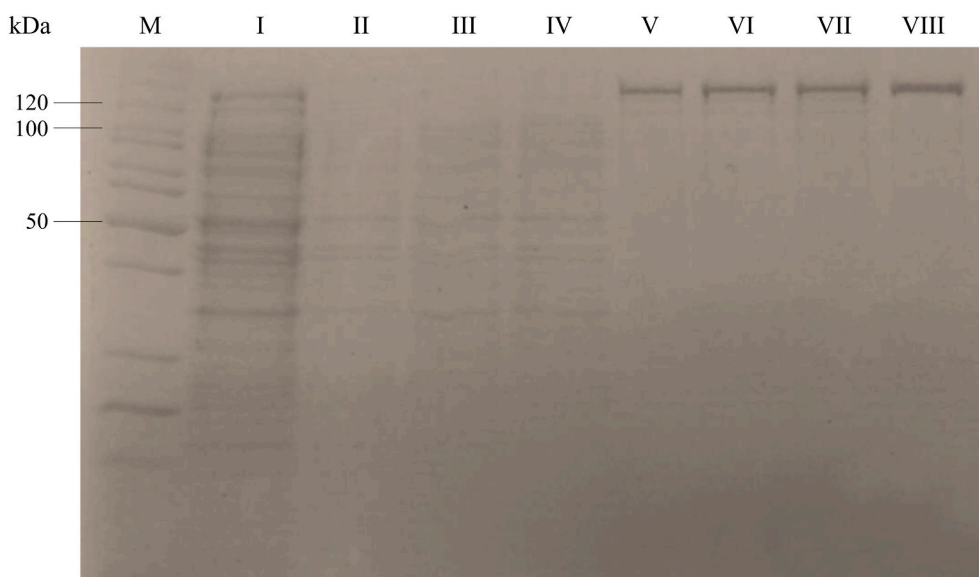
3.2. Textural and morphological assessment of the cellulosic supports and the derivatives produced

Table S2 shows the textural characteristics of the surface area and pore volume of the cellulosic supports (MCC, ALNC, and ACNC) and the derivatives obtained (MCC-Gal, ALNC-Gal, and ACNC-Gal). Their adsorption-desorption isotherms are presented in Fig. S1. The cellulosic supports and the derivatives produced during the oriented immobilization process showed a type II isotherm, typical of non-porous materials (Fig. S1) [37], with small surface areas. According to Trache et al. [38], the surface area and microcrystalline cellulose morphology depend on the biopolymer origin and the treatment used to obtain it. The MCC used in this study showed a surface area of about 1.3 m^2/g that did not change after the treatments used to obtain ALNC and ACNC. After immobilization of the recombinant β-galactosidase, the surface area of the derivatives MCC-Gal and ACNC-Gal slightly decreased.

Fig. 2 shows the morphological characteristics of the supports MCC, ALNC, and ACNC and derivatives MCC-Gal, ALNC-Gal, and ACNC-Gal. After the alkaline (Fig. 2B) and acid treatment (Fig. 2C), the surface area of cellulose changed: it displayed fragments in blocks and fibers, respectively. After the oriented immobilization processes of the recombinant β-galactosidase (Fig. 2D, E, and F), different agglomerates appeared on supports' surfaces. Verma and Raghav [39] also observed that cellulose used as a support for the immobilization of α-amylase acquired an irregular surface. Verma and Raghav [39] verified through scanning electron microscopy that the polymeric supports used in the study acquired an irregular surface due to the severe chemical treatments used to obtain cellulose derivatives.

Table S3 shows the elemental composition of the cellulosic supports used in the oriented immobilization of recombinant β-galactosidase and derivatives obtained. The treatments used to synthesize the supports ALNC and ACNC increased the percentage of oxygen in the materials. Also, the percentage of carbon increased in the three derivatives obtained after the immobilization processes due to enzyme binding to the support [21].
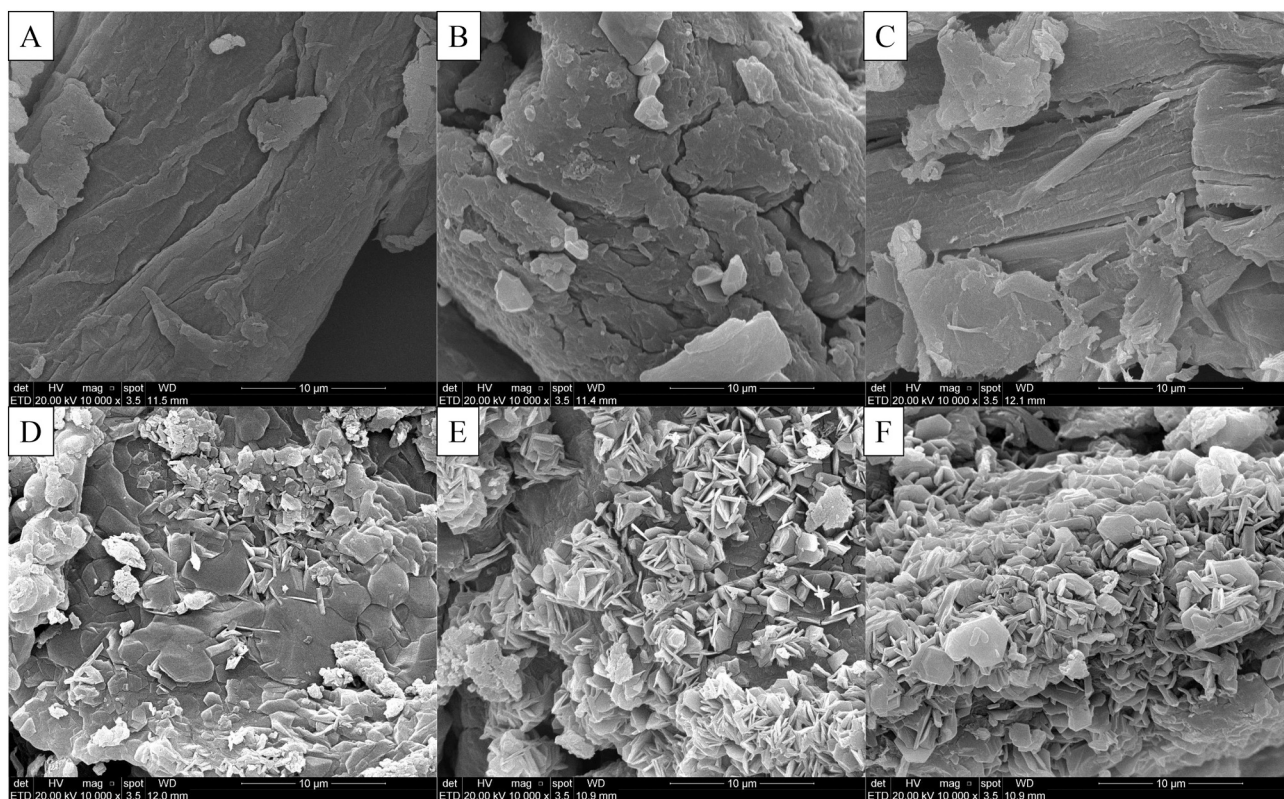

Fig. 1. Polyacrylamide gel electrophoresis of the proteins present in different solutions before and after the immobilization on cellulose. M: Page Ruler molecular weight marker; I: crude enzymatic extract; II, III, and IV: supernatant after immobilization with MCC, ALNC, and ACNC, respectively; V, VI, and VII: derivative MCC-Gal, ALNC-Gal, and ACNC-Gal, respectively; VIII: free β-galactosidase (cleaved from the derivative MCC-Gal with Factor Xa).

Fig. 2. Images obtained using SEM-FE with magnification of 10.000 ×. Cellulosic supports: (A) MCC, (B) ALNC, and (C) ACNC. Derivatives obtained after the oriented immobilization process of the recombinant β-galactosidase: (D) MCC-Gal, (E) ALNC-Gal, and (F) ACNC-Gal.

3.3. Characterization of the catalytic properties of free and immobilized β-galactosidase

3.3.1. Determination of reaction conditions (pH and temperature) and kinetic parameters

The CCD method was used to determine the effect of pH (5 to 9) and temperature (25 to 75 °C) values in the activity of free and immobilized β-galactosidases. Table S1 shows the CCD experimental matrix with real and codified values of the assessed conditions and their respective values of enzymatic activity obtained in the 11 tests.

The significance values for the enzymatic activity models of Free Gal, ALNC-Gal, ACNC-Gal, and MCC-Gal were p-value<0.0169, p-value<0.0543, p-value<0.0103, and p-value<0.0162, respectively. The response surface of free and immobilized enzymes was obtained based on the models (Eqs. (1)–(4)) (Fig. S2), which demonstrates the relationship between the enzymatic activity and the reaction conditions of pH and temperature assessed. The optimal values of pH and temperature for the activities of Free Gal, MCC-Gal, ALNC-Gal, and ACNC-Gal and their respective predicted values of enzymatic activity were determined using optimized mathematical models (Table 2). To validate the models, the activities of free and immobilized β-galactosidases in the different cellulosic supports were carried out under optimal pH and temperature

conditions. Table 2 shows the experimental values. The results show that the theoretical values predicted by the models for the activities of β-galactosidases (Free Gal, MCC-Gal, ALNC-Gal, and ACNC-Gal) and those determined in the experiments have a similarity of about 90%.

As shown in Table 2, the optimal pH values for the activity of recombinant free and immobilized β-galactosidases on different cellulosic supports are close to neutrality. The derivatives MCC-Gal and ACNC-Gal showed optimal activity conditions at 43 °C, while free Gal and ALNC-Gal showed higher optimal temperature values, approximately 5 °C. According to Bayramoglu et al. [40], variations in optimal temperatures of enzymatic activity may be related to distinct interactions between the

Table 2
Optimal conditions of reaction pH and temperature obtained by the models for the activity of free and immobilized β-galactosidases and their respective predicted and observed values.

	Free Gal	MCC-Gal	ALNC-Gal	ACNC-Gal
pH	6.90	6.49	6.82	6.72
Temperature (°C)	48.16	42.61	48.11	42.99
Predicted activity[a]	22.06	111.56	126.98	123.31
Experimental activityy[a]	19.83	110.63	119.48	117.36

[a] Free Gal (U/mL); MCC-Gal, ALNC-Gal, ACNC-Gal (IU/g).

Table 3
Kinetic parameters of free and immobilized β-galactosidases in different substrate solutions.

	K_M (mM)	V_{max} (mM/min)	k_{cat} (min^{-1})
ONPG			
Free Gal	12.70 ± 0.68 [a]	189.67 ± 2.65 [ab]	14.96 ± 0.61 [ab]
MCC-Gal	9.96 ± 0.33 [b]	183.33 ± 1.46 [ab]	18.41 ± 0.45 [a]
ALNC-Gal	14.94 ± 0.85 [a]	216.33 ± 5.45 [a]	14.50 ± 0.49 [b]
ACNC-Gal	9.93 ± 0.53 [b]	176.17 ± 2.59 [b]	17.77 ± 0.67 [ab]
Lactose			
Free Gal	46.19 ± 0.73 [b]	338.57 ± 1.80 [a]	7.33 ± 0.09 [b]
MCC-Gal	48.98 ± 1.29 [b]	371.10 ± 3.12 [a]	7.58 ± 0.14 [b]
ALNC-Gal	56.15 ± 1.30 [a]	354.23 ± 1.60 [a]	6.31 ± 0.12 [c]
ACNC-Gal	41.34 ± 1.08 [b]	365.10 ± 1.76 [a]	8.83 ± 0.18 [a]
Milk			
Free Gal	86.85 ± 2.09 [a]	286.63 ± 1.39 [c]	3.30 ± 0.07 [c]
MCC-Gal	79.46 ± 2.69 [a]	323.70 ± 2.43 [ab]	4.08 ± 0.11 [ab]
ALNC-Gal	80.28 ± 2.12 [a]	309.73 ± 2.15 [bc]	3.86 ± 0.08 [b]
ACNC-Gal	70.25 ± 1.55 [a]	349.70 ± 1.51 [a]	4.41 ± 0.07 [a]

Different letters in the same column for each substrate solution represent a significant difference (p<0.05). Each value represents the mean of three experiments conducted in duplicate and the standard deviation.

enzyme and the cellulosic support, especially by hydrogen bonds.

The results obtained for optimal pH and temperature conditions of the free and immobilized enzymes are congruent with those reported by Wang et al. [36], who immobilized a β-galactosidase with the CBD tag on cellulose. Wang et al. [36] observed that the optimal conditions of enzymatic activity did not change after immobilization. This indicates that the cellulose-binding domain added to the enzyme does not alter the natural conformation of β-galactosidase after it binds to the cellulose.

Table 3 and Fig. S3 show the kinetic parameters of free and immobilized β-galactosidases in different substrate solutions. Using ONPG, K_M values of the derivatives MCC-Gal and ACNC-Gal decreased approximately 25% when compared to free Gal. This indicates a significant increase ($p < 0.05$) in the affinity of these enzymes immobilized by the substrate. Regarding the catalytic constant, free and immobilized β-galactosidases did not differ in the ONPG hydrolysis reaction. Although V_{max} values did not show any significant difference between the β-galactosidases with the lactose solution, the affinity of the derivative ALNC-Gal was significantly lower (p < 0.05) than that of the other enzymatic preparations, resulting in 10 to 30% lower k_{cat} values. These results suggest that, in comparison with other supports, the cellulose structural form after conjugated double bonds (ALNC) negatively affects the catalytic activity of immobilized β-galactosidase in ONPG and lactose hydrolysis reactions.

Kinetic parameters were determined in a milk solution (with 5% of lactose) to enable the application of the immobilized enzymes in the lactose hydrolysis of dairy products (Table 3). Significant higher values of V_{max} and k_{cat} indicate that immobilized β-galactosidases (MCC-Gal, ALNC-Gal, and ACNC-Gal) are more efficient in the lactose hydrolysis of milk. The comparative results of the kinetic parameters for the lactose and milk solutions show an increase in K_M and a decrease in V_{max} and k_{cat} values. This variation is probably associated with the components present in milk, like proteins and mineral salts, which can hinder the association between lactose and β-galactosidase's active site [41].

Liu et al. [28] produced and purified a recombinant β-galactosidase from *Bacillus velezensis*. Their soluble β-galactosidase showed a K_M value 9.5 × higher and V_{max} 33% lower in the lactose hydrolysis reaction in comparison with ONPG. In the present study, free and immobilized β-galactosidases showed an increase of 4 × in the affinity value and 2 × in the V_{max} value in the lactose hydrolysis in comparison with ONPG. These results may be related to β-galactosidase structural characteristics, as the enzyme reported by Liu et al. [28] is a dimer and the enzyme of this study is a tetramer with CBDs [20].

Lu et al. [35] also verified that the recombinant β-galactosidase from *L. bulgaricus* L3 immobilized on microcrystalline cellulose via CBD (CBD-BgaL3) showed different values of affinity with ONPG and lactose. After immobilization, the K_M values of CBD-BgaL3 for the substrate ONPG decreased (from 2.50 to 1.51 mM), and the values for lactose remained constant (~30 mM). V_{max} values decreased approximately 25% for both substrates.

3.3.2. Assessment of the presence of galactose and different ions in the enzymatic activity

The inhibition of biocatalysis reactions by the product formed is one of the main limitations of the application of enzymes in industrial processes. The monosaccharide galactose, one of the products of lactose hydrolysis, is a competitive inhibitor of β-galactosidase [42]. In the present study, the enzymatic activities were determined under increasing galactose concentrations to assess their effect on free (free Gal) and immobilized (MCC-Gal, ALNC-Gal, and ACNC-Gal) β-galactosidases (Fig. 3).

Fig. 3 shows that the enzymatic activity of free Gal is more affected by the inhibition of galactose in a dose-dependent manner, decreasing approximately 45% of its initial activity under galactose concentration of 5%. In the same condition, immobilized β-galactosidases (ALNC-Gal and MCC-Gal) showed higher values of relative activity (lower inhibitory effect) in comparison with the commercial *K. lactis* β-galactosidase and free Gal. The free and immobilized enzymes in this study were less inhibited by galactose in comparison with the commercial *Aspergillus oryzae* β-galactosidase immobilized on silver nanoparticles [43]. The fungal enzyme in its free form retained only 25% of its activity in the presence of 5% of galactose, while the immobilized enzyme retained 52% of its activity. According to Mateo et al. [44], the decreased inhibition of the enzymatic activity of immobilized β-galactosidases by galactose may be related to conformational changes in the enzyme. These changes are caused by adhesion to the support and may limit the interaction of the enzyme with the inhibitor.

In the industrial sector, β-galactosidase is used to hydrolyze the lactose present in milk and dairy products. However, these products have different mineral salts, like sodium, potassium, magnesium, and calcium. The effect of these ions on the enzymatic activity has been assessed, as they directly affect the interaction between the substrate and the enzyme active site [45]. Information about the action of metallic ions in enzymatic reactions can enhance catalytic efficiency by the
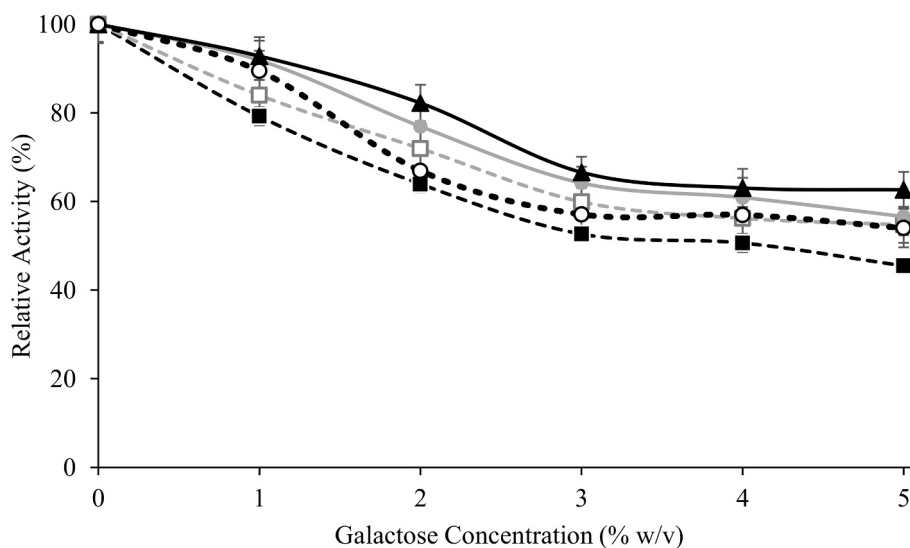

Fig. 3. Effect of different galactose concentrations (% m/v) in the activities of free and immobilized β-galactosidases on different cellulosic supports. (■) Free Gal; (□) Commercial *K. lactis* β-galactosidase; (●) MCC-Gal; (▲) ALNC-Gal; (○) ACNC-Gal.

selection of the ionic specificity of the enzyme [46]. Therefore, the enzymatic activity of free and immobilized β-galactosidases (Table S4) was assessed in the presence of the ions Na^+, K^+, Mg^{2+}, Ca^{2+}, Cl^-, and SO_4^{2-} after 30 min of contact with the enzymes (Table S5).

The assessed ions, except Ca^{2+}, caused an activation effect on the enzymatic activities of the recombinant soluble β-galactosidases (free Gal) and β-galactosidases immobilized in an oriented way on cellulosic supports (MCC-Gal, ALNC-Gal, and ACNC-Gal). The cation Mg^{2+} increased the enzymatic activity above 40% (Table S4) and up to 25% (Table S5) for β-galactosidases immobilized on cellulosic supports. Lo et al. [47] showed that Mg^{2+} interacts with some regions adjacent to the active site of β-galactosidase, increasing its enzymatic activity.

Liu et al. [27] assessed the effects of different metallic ions on the enzymatic activity of the recombinant β-galactosidase from *B. velezensis* (BsGal1332). Liu et al. [28] observed that, among the different metallic ions assessed (Na^+, K^+, Ag^+, Mg^{2+}, Ba^{2+}, Ca^{2+}, Mn^{2+}, Co^{2+}, Zn^{2+}, Cu^{2+}, Fe^{2+}, and Fe^{3+}), only K^+ increased the activity of BsGal1332. The presence of Mg^{+2} decreased the enzymatic activity by 15%, and the ion Ca^{2+} did not affect the activity variation of the recombinant β-galactosidase. Khan and Husain [48] investigated how metallic ions present in milk affect the functional structure of the immobilized β-galactosidase from *Aspergillus oryzae* on native and polyaniline chitosan nanocomposites. The authors observed an increase in the enzymatic activity on both nanocomposites in the presence of the cations K^+ and Ca^{2+}. The results reported by Liu et al. [28], Khan and Husain [47], and this study show different effects for the same ions, suggesting that the β-galactosidase source and the immobilization protocol (bond type to the support) affect the interaction between the enzyme and metallic ions.

3.3.3. Assessment of thermal and storage stabilities

The enzymes used in industrial processes should be stable under the operational conditions. Therefore, free Gal, MCC-Gal, ALNC-Gal, and ACNC-Gal were incubated at different temperatures (55, 60, 65, and 70 °C), and their parameters of thermal inactivation were assessed (Table 4).

The inactivation kinetic constant (k) values showed a linear increase with higher temperatures under the assessed conditions. Consequently, the half-life time decreased progressively. The β-galactosidase immobilized on cellulose in an oriented way showed higher thermal stability than the soluble recombinant enzyme at all temperatures assessed. At lower temperatures (55 and 60 °C), the derivatives ALNC-Gal and ACNC-Gal showed the highest thermal stability values. However, at 65 and 70 °C, β-galactosidase immobilized on MCC was more stable. De Andrade et al. [17] used His-tag for the oriented immobilization of a β-galactosidase on magnetic nanoparticles with nickel. They also observed an increase in the thermal stability of the immobilized enzyme. These results possibly indicate that enzymes immobilized by oriented bonds are less susceptible to conformational changes and consequently have higher thermostability.

Information about the stability of the enzyme stored for long periods is also relevant for its application in industrial processes. Thus, the free enzyme and the three derivatives produced were stored for 90 days at 4 °C (Fig. S4).

After two months of storage, the enzymes MCC-Gal and ALNC-Gal retained approximately 70% of their initial activity. After 90 days, the

three derivatives showed relative enzymatic activity values above 50%. However, free Gal showed lower stability, retaining 37% of its initial activity. According to Zhang et al. [49], the immobilization process increases enzymes' tridimensional structure rigidity, resulting in higher storage stability.

3.4. Cytotoxicity investigation of the obtained supports and derivatives

The evaluation of the cytotoxicity of the supports and immobilized β-galactosidases (Fig. 4) is necessary for their application in the food industry [50]. Therefore, MTT and NR assays were performed to verify the cell viability of HepG2 and Vero cultures after their incubation in the presence of the supports and derivatives produced. The MTT technique is based on induced damage to mitochondria, evaluating the activity of mitochondrial dehydrogenases, quantified by the reduction of MTT to formazan [30]. NR, on the other hand, measures cell viability based on lysosomal activity, in which viable cells, with their lysosome functioning, retain the dye in their structures [31]. The two methods studied (MTT and NR), in all preparations (supports, derivatives and free enzyme), revealed the cell viability of HepG2 and Vero was greater than 90%, compared to the negative control (DMEM). According to Dioguardi et al. [51] and Dahl et al. [52], cell viability responses >90% are classified as non-cytotoxic. In the present study, even with the chemical modifications carried out on cellulose, the supports (MCC, ALNC, and ACNC) used for the immobilization of β-galactosidase proved to be non-toxic. The incorporation of the recombinant enzyme to these supports (MCC-Gal, ALNC-Gal, and ACNC-Gal) resulted in no significant changes ($p < 0.05$) in the values obtained. Therefore, the derivatives produced can be safely used in the food industry, as well as in other biotechnology sectors.

3.5. Assessment of the reusability of immobilized β-galactosidases

The possibility to reuse enzymes is one of the main advantages of immobilization. For that reason, the derivatives produced in this study were used in sequential hydrolysis reactions of milk lactose (5% of lactose). Fig. 5 shows the results of relative lactose hydrolysis of the derivatives MCC-Gal, ALNC-Gal, and ACNC-Gal.

After five reaction cycles, the three derivatives assessed retained 80% of their capacity to hydrolyze lactose, and this capacity remained constant until the 20th reuse cycle. The three derivatives produced were used in lactose hydrolysis processes for 40 reuse cycles and retained the hydrolysis values between 53 and 64%. After 40 batches of reuse, the derivatives MCC-Gal, ALNC-Gal, and ACNC-Gal showed higher relative enzymatic activities of 59.23, 60.78, and 51.47%, respectively. Between the 20th and 40th reuse cycles, the derivative MCC-Gal showed the highest potential to hydrolyze skim milk lactose under the conditions assessed. Probably, the decrease in the hydrolysis capacity of the derivatives along the reuse cycles is due to enzymatic inactivity, enzyme detachment, and loss of derivative mass during the washing processes after each cycle [53].

Lu et al. [35] used a β-galactosidase-CBD immobilized on MCC to hydrolyze a lactose solution (40% m/v) at 45 °C for 75 min. After 20 reuse cycles, the derivative retained approximately 85% of its initial capacity. Those values are similar to those obtained in this study. Wang

Table 4
Parameters of thermal inactivation of free (free Gal) and immobilized β-galactosidases on cellulosic supports.

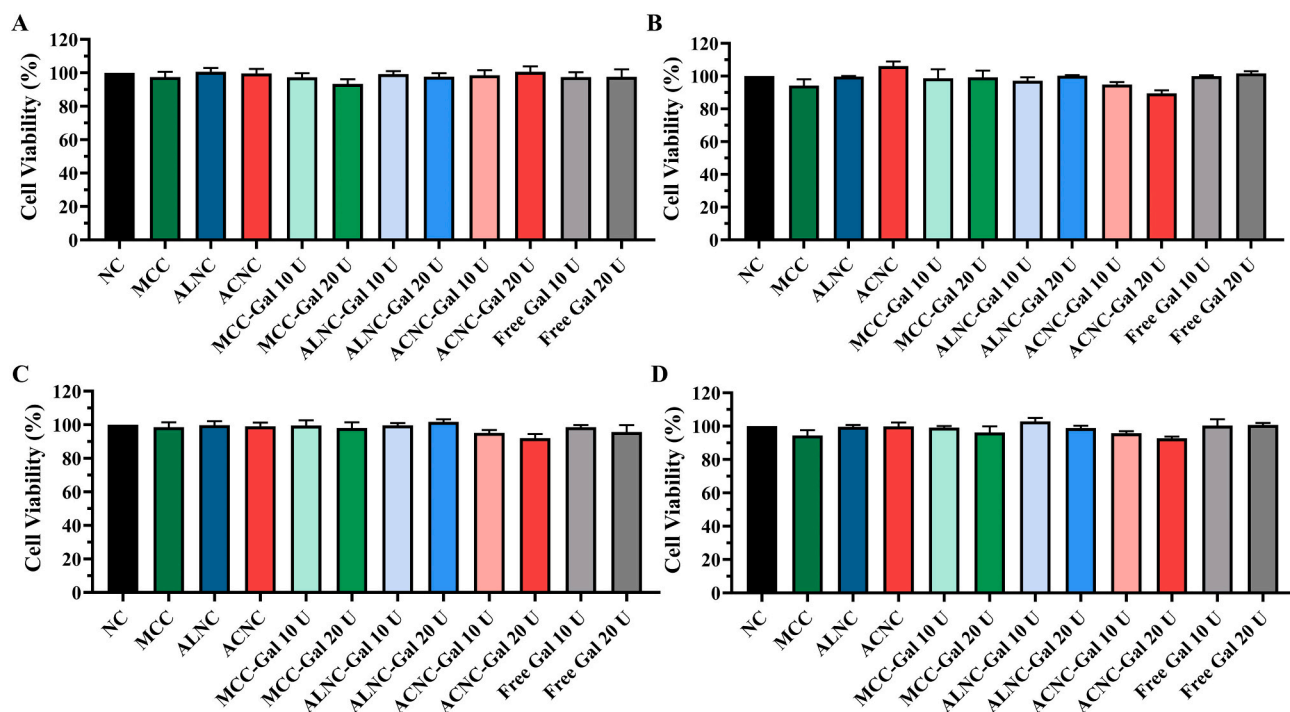| β-galactosidase	55 °C			60 °C			65 °C			70 °C		
	k (min⁻¹)	t₁/₂ (min)	SF	k (min⁻¹)	t₁/₂ (min)	SF	k (min⁻¹)	t₁/₂ (min)	SF	k (min⁻¹)	t₁/₂ (min)	SF
Free Gal	2.14	0.32	–	2.28	0.30	–	2.80	0.25	–	2.86	0.24	–
MCC-Gal	1.24	0.56	1.73	1.42	0.47	1.57	1.55	0.45	1.81	1.60	0.43	1.78
ALNC-Gal	1.16	0.60	1.85	1.23	0.56	1.86	1.68	0.41	1.66	1.85	0.37	1.54
ACNC-Gal	1.17	0.59	1.84	1.57	0.46	1.53	1.68	0.41	1.67	2.01	0.35	1.43

Fig. 4. Cell viability employing MTT and NR assays, from HepG2 and Vero cells treated with the produced supports, derivatives, and enzyme preparations. (A) HepG2 - MTT; (B) Vero - MTT; (C) HepG2 - NR e (D) Vero - NR. NC: Negative control.
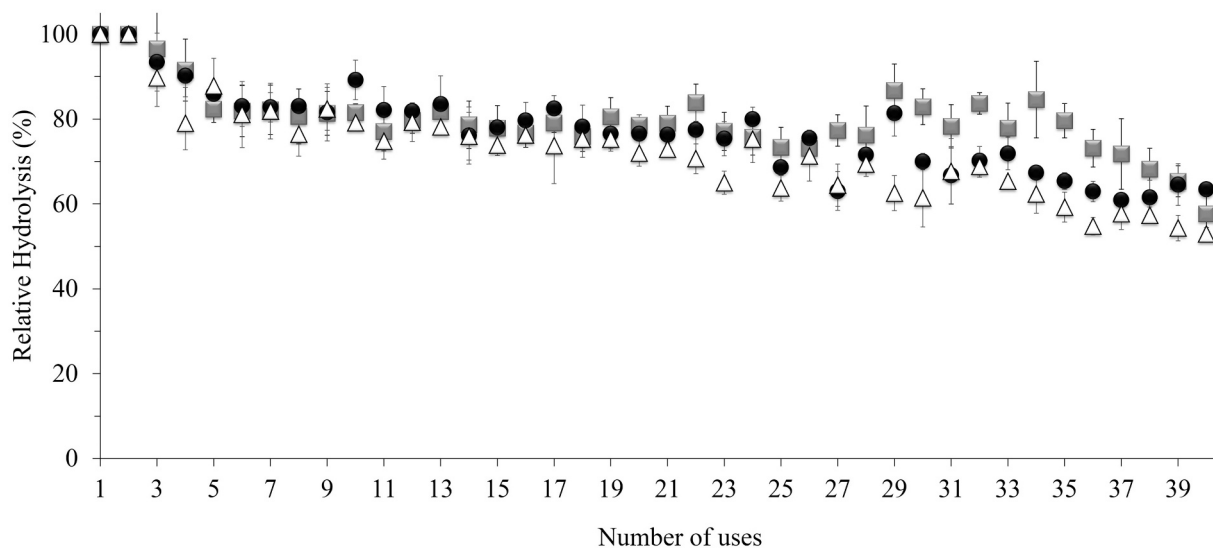

Fig. 5. Reuse of immobilized β-galactosidases on cellulosic supports in hydrolysis reactions of skim milk lactose (5% of lactose) at 25 °C for 30 min. (■) MCC-Gal; (●) ALNC-Gal; (△) ACNC-Gal.

et al. [36] assessed the reusability of a β-galactosidase-CBD immobilized on cellulosic filter paper and observed that the enzyme retained only 30% of its initial capacity after 9 reuse cycles. Probably, higher stabilities to reuse processes of the immobilized β-galactosidases obtained in this study are related to the origin and preparation of the cellulose used (chemically treated commercial MCC).

4. Conclusion

This is the first study that purifies and immobilizes a β-galactosidase from *Kluyveromyces* sp. with the CBD tag in one step by binding it to different cellulosic supports. By using enzymatic loads of 150 U/g$_{support}$,

approximately 70% of β-galactosidase-CBD were immobilized on microcrystalline cellulose, nanocellulose obtained from alkaline hydrolysis, and nanocellulose obtained from acid hydrolysis in 15 min of reaction, showing expressed activity values of 106 to 55 IU/g. β-Galactosidase-CBD retained 100% of its catalytic activity. The strong presence of β-galactosidase-CBD in the derivatives showed the high efficiency of the CBD tag in the purification and immobilization processes of the recombinant enzyme on the supports. The kinetic parameters (V_{max} and k_{cat}) indicate that the β-galactosidases immobilized on the three supports are more efficient to hydrolyze milk lactose than to hydrolyze its pure substrates (lactose and ONPG). The β-galactosidase-CBD immobilized in an oriented way on microcrystalline cellulose and

nanocellulose obtained from alkaline hydrolysis was less inhibited by galactose in comparison with the commercial *Kluyveromyces lactis* β-galactosidase and the soluble β-galactosidase. The ions Na^+, K^+, Mg^{2+}, Cl^-, and SO_4^{2-} showed an inhibition effect on the enzymatic activity of β-galactosidases immobilized in an oriented way and recombinant soluble β-galactosidases on cellulosic supports. Cellulosic supports and obtained derivatives showed no cytotoxic effect on cell viability of HepG2 and Vero cultures. The three derivatives produced showed high operational stability in the hydrolysis of milk lactose and retained from 53 to 64% of their hydrolysis capacity after 40 reuse cycles. The processes of purification and oriented immobilization of the recombinant β-galactosidase via CBD-binding to micro and nanocellulosic materials developed in this study contribute to the development of sustainable and economically viable bioprocesses.

CRediT authorship contribution statement

- Adriano Gennari: Conceptualization, Investigation, Methodology, Formal Analysis, Writing-Original Draft Preparation, and Writing-Reviewing and Editing.
- Renate Simon: Investigation, and Methodology.
- Nathalia Denise de Moura Sperotto: Methodology, Formal Analysis, Writing-Original Draft Preparation, and Writing-Reviewing and Editing.
- Cristiano Valim Bizarro: Formal Analysis, and Writing-Original Draft Preparation.
- Luiz Augusto Basso: Formal Analysis, and Writing-Original Draft Preparation.
- Pablo Machado: Formal Analysis, and Writing-Original Draft Preparation.
- Edilson Valmir Benvenutti: Methodology, Formal Analysis, Writing-Original Draft Preparation, and Writing-Reviewing and Editing.
- Gaby Renard: Conceptualization, Methodology, Formal Analysis, Writing-Original Draft Preparation, and Writing-Reviewing and Editing.
- Jocelei Maria Chies: Resources, and Supervision.
- Giandra Volpato: Conceptualization, Formal Analysis, Resources, Supervision, Writing-Original Draft Preparation, and Writing-Reviewing and Editing.
- Claucia Fernanda Volken de Souza: Conceptualization, Investigation, Methodology, Formal Analysis, Resources, Supervision, Writing-Original Draft Preparation, and Writing-Reviewing and Editing.

Declaration of competing interest

The authors declare they do not have any conflicts of interest.

Acknowledgements

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijbiomac.2022.01.006.

References

[1] S. Haghju, M.R. Bari, M.A. Khaled-Abad, Affecting parameters on fabrication of β-D-galactosidase immobilized chitosan/poly (vinyl alcohol) electrospun nanofibers, Carbohydr. Polym. 200 (2018) 137–143, https://doi.org/10.1016/j.carbpol.2018.07.096.

[2] P. Torres, F. Batista-Viera, Improved biocatalysts based on Bacillus circulans β-galactosidase immobilized onto epoxy-activated acrylic supports: applications in whey processing, J. Mol. Catal. B Enzym. 83 (2012) 57–64, https://doi.org/10.1016/j.molcatb.2012.07.004.

[3] D. ZieliNska, D. Kolohyn-Krajewska, Food-origin lactic acid bacteria may exhibit probiotic properties: review, Biomed. Res. Int. 2018 (2018).

[4] C. Picard, J. Fioramonti, A. Francois, T. Robinson, F. Neant, C. Matuchansky, Review article: Bifidobacteria as probiotic agents - physiological effects and clinical benefits, Aliment. Pharmacol. Ther. 22 (2005) 495–512, https://doi.org/10.1111/j.1365-2036.2005.02615.x.

[5] S.C. Spohner, V. Schaum, H. Quitmann, P. Czermak, Kluyveromyces lactis: an emerging tool in biotechnology, J. Biotechnol. 222 (2016) 104–116, https://doi.org/10.1016/j.jbiotec.2016.02.023.

[6] M.H. Khani, M. Bagheri, Introducing a cost-effective method for purification of bioactive flagellin from several flagellated gram-negative bacteria, Protein Expr. Purif. 155 (2019) 48–53, https://doi.org/10.1016/j.pep.2018.11.007.

[7] G.L. Rosano, E.A. Ceccarelli, Recombinant protein expression in Escherichia coli: advances and challenges, Front. Microbiol. 5 (2014) 1–17, https://doi.org/10.3389/fmicb.2014.00172.

[8] A.I. Freitas, L. Domingues, T.Q. Aguiar, Tag-mediated single-step purification and immobilization of recombinant proteins toward protein-engineered advanced materials, J. Adv. Res. (2021), https://doi.org/10.1016/j.jare.2021.06.010.

[9] C. Oliveira, L. Domingues, Guidelines to reach high-quality purified recombinant proteins, Appl. Microbiol. Biotechnol. 102 (2018) 81–92, https://doi.org/10.1007/s00253-017-8623-8.

[10] S. Costa, A. Almeida, A. Castro, L. Domingues, Fusion tags for protein solubility, purification, and immunogenicity in Escherichia coli: the novel Fh8 system, Front. Microbiol. 5 (2014), https://doi.org/10.3389/fmicb.2014.00063.

[11] K.J. Chen, Y.T. Wu, C.K. Lee, Cellulose binding domain fusion enhanced soluble expression of fructosyl peptide oxidase and its simultaneous purification and immobilization, Int. J. Biol. Macromol. 133 (2019) 980–986, https://doi.org/10.1016/j.ijbiomac.2019.04.171.

[12] I. Levy, O. Shoseyov, Cellulose-binding domains: biotechnological applications, Biotechnol. Adv. 20 (2002) 191–213, https://doi.org/10.1016/S0734-9750(02)00006-X.

[13] D.M. Liu, J. Chen, Y.P. Shi, Advances on methods and easy separated support materials for enzymes immobilization, TrAC -Trends Anal. Chem. 102 (2018) 332–342, https://doi.org/10.1016/j.trac.2018.03.011.

[14] Z. Su, J. Luo, S.B. Sigurdardóttir, T. Manferrari, K. Jankowska, M. Pinelo, An enzymatic membrane reactor for oligodextran production: effects of enzyme immobilization strategies on dextranase activity, Carbohydr. Polym. 271 (2021), https://doi.org/10.1016/j.carbpol.2021.118430.

[15] J. Zdarta, A.S. Meyer, T. Jesionowski, M. Pinelo, A general overview of support materials for enzyme immobilization: characteristics, properties, practical utility, Catalysts 8 (2018), https://doi.org/10.3390/catal8020092.

[16] A. Żywicka, A. Banach, A.F. Junka, R. Drozd, K. Fijałkowski, Bacterial cellulose as a support for yeast immobilization – correlation between carrier properties and process efficiency, J. Biotechnol. 291 (2019) 1–6, https://doi.org/10.1016/j.jbiotec.2018.12.010.

[17] B.C. de Andrade, A. Gennari, G. Renard, B.D.R. Nervis, E.V. Benvenutti, T.M. H. Costa, S. Nicolodi, N.P. da Silveira, J.M. Chies, G. Volpato, C.F. Volken de Souza, Synthesis of magnetic nanoparticles functionalized with histidine and nickel to immobilize His-tagged enzymes using β-galactosidase as a model, Int. J. Biol. Macromol. 184 (2021) 159–169, https://doi.org/10.1016/j.ijbiomac.2021.06.060.

[18] C. Oliveira, V. Carvalho, L. Domingues, F.M. Gama, Recombinant CBM-fusion technology - applications overview, Biotechnol. Adv. 33 (2015) 358–369, https://doi.org/10.1016/j.biotechadv.2015.02.006.

[19] A. Bernardes, V.O.A. Pellegrini, F. Curtolo, C.M. Camilo, B.L. Mello, M.A. Johns, J. L. Scott, F.E.C. Guimaraes, I. Polikarpov, Carbohydrate binding modules enhance cellulose enzymatic hydrolysis by increasing access of cellulases to the substrate, Carbohydr. Polym. 211 (2019) 57–68, https://doi.org/10.1016/j.carbpol.2019.01.108.

[20] A. Gennari, R. Simon, B.C. de Andrade, L.F.Saraiva Macedo Timmers, V.L. Milani Martins, G. Renard, J.M. Chies, G. Volpato, C.F.Volken de Souza, Production of beta-galactosidase fused to a cellulose-binding domain for application in sustainable industrial processes, Bioresour. Technology. 326 (2021), 124747, https://doi.org/10.1016/j.biortech.2021.124747.

[21] A. Gennari, F.H. Mobayed, B. da Rolt Nervis, E.V. Benvenutti, S. Nicolodi, N.P. da Silveira, G. Volpato, C.F.Volken De Souza, Immobilization of β-galactosidases on magnetic nanocellulose: textural, morphological, magnetic, and catalytic properties, Biomacromolecules 20 (2019), https://doi.org/10.1021/acs.biomac.9b00285.

[22] C. Carrick, M. Ruda, B. Pettersson, P.T. Larsson, L. Wågberg, Hollow cellulose capsules from CO2 saturated cellulose solutions - their preparation and characterization, RSC Adv. 3 (2013) 2462–2469, https://doi.org/10.1039/c2ra22020b.

[23] R.A. Sheldon, S. van Pelt, Enzyme immobilisation in biocatalysis: why, what and how, Chem. Soc. Rev. 42 (2013) 6223–6235, https://doi.org/10.1039/c3cs60075k.

[24] A. Hill, S. Karboune, C. Mateo, Investigating and optimizing the immobilization of levansucrase for increased transfructosylation activity and thermal stability, Process Biochem. 61 (2017) 63–72, https://doi.org/10.1016/j.procbio.2017.06.011.

[25] R. Rech, C.F. Cassini, A. Secchi, M.A.Z. Ayub, Utilization of protein-hydrolyzed cheese whey for production of β-galactosidase by Kluyveromyces marxianus, J. Ind. Microbiol. Biotechnol. 23 (1999) 91–96, https://doi.org/10.1038/sj.jim.2900692.

[26] M.M. Bradford, A rapid and sensitive method for the quantitation microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem. 72 (1976) 248–254.

[27] J. Sambrook, E. Fritsch, T. Maniatis, Molecular Cloning: A Laboratory Manual, 1989. Second, New York.

[28] P.A. Webb, C. Orr, Analytical Methods in Fine Particle Technology, Micromeritics Ins. Corp., 1997.

[29] A. Gennari, F.H. Mobayed, G. Volpato, C.F.V. de Souza, Chelation by collagen in the immobilization of Aspergillus oryzae β-galactosidase: a potential biocatalyst to hydrolyze lactose by batch processes, Int. J. Biol. Macromol. 109 (2018) 303–310, https://doi.org/10.1016/j.ijbiomac.2017.12.088.

[30] Y. Liu, Z. Wu, X. Zeng, P. Weng, X. Zhang, C. Wang, A novel cold-adapted phospho-beta-galactosidase from Bacillus velezensis and its potential application for lactose hydrolysis in milk, Int. J. Biol. Macromol. 166 (2021) 760–770, https://doi.org/10.1016/j.ijbiomac.2020.10.233.

[31] M.de F.M.de Freitas, L.C. Hortêncio, T.L. de Albuquerque, M.V.P. Rocha, L.R. B. Gonçalves, Simultaneous hydrolysis of cheese whey and lactulose production catalyzed by β-galactosidase from Kluyveromyces lactis NRRL Y1564, Bioprocess Biosyst. Eng. 43 (2020) 711–722, https://doi.org/10.1007/s00449-019-02270-y.

[32] J. van Meerloo, G.J.L. Kaspers, J. Cloos, Cell sensitivity assays: the MTT assay, in: Cancer Cell Culture: Methods and Protocols, 2011, pp. 237–245, https://doi.org/10.1385/1592594069.

[33] G. Repetto, A. del Peso, J.L. Zurita, Neutral red uptake assay for the estimation of cell viability/cytotoxicity, Nat. Protoc. 3 (2008) 1125–1131, https://doi.org/10.1038/nprot.2008.75.

[34] M.H.G. Canteri, L. Moreno, G. Wosiacki, A.P. Scheer, Pectin: from raw material to the final product, Polímeros 22 (2012) 149–157, https://doi.org/10.1590/S0104-14282012005000024.

[35] J. Singthong, S.W. Cui, S. Ningsanond, H. Douglas Goff, Structural characterization, degree of esterification and some gelling properties of Krueo Ma Noy (Cissampelos pareira) pectin, Carbohydr. Polym. 58 (2004) 391–400, https://doi.org/10.1016/j.carbpol.2004.07.018.

[36] A. Potthast, T. Rosenau, R. Buchner, T. Röder, G. Ebner, H. Bruglachner, H. Sixta, P. Kosma, The cellulose solvent system N, N-dimethylacetamide/lithium chloride revisited: the effect of water on physicochemical properties and chemical stability, Cellulose 9 (2002) 41–53, https://doi.org/10.1023/A:1015811712657.

[37] L. Lu, S. Xu, R. Zhao, D. Zhang, Z. Li, Y. Li, M. Xiao, Synthesis of galactooligosaccharides by CBD fusion β-galactosidase immobilized on cellulose, Bioresour. Technol. 116 (2012) 327–333, https://doi.org/10.1016/j.biortech.2012.03.108.

[38] Z. Wang, J. Qi, T.C. Hinkley, S.R. Nugen, J.M. Goddard, Recombinant lactase with a cellulose binding domain permits facile immobilization onto cellulose with retained activity, Food Bioprod. Process. 126 (2021) 207–214, https://doi.org/10.1016/j.fbp.2021.01.010.

[39] K.V. Kumar, C. Valenzuela-Calahorro, J.M. Juarez, M. Molina-Sabio, J. Silvestre-Albero, F. Rodriguez-Reinoso, Hybrid isotherms for adsorption and capillary condensation of N2 at 77K on porous and non-porous materials, Chem. Eng. J. 162 (2010) 424–429, https://doi.org/10.1016/j.cej.2010.04.058.

[40] D. Trache, M.H. Hussin, C.T. Hui Chuin, S. Sabar, M.R.N. Fazita, O.F.A. Taiwo, T. M. Hassan, M.K.M. Haafiz, Microcrystalline cellulose: isolation, characterization and bio-composites application-a review, Int. J. Biol. Macromol. 93 (2016) 789–804, https://doi.org/10.1016/j.ijbiomac.2016.09.056.

[41] N.K. Verma, N. Raghav, Comparative study of covalent and hydrophobic interactions for α-amylase immobilization on cellulose derivatives, Int. J. Biol. Macromol. 174 (2021) 134–143, https://doi.org/10.1016/j.ijbiomac.2021.01.033.

[42] G. Bayramoglu, B. Filiz Senkal, M. Yilmaz, M. Yakup Arica, Immobilization and stabilization of papain on poly(hydroxyethyl methacrylate-ethylenglycol dimethacrylate) beads grafted with epoxy functional polymer chains via surface-initiated-atom transfer radical polymerization (SI-ATRP), Bioresour. Technol. 102 (2011) 9833–9837, https://doi.org/10.1016/j.biortech.2011.08.042.

[43] M.L. Verma, C.J. Barrow, J.F. Kennedy, M. Puri, Immobilization of β-d-galactosidase from Kluyveromyces lactis on functionalized silicon dioxide nanoparticles: characterization and lactose hydrolysis, Int. J. Biol. Macromol. 50 (2012) 432–437, https://doi.org/10.1016/j.ijbiomac.2011.12.029.

[44] Z. Zhang, F. Zhang, L. Song, N. Sun, W. Guan, B. Liu, J. Tian, Y. Zhang, W. Zhang, Site-directed mutation of β-galactosidase from Aspergillus candidus to reduce galactose inhibition in lactose hydrolysis, 3Biotech 8 (2018), https://doi.org/10.1007/s13205-018-1418-5.

[45] A. Arsalan, M.F. Alam, S.F.Farheen Zofair, S. Ahmad, H. Younus, Immobilization of β-galactosidase on tannic acid stabilized silver nanoparticles: a safer way towards its industrial application, Spectrochim. Acta A Mol. Biomol. Spectroscpy. 226 (2020), 117637, https://doi.org/10.1016/j.saa.2019.117637.

[46] C. Mateo, J.M. Palomo, G. Fernandez-Lorente, J.M. Guisan, R. Fernandez-Lafuente, Improvement of enzyme activity, stability and selectivity via immobilization techniques, Enzym. Microb. Technol. 40 (2007) 1451–1463, https://doi.org/10.1016/j.enzmictec.2007.01.018.

[47] P.K.A. Jagtap, S.K. Verma, N. Vithani, V.S. Bais, B. Prakash, Crystal structures identify an atypical two-metal-ion mechanism for uridyltransfer in GlmU: its significance to sugar nucleotidyl transferases, J. Mol. Biol. 425 (2013) 1745–1759, https://doi.org/10.1016/j.jmb.2013.02.019.

[48] M.J. Page, E. di Cera, Role of Na+ and K+ in enzyme function, Physiol. Rev. 86 (2006) 1049–1092, https://doi.org/10.1152/physrev.00008.2006.

[49] S. Lo, M.L. Dugdale, N. Jeerh, T. Ku, N.J. Roth, R.E. Huber, Studies of Glu-416 variants of β-galactosidase (E. coli) show that the active site Mg2+ is not important for structure and indicate that the main role of Mg2+ is to mediate optimization of active site chemistry, Protein J. 29 (2010) 26–31, https://doi.org/10.1007/s10930-009-9216-x.

[50] M. Khan, Q. Husain, Effect of metal ions present in milk on the structure and functional integrity of native and polyaniline chitosan nanocomposites bound β-galactosidase: a multi-spectroscopic approach, Food Chem. 279 (2019) 312–320, https://doi.org/10.1016/j.foodchem.2018.12.022.

[51] W. Zhang, A. Abbaspourrad, D. Chen, E. Campbell, H. Zhao, Y. Li, Q. Li, D.A. Weitz, Osmotic pressure triggered rapid release of encapsulated enzymes with enhanced activity, Adv. Funct. Mater. 27 (2017) 1–7, https://doi.org/10.1002/adfm.201700975.

[52] M. Khan, Q. Husain, N. Ahmad, Elucidating the binding efficacy of β-galactosidase on polyaniline–chitosan nanocomposite and polyaniline–chitosan–silver nanocomposite: activity and molecular docking insights, J. Chem. Technol. Biotechnol. 94 (2019) 837–849, https://doi.org/10.1002/jctb.5831.

[53] M. Dioguardi, D. Perrone, G. Troiano, L. Laino, F. Ardito, F. Lauritano, M. Cicciù, L. lo Muzio, Cytotoxicity evaluation of five different dual-cured resin cements used for fiber posts cementation, Int. J. Clin. Exp. Med. 8 (2015) 9327–9333.