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One-step purification of a recombinant beta-galactosidase using magnetic cellulose as a support: Rapid immobilization and high thermal stability

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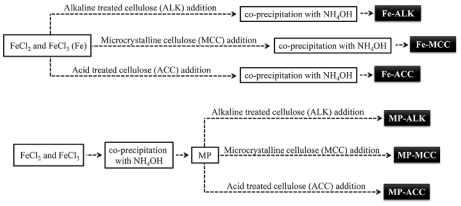
HIGHLIGHTS

- One-step purification and targeted immobilization process of a β-galactosidase.
- These bioprocesses were carried out efficiently on the magnetic cellulose supports.
- Derivatives showed a typical response of a predominantly superparamagnetic system.
- Immobilization process on magnetic supports increased the enzyme thermal stability.
- The materials used in the immobilization processes did not show a cytotoxic effect.

GRAPHICAL ABSTRACT



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ABSTRACT

For the first time, this work reported the one-step purification and targeted immobilization process of a β-galactosidase (Gal) with the Cellulose Binding Domain (CBD) tag, by binding it to different magnetic cellulose supports. The process efficiency after β-galactosidase-CBD immobilization on magnetic cellulose-based supports showed values of approximately 90% for all evaluated enzymatic loads. Compared with free Gal, derivatives showed affinity values between β-galactosidase and the substrate 1.2 × higher in the lactose hydrolysis of milk. β-Galactosidase-CBD's oriented immobilization process on supports increased the thermal stability of the

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immobilized enzyme by up to 7 × . After 15 cycles of reuse, both enzyme preparations showed a relative hydrolysis percentage of 50% of lactose in milk. The oriented immobilization process developed for purifying recombinant proteins containing the CBD tag enabled the execution of both steps simultaneously and quickly and the obtention of β-galactosidases with promising catalytic characteristics for application in the food and pharmaceutical industries.

1. Introduction

Tag-oriented immobilization processes enable obtaining high-stability biocatalysts, increased catalytic activation, reduced enzymatic activity inhibition, and protein purification, in addition to facilitating biocatalyst recovery (Fan et al., 2018; Sheldon and van Pelt, 2013). Furthermore, enzymes immobilized on magnetic supports are easier to separate from the reaction medium by applying a magnetic field, improving the operational performance of this recovery process (Gennari et al., 2019a, Gennari et al., 2019b). In this context, magnetic particles represent a class of materials increasingly used in the targeted immobilization of recombinant enzymes (De Andrade et al., 2021).

β-Galactosidase (lactase, EC 3.2.1.23) is an important commercial enzyme used in the food and pharmaceutical industry to remove lactose from dairy products, making them consumable for people with lactose intolerance (Guerrero et al., 2019; You et al., 2017). Purification processes are still considered a limitation in the large-scale production of β-galactosidases (Yu and Liu, 2012, Zhang et al., 2019). However, the recombinant production of these enzymes containing CBD can reduce these difficulties (Gennari et al., 2021).

Several molecular biology techniques have been used to produce recombinant enzymes, among which the inclusion of tags stands out. These tags are widely used for protein purification through affinity chromatography (Rezaei et al., 2017). The main limitations of this chromatographic purification process include the high cost of affinity resins, low yield, and reduced enzymatic activity due to dilution (Fan et al., 2018).

Cellulose Binding Domain (CBD) tag incorporation can be a strategy for one-step purification and immobilization of proteins on cellulose-based supports (Gennari et al., 2020; Lu et al., 2012). Thus, it is possible to overcome the above mentioned limitations of purification processes, mainly due to the low cost of obtaining cellulose (Chen et al., 2019). Furthermore, the synthesis of nanocellulose increases this biopolymer's immobilization process efficiency due to its high surface area (Gennari et al., 2020).

In continuation to our previous study (Gennari et al., 2021), the present work aimed to develop a one-step purification and targeted immobilization process of a β-galactosidase (Gal) from *Kluyveromyces* sp. with the CBD tag by binding it to different magnetic cellulose supports. The soluble purified enzyme and biocatalysts obtained were characterized according to catalytic, kinetic and thermal parameters. In addition, the immobilized enzyme's operational stability during the hydrolysis of skim milk lactose through a batch process was investigated. This study introduces a one-step purification strategy that could be useful for many industrial bioprocesses.

2. Materials and methods

2.1. Materials

Immobilization experiments were carried out using a crude enzymatic extract with β-galactosidase-CBD (Gennari et al., 2021). ONPG, activated factor X (Xa) from bovine plasma, and microcrystalline cellulose (MCC) were acquired from Sigma-Aldrich® (Missouri, US). The other substances used were analytical-grade reagents (Sigma-Aldrich®, Missouri, US).

2.2. Methods

2.2.1. Synthesis of magnetic cellulose supports

Microcrystalline cellulose (MCC) was used as a cellulosic matrix to support β-galactosidase-CBD immobilization. This material was subjected to alkaline (ALK) and acid (ACC) treatments. Subsequently, two methods were used to obtain the magnetic particles: (i) by mixing the previously obtained cellulosic matrix with iron salts (Fe) and carrying out the joint co-precipitation of this suspension; (ii) by coating the magnetic particles (MP) co-precipitated using only iron salts with the cellulosic matrices.

MCC magnetic particles (Fe-MCC) were synthesized using an adaptation of the methodology described by Hassan et al. (2019): 1.80 g of $FeCl_2$ were added to 4.86 g of $FeCl_3$ in 100 mL of ultrapure water containing 1 g of MCC and incubated under a N_2 atmosphere and constant magnetic stirring for 2 min. Then the mixture was heated at 85 °C for 5 min, 40 mL of NH_4OH (30% v/v) were added, and the resulting solution was kept under magnetic stirring for 60 min. The production of nanocellulose-based MP obtained through alkaline hydrolysis (Fe-ALK) was based on the combination of synthesis processes reported by Gennari et al. (2019a); Gennari et al. (2019b) and Hassan et al. (2019). Initially, alkaline cellulose (ALK) was obtained by following the treatment described by Gennari et al. (2019a); Gennari et al. (2019b). Then, using a N_2 atmosphere, 1 g of ALK was mixed with $FeCl_2$ (1.80 g) and $FeCl_3$ (4.86 g), and this mixture was magnetically stirred for 2 min. The mixture was heated for 5 min at 85 °C, 40 mL of NH_4OH (30% v/v) were added, and the synthesized Fe-ALK particles were kept under magnetic stirring for 60 min. The preparation of nanocellulose-based magnetic particles obtained by acid hydrolysis (Fe-ACC) was performed using the methods described by Gennari et al. (2019a); Gennari et al. (2019b) and Huang et al. (2018). To do so, 4 g of MCC were mixed with 100 mL of HCl 6 M and heated at 90 °C under magnetic stirring for 90 min. The solution was cooled in an ice bath and centrifuged (Hettich®, Universal 320R, Germany) (2790 × *g*, 4 °C, 2 min). The acid hydrolyzed cellulose (ACC) was washed with ultrapure water. Then, under a N_2 atmosphere, 1 g of ACC was mixed with 100 mL of iron salts solution (1.80 g of $FeCl_2$ and 4.86 g of $FeCl_3$). To form Fe-ACC particles, the mixture was heated for 5 min at 85 °C, 40 mL of NH_4OH (30% v/v) were added, and the resulting suspension was kept under magnetic stirring for 60 min.

MP-MCC synthesis was performed by coating MPs with MCC according to the method adapted from Cao et al. (2017). First, the magnetic iron particles were prepared as described above. The mixture remained under magnetic stirring for 60 min. At the end of this period, MPs were separated using a magnet and washed with ultrapure water. 150 mg of MP was dispersed in 30 mL of 7% NaOH (w/v) and 12% urea (w/v), and this suspension was kept for 1 h at −20 °C. Then 500 mg of MCC were added to the above suspension, and the mixture was again kept for 1 h at −20 °C. After that, 150 mL of ultrapure water were added to the MP-MCC. The MP-ALK and MP-ACC magnetic cellulose particles were synthesized following the methodologies described by Gennari et al. (2019a); Gennari et al. (2019b). The MP-ALK particles were produced by mixing MP and cellulose treated with NaOH and urea. For the preparation of MP-ACC, cellulose was hydrolyzed with HCl and then mixed with magnetic particles.

At the end of all the support synthesis processes (Fe-MCC, Fe-ALK, Fe-ACC, MP-MCC, MP-ALK, and MP-ACC), the magnetic cellulose particles were separated from the solution using a magnet, washed three times with ultrapure water, and oven-dried at 60 °C (Ethik

Technology®, 403-3D, Brazil) for 12 h.

2.2.2. Immobilizing recombinant β-galactosidase-CBD on magnetic cellulose

The targeted immobilization processes of β-galactosidase-CBD (Gal) included the incubation of 6 mL of the immobilization solution (20 to 60 $U_{enzyme}/g_{support}$ in a 50 mM sodium phosphate buffer solution with 3 mM of $MgCl_2$, pH 7.0) containing 100 mg of synthesized magnetic cellulose supports (Fe-MCC, Fe-ALK, Fe-ACC, MP-MCC, MP-ALK, and MP-ACC). The solutions were kept under rocking and rolling motion (Didática SP®, Roller Mixer MRII, Brazil) of 100 rpm at 25 °C for 60 min. During this process, samples of the supernatant (at 0, 15, 30, and 60 min) were periodically collected to monitor the immobilization yield (Sheldon and van Pelt, 2013). At the end of the process, the β-galactosidase-CBD immobilized on cellulose was separated using a magnet. The derivatives obtained (Fe-MCC-Gal, Fe-ALK-Gal, Fe-ACC-Gal, MP-MCC-Gal, MP-ALK-Gal, and MP-ACC-Gal) were washed 5 × with 6 mL of Tris-HCl buffer (50 mM, pH 7.5) and analyzed to determine efficiency and recovered activity, as proposed by Sheldon and Van Pelt (2013). All experiments were carried out in triplicate. Statistical verification of the immobilization parameters was performed through a one-way analysis of variance (ANOVA). Fisher's F-test determined the significance of the model. In the significant models, the Tukey Test (with a significance level of 0.05 (p-value < 0.05)) was performed to compare the means. The SPSS® Statistics 26.0 software was used for the statistical analysis.

To obtain β-galactosidase in its free form, a cleavage sequence was added to construct the recombinant protein (Gennari et al., 2021). Thus, the enzyme immobilized on magnetic cellulose was incubated in a drying oven (Ethik Technology®, 403-3D, Brazil) at a ratio 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 × *g*, 4 °C, 5 min), and the free enzyme (free Gal) was separated from the solution supernatant for characterization.

2.2.3. Analytical determinations

β-Galactosidase-CBD's enzymatic activity was determined using the ONPG substrate at 41 °C for 1 min according to Gennari et al. (2021). The protein profile of the crude enzyme extract containing β-galactosidase-CBD, the supernatant after immobilizations with Fe-ALK and MP-MCC, the Fe-ALK-Gal and MP-MCC-Gal derivatives, and the free Gal were evaluated using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE 12% w/v).

2.2.4. Textural, morphological, and magnetic evaluation of magnetic cellulose supports and produced derivatives

The textural characterization of the supports based on magnetic cellulose (Fe-ALK and MP-MCC) and of the derivatives obtained (Fe-ALK-Gal and MP-MCC-Gal) was performed using N_2 adsorption–desorption isotherms at liquid N_2 boiling temperature using a Micromeritics device (Micromeritics®, Tristar II Kr 3020, USA) according to (Gennari et al., 2019a, Gennari et al., 2019b). The structural morphology and the Energy Dispersive Spectroscopy (EDS) of the supports (Fe-ALK and MP-MCC) and of the derivatives (Fe-ALK-Gal and MP-MCC-Gal) was obtained using a Scanning Electronic Microscope with Field Emission (SEM-FE) (FEI®, Inspect F50, Japan) (Gennari et al., 2019a, Gennari et al., 2019b). The magnetic properties of the magnetic cellulose preparations (Fe-ALK and MP-MCC) and of the produced derivatives (Fe-ALK-Gal and MP-MCC-Gal) were evaluated using a vibrating sample magnetometer (VSM) (MicroSense®, EZ9, USA) (De Andrade et al., 2021).

2.2.5. Characterization of the catalytic properties of free and immobilized β-galactosidases

2.2.5.1. Determining the reaction conditions of pH and temperature and kinetic parameters. The optimal pH and temperature values for the activity of free (free Gal) and immobilized (Fe-ALK-Gal and MP-MCC-Gal) β-galactosidases were determined using the Central Composite Design (CCD) methodology (see supplementary materials) as per Gennari et al. (2021). The effect of different temperatures (25 to 75 °C) and pH (5 to 9) on the catalytic activity of enzyme preparations was evaluated. The experiments were designed and analyzed using the Statistica 13.1 software (Dell Statistica®, USA). The kinetic parameters (Michaelis constant (K_M), maximum reaction speed (V_{max}), and catalytic specificity constants (k_{cat})) of free and immobilized β-galactosidases were determined using the Michaelis-Menten model and the Lineweaver-Burk linearization method by varying the concentration 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 lactose) (Gennari et al., 2018; Liu et al., 2021). All substrate solutions were prepared in a sodium phosphate buffer solution (50 mM with 3 mM of $MgCl_2$, pH 7.5). The reactions with ONPG were conducted for 1 min at 41 °C, as described in item 2.2.3. The hydrolysis of lactose solutions was conducted for 5 min at 41 °C, with subsequent determination of glucose concentration using a glucose oxidase kit (Labtest®, Minas Gerais, Brazil). Kinetic parameters were analyzed using ANOVA, and the significance of the model was determined by Fisher's F-test. In the significant models, the Tukey Test (with a significance level of 0.05 (p-value < 0.05)) was performed to compare the means. The SPSS® Statistics 26.0 software was used for the statistical analysis.

2.2.5.2. Determining the effect of the presence of galactose and ions. To evaluate galactose's effect on the catalysis of free (free Gal) and immobilized (Fe-ALK-Gal and MP-MCC-Gal) (30 U) enzymes, enzyme activity was analyzed (item 2.2.3) in the presence of different concentrations of galactose (1 to 5%, w/v) in the ONPG solution (Shafi et al., 2021). In order to evaluate the effect of galactose on the activity of an industrially used enzyme, the commercial β-galactosidase from *Kluyveromyces lactis* (30 U) (Prozyn®, Lactomax Pure, Brazil) was subjected to the same conditions. The effect of the presence of ions on the activity of free (free Gal) and immobilized β-galactosidases (Fe-ALK-Gal and MP-MCC-Gal) was evaluated as follows: (i) for 1 min under reaction conditions to determine enzyme activity, and (ii) for 30 min in a Tris-HCl buffer solution (50 mM, pH 7.5) (De Freitas et al., 2020; Gennari et al., 2021). In (i), the test was conducted using 10 to 20 U of β-galactosidases with the ONPG substrate (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) containing different cation concentrations (of 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). The solution also contained different anion concentrations (magnesium salts): Cl^- (8 and 16 mM), $(SO_4)^{2-}$ (8 and 16 mM) and $(PO_4)^{3-}$ (21 and 42 mM). The activity of β-galactosidase in the absence of ions was used as control (100%) to determine the enzyme's relative activity in the presence of ions. In this condition (ii), the assay was conducted with 10 and 20 U of β-galactosidases incubated for 30 min at 25 °C in Tris-HCl buffer (50 mM, pH 7.5), containing the different concentrations of ions described above. After that, the enzymatic activity of each solution was determined by following the methods described in item 2.2.3.

2.2.5.3. Determining thermal, storage and operational stabilities. To evaluate the thermal stability of free (free Gal) and immobilized (Fe-ALK-Gal and MP-MCC-Gal) β-galactosidases, enzymatic preparations (2 U) were incubated in a thermostated bath (Marconi®, MA 156, Brazil) at temperatures of 55, 60, 65, and 70 °C. Aliquots were collected periodically to determine the residual enzyme activity. The kinetic inactivation

constant (k), the half-life time ($t_{1/2}$), and the stabilization factor (SF) of β-galactosidases were calculated according to Gennari et al. (2018). The storage stability (4 °C) was evaluated by incubating free and immobilized enzymes (15 U) in 50 mM of Tris-HCl buffer (pH 7.5) for 90 days. Samples were collected periodically to determine enzyme activity. The activity of each β-galactosidase in the first days of storage was used as control (100%) to calculate the residual activity. The reusability of β-galactosidase immobilized on magnetic cellulose (200 mg) was evaluated in batch process using the hydrolysis reaction of lactose from skim milk (5% w/v lactose) (10 mL) following the methodology reported by Gennari et al. (2018).

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

The cytotoxicity of the synthesized magnetic supports (Fe-ALK and MP-MCC), derivatives (Fe-ALK-Gal and MP-MCC-Gal, 10 and 20 U/mL), and the free Gal (10 and 20 U/mL) was determined by cell viability of the HepG2 and Vero cultures employing two different methods: the Methyl Thiazol Tetrazolium (MTT) and neutral red (NR) uptake assays (van Meerloo et al., 2011; Repetto et al., 2008). The cell cultures were grown in Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen, US) and 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 were incubated overnight. Before incubation, support and enzyme samples were solubilized in 6 M H_2SO_4, and the mixture was neutralized with 12 M NaOH until pH 7.0. The solutions of solubilized samples were incubated with the cell lines for 72 h at 37 °C under 5% CO_2.

For the MTT assay, the cultures were incubated (72 h at 37 °C under 5% CO_2) with an MTT reagent (2 mg/mL) for 4 h, and the absorbance was measured at 570 nm using an EZ Read 400 microplate reader (Biochrom®, US). The 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, the cells were washed with Phosphate-Buffered Saline (PBS) before the addition of 200 μL of NR dye solution (25 μg/mL) prepared in a serum-free medium, and the plate was incubated for additional 3 h at 37 °C under 5% CO_2. The 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 the viable cells. Absorbance was measured at 562 nm using an EZ Read 400 microplate reader (Biochrom®, US). Cell viability was expressed as a percentage, considering the vehicle control cell as 100% cell viability. Statistical analysis was performed using ANOVA followed by Dunnett's Multiple Comparison Test post-test using GraphPad Prism 9 (San Diego, US).

3. Results and discussion

3.1. Immobilizing β-galactosidase-CBD on magnetic cellulose

Table 1 shows the results obtained in the targeted immobilization of β-galactosidase-CBD on different magnetic cellulose supports after 15 min of reaction. The yield, efficiency, and recovered activity results did not significantly increase (data not shown) between the immobilization times evaluated (15, 30, and 60 min). The highest yields (~63%) were obtained with loads of 20 and 30 U/$g_{support}$. The reduction in the immobilization parameters with the increase in the enzymatic load is probably associated with the enzymatic saturation of the support surface and changes in the enzyme tridimensional structure after immobilization (Neto et al., 2021; Sheldon and van Pelt, 2013). Efficiency after β-galactosidase-CBD immobilization in the six supports, and for all the evaluated enzymatic loads, was approximately 90%.

The immobilization yield indicated that the characteristics of the supports, produced with different methods, caused different interactions between the β-galactosidase-CBD and the magnetic cellulose particles. Cellulose forms (MCC, ALK, and ACC) have distinct structural characteristics due to the treatments used for their preparations. These treatments generate functional groups with different interaction possibilities with β-galactosidase-CBD (Bahrami and Hejazi, 2013). CBD is formed by sequences rich in proline and hydroxyproline amino acids, which enable the recombinant enzyme to bind to cellulose (Zhang et al., 2019). After the fast binding of the β-galactosidase CBD tag with magnetic cellulose, probably other interactions were formed, such as hydrogen bonds between the supports' hydroxyl groups with the enzyme's amine groups, and between the cellulose hydrogen bond network with carboxyl groups of the β-galactosidase amino acid side chains (Huang et al., 2011).

Based on the results in the present study (Table 1), the Fe-ALK and MP-MCC magnetic cellulose supports showed the best results in the immobilization parameters. Therefore, Fe-ALK-Gal and MP-MCC-Gal derivatives, with a load of 30 U/g $_{support}$, were selected for characterization assays.

Fig. 1 shows the crude enzymatic extract's protein profile, the

Table 1
Immobilization parameters of β-galactosidase-CBD using different activity loads on magnetic cellulose supports after 15 min of immobilization.

Derivative	Immobilization Parameter	Activity Load (U/$g_{support}$)				
		20	30	40	50	60
Fe-MCC-Gal	Yield (%)	56.62 ± 3.64 ^{bcd}	56.30 ± 3.53 ^{bcd}	46.66 ± 3.58 ^{efg}	38.98 ± 2.53 ^{gh}	30.97 ± 3.72 ^{ij}
	Efficiency (%)	91.62 ± 3.26 ^{a}	92.17 ± 2.70 ^{a}	86.43 ± 3.40 ^{a}	90.15 ± 2.67 ^{a}	91.24 ± 1.66 ^{a}
	Activity Recovery (%)	51.91 ± 4.60 ^{bcd}	51.87 ± 2.96 ^{bcd}	40.36 ± 4.01 ^{efghi}	35.10 ± 1.23 ^{ghijk}	28.22 ± 2.87 ^{jkl}
Fe-ALK-Gal	Yield (%)	70.04 ± 4.07 ^{a}	64.40 ± 4.17 ^{ab}	46.48 ± 2.60 ^{efg}	39.30 ± 2.04 ^{gh}	32.01 ± 3.51 ^{hij}
	Efficiency (%)	90.68 ± 3.40 ^{a}	90.62 ± 4.48 ^{a}	92.67 ± 3.56 ^{a}	90.39 ± 2.31 ^{a}	85.87 ± 4.09 ^{a}
	Activity Recovery (%)	63.61 ± 6.12 ^{a}	58.23 ± 0.93 ^{ab}	43.02 ± 1.60 ^{defgh}	35.50 ± 0.93 ^{ghijk}	27.58 ± 4.33 ^{kl}
Fe-ACC-Gal	Yield (%)	50.01 ± 2.77 ^{def}	48.20 ± 1.91 ^{defg}	41.91 ± 2.45 ^{fgh}	33.54 ± 1.96 ^{hi}	23.85 ± 3.50 ^{j}
	Efficiency (%)	89.48 ± 3.96 ^{a}	92.66 ± 2.42 ^{a}	90.11 ± 1.51 ^{a}	86.18 ± 2.58 ^{a}	87.61 ± 3.96 ^{a}
	Activity Recovery (%)	44.82 ± 4.43 ^{cdefg}	44.70 ± 2.94 ^{cdefg}	37.79 ± 2.83 ^{fghij}	28.87 ± 0.87 ^{jkl}	20.98 ± 4.01 ^{l}
MP-MCC-Gal	Yield (%)	64.51 ± 3.96 ^{ab}	62.65 ± 3.56 ^{abc}	51.80 ± 4.45 ^{cde}	40.15 ± 3.44 ^{gh}	36.18 ± 3.35 ^{hi}
	Efficiency (%)	91.54 ± 2.92 ^{a}	92.39 ± 3.61 ^{a}	90.55 ± 2.08 ^{a}	90.48 ± 2.28 ^{a}	89.13 ± 4.30 ^{a}
	Activity Recovery (%)	58.97 ± 1.79 ^{ab}	57.97 ± 5.46 ^{ab}	46.96 ± 5.09 ^{cdef}	36.38 ± 4.01 ^{ghijk}	32.15 ± 1.46 ^{ijk}
MP-ALK-Gal	Yield (%)	54.48 ± 4.07 ^{bcde}	53.07 ± 1.28 ^{cde}	46.54 ± 2.38 ^{efg}	36.83 ± 1.82 ^{hi}	29.65 ± 2.00 ^{ij}
	Efficiency (%)	90.71 ± 2.20 ^{a}	87.52 ± 1.78 ^{a}	90.17 ± 3.55 ^{a}	89.46 ± 3.16 ^{a}	90.66 ± 2.23 ^{a}
	Activity Recovery (%)	49.36 ± 2.61 ^{bcde}	46.46 ± 2.05 ^{cdef}	42.02 ± 3.77 ^{defgh}	32.98 ± 2.78 ^{ijk}	26.86 ± 1.17 ^{kl}
MP-ACC-Gal	Yield (%)	56.76 ± 3.53 ^{bcd}	60.05 ± 4.12 ^{bc}	47.10 ± 2.51 ^{efg}	37.94 ± 3.53 ^{ghi}	28.93 ± 3.41 ^{ij}
	Efficiency (%)	90.64 ± 2.16 ^{a}	88.93 ± 4.50 ^{a}	89.71 ± 4.90 ^{a}	89.57 ± 3.11 ^{a}	89.90 ± 3.43 ^{a}
	Activity Recovery (%)	51.49 ± 4.18 ^{bcd}	53.52 ± 6.39 ^{bc}	42.20 ± 1.87 ^{defgh}	33.91 ± 1.96 ^{hijk}	26.09 ± 4.08 ^{kl}

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.

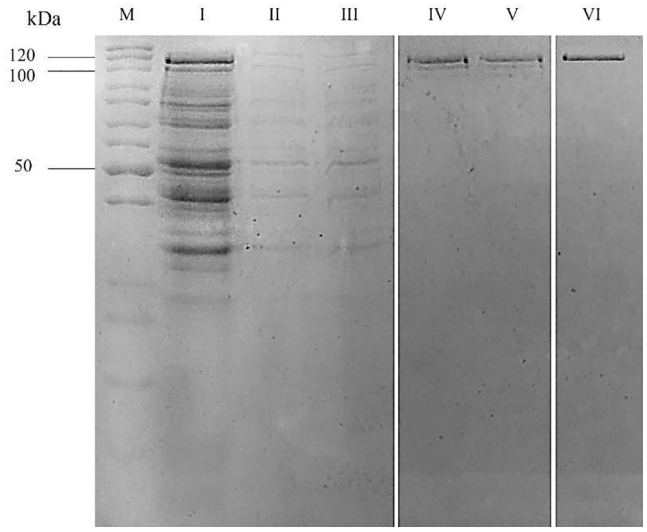

Fig 1. Polyacrylamide gel electrophoresis of the proteins present in different solutions before and after immobilization on cellulose M: Page Ruler molecular weight marker; I: crude enzyme extract; II and III: supernatant after immobilization with Fe-ALK and MP-MCC, respectively; IV and V: Fe-ALK-Gal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

supports Fe-ALK and MP-MCC's immobilization supernatants, the Fe-ALK-Gal and MP-MCC-Gal derivatives, and the free Gal obtained by cleaving the MP-MCC-derivative Gal with activated factor X. It shows the presence of β-galactosidase-CBD (~120 kDa) in the crude enzyme extract (I), in the derivatives (IV and V), and in the soluble enzyme sample (VI). The enzyme's targeted immobilization was efficiently performed on both magnetic cellulose supports tested, resulting in a reduction in the enzyme band's intensity in the solution supernatant (II and III). The strong presence of β-galactosidase-CBD in Fe-ALK-Gal and MP-MCC-Gal derivatives (IV and V) evidenced the one-step immobilization and purification of enzyme. The results shown in Fig. 1, together with the rapid binding of the recombinant protein to different cellulose supports (15 min), demonstrate the functionality and high specificity of the CBD tag.

Peprah Addai et al. (2020) purified a β-galactosidase from *Lactobacillus* sp. B164 fused to an elastin-like polypeptide (ELP) sequence and modified with 6x-Histidine, employing inverse thermal cycling (ITC) and nickel-nitrilotriacetic acid (Ni-NTA) resin. Through ITC, they obtained the pure enzyme with better catalytic properties, compared to the commercial Ni-NTA resin system. Although affinity chromatography is considered a promising technique to purify recombinant proteins, this system still has limitations for the purification of enzymes on an industrial scale (Fan et al., 2018; Fang et al., 2018).

Some of the advantages of CBD compared to other tags used in the immobilization and purification of β-galactosidases (Liu et al., 2021; Lu et al., 2012) include high binding affinity, low cost of inputs used in enzyme purification, simultaneous and fast immobilization and purification of β-galactosidase, and a simple application process.

3.2. Textural, morphological, and magnetic evaluation of magnetic cellulose supports and their derivatives

The values of surface area and pore volume of magnetic cellulose supports (Fe-ALK and MP-MCC) and their derivatives with recombinant β-galactosidase (Fe-ALK-Gal and MP-MCC-Gal) were obtained through adsorption and desorption isotherms. Fe-ALK particles had higher surface area values (101 m^2/g) than MP-MCC particles (94 m^2/g). As verified by de Andrade et al. (2021), the supports' micropores (diameter < 20 nm) contributed to the materials' high surface area. After β-galactosidase-CBD immobilization, surface area (12 to 25%) and pore volume (18 to 41%) decreased. For Fe-ALK-Gal and MP-MCC-Gal particles, the maximum values of the pore diameter distribution curves (see supplementary materials) moved to smaller sizes. These variations in isotherms and micropore size distribution are the result of incorporating the enzyme into the cellulose particles, covering their surface and blocking access to the pores. Average surface area values of approximately 90 m^2/g were obtained in this study.

Fe-ALK, MP-MCC, Fe-ALK-Gal, and MP-MCC-Gal particles were observed by SEM-FE with a magnification of 100,000 × . The supports' morphology did not change after immobilization with β-galactosidase-CBD. Fe-ALK, MP-MCC, Fe-ALK-Gal, and MP-MCC-Gal particles showed spherical shapes with diameters of 92.03, 88.47, 94.65, and 91.81 nm, respectively. SEM-FE images showed that all preparations formed blocks of agglomerates. This characteristic can be attributed to the preparation of samples for the images and to particles' natural attraction as a function of magnetism (Gennari et al., 2019a, Gennari et al., 2019b).

Regarding the elementary composition of the magnetic particles used as supports, iron was the main component, with approximately 33% and 26% in Fe-ALK and MP-MCC particles, respectively. The presence of carbon and oxygen was observed in all samples as they are present in cellulose. The percentage of Fe reduced after the immobilization of β-galactosidase-CBD due to carbon and oxygen incorporation from the protein structures.

Fig. 2A shows the magnetization curves of supports (Fe-ALK and MP-MCC) and derivatives (Fe-ALK-Gal and MP-MCC-Gal), showing the responsiveness of these samples to the applied magnetic field. The saturation magnetization (M_S) of Fe-ALK and MP-MCC magnetic cellulose supports was 32.51 and 31.13 emu/g, respectively. After β-galactosidase-CBD immobilization, M_S decreased to 30.03 emu/g in Fe-ALK-Gal and 28.58 emu/g in MP-MCC-Gal. The results showed a typical response of a predominantly superparamagnetic system (John and Mathew, 2019), with low coercivity in all tested samples (0.19 to 4.16 kOe). Based on these results and due to the presence of narrow hysteresis cycles (Fig. 2A), the nanoparticles of the supports and derivatives were classified as soft (Netto et al., 2013). The immobilization of β-galactosidase-CBD on magnetic cellulose supports did not affect the materials' magnetic response. The small reductions observed can be attributed to the increase in the mass of enzyme molecules (non-magnetic) in relation to the magnetic supports' mass. These results demonstrate that β-galactosidase-CBD immobilized on magnetic cellulose can be quickly separated from the reaction medium through a magnetic field. This is a promising feature for the application of the enzyme in large-scale lactose hydrolysis processes.

3.3. Determining the reaction conditions of pH and temperature and kinetic parameters of free and immobilized β-galactosidase

The investigation of optimum pH and temperature values for catalysis with free and immobilized enzymes was carried out using CCD, with pH varying from 5 to 9 and the temperature from 25 to 75 °C. For the enzymatic activity of Fe-ALK-Gal and MP-MCC-Gal derivatives, the linear regression coefficient of the pH variable (x_1) was significant (p-value < 0.05). As for the enzymatic activity of free Gal and its derivatives (Fe-ALK-Gal and MP-MCC-Gal), the second-order regression coefficients of pH ($x_1.x_1$) and temperature ($x_2.x_2$) showed significance (p-value < 0.05), indicating that small changes in the values of these variables produce significant changes in the activity of free and immobilized β-galactosidase. Furthermore, the regression coefficient of the interaction between pH and temperature ($x_1.x_2$) showed significance (p-value < 0.05) in the activity of free Gal. These differences observed between the free Gal and β-galactosidase-CBD derivatives may be due to a change in the microenvironment's dielectric constant generated after the bonds to magnetic celluloses, modifying the acid constants of basic or acid groups of the immobilized enzyme (Alptekin et al., 2010).

The significance values for the obtained models of free Gal, Fe-ALK-

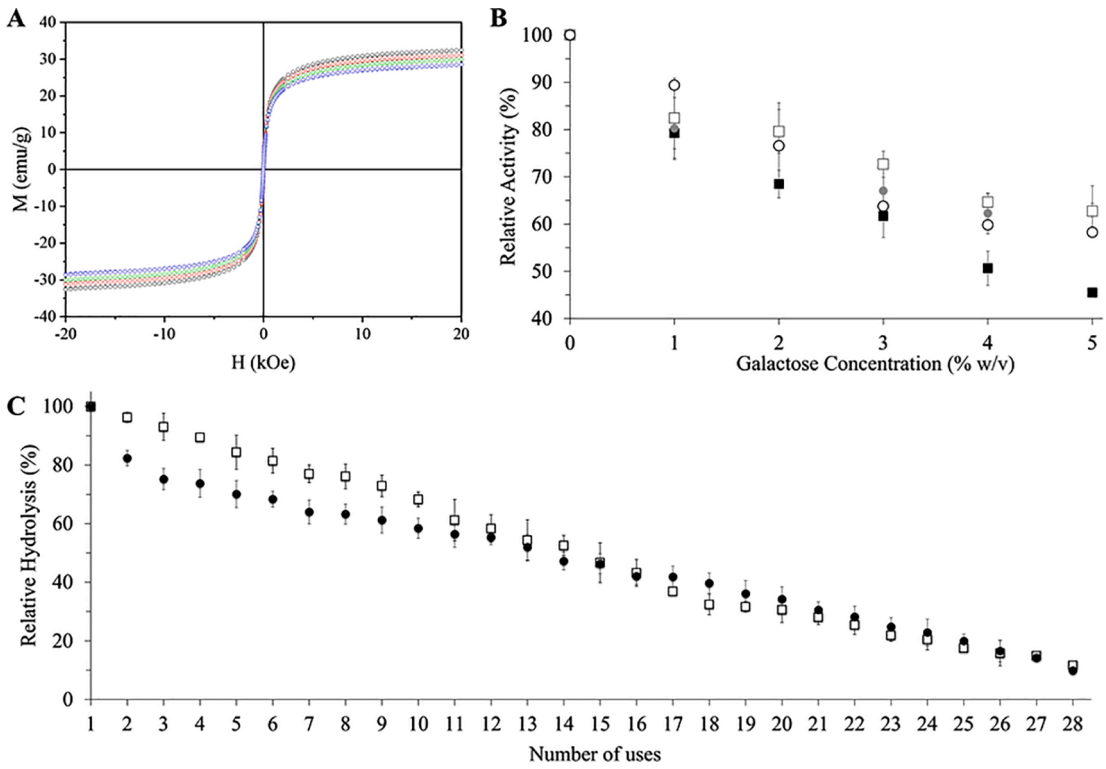

Fig 2. (A) Magnetization curves obtained using a vibrating sample magnetometer of the magnetic cellulose supports (○) Fe-ALK and (○) MP-MCC, and the derivatives after the immobilization of β-galactosidase-CBD (○) Fe-ALK-Gal and (○) MP-MCC-Gal; (B) Effect of different galactose concentrations (% w/v) on β-galactosidase activities: (■) free Gal, (○) Commercial *K. lactis* β-galactosidase, (●) Fe-ALK-Gal, (□) MP-MCC-Gal; (C) Reuse of β-galactosidase immobilized on magnetic cellulose supports: (□) Fe-ALK-Gal, (●) MP-MCC-Gal.

Gal, and MP-MCC-Gal enzymatic activity were p-value < 0.0360, p-value < 0.0088, and p-value < 0.0018, respectively. The coefficients of determination (R^2) ranged from 0.86 to 0.96. Based on the models, the response surfaces of free and immobilized enzymes were obtained (see supplementary materials), which demonstrates the relationship between enzymatic activity and the assessed pH and temperature reaction conditions. From the optimized mathematical models (see supplementary materials), the optimum pH and temperature values were determined for the activities of free and immobilized enzymes and the respective predicted values of enzymatic activity. In order to validate the models, the activities of free and immobilized β-galactosidases on magnetic cellulose supports were carried out under the optimum conditions of pH and temperature (see supplementary materials). The results showed a similarity of approximately 90% between the theoretical values predicted by the models for the activities of β-galactosidases (free Gal, Fe-ALK-Gal, and MP-MCC-Gal) and those determined experimentally.

The optimal pH values for free and immobilized enzymes were similar, maintaining their optimal biocatalysis range at values close to 7.0, indicating that the recombinant β-galactosidase from *Kluyveromyces* sp. immobilization did not change this operating parameter (Gennari et al., 2019a, Gennari et al., 2019b). The optimum temperature of the MP-MCC derivative was approximately 5 °C higher than the temperatures of the other enzymatic preparations (free Gal and Fe-ALK-Gal). According to Jordan et al. (2011), changes in pH and temperature for maximum enzymatic activity can be attributed to the different interactions formed, mainly hydrogen bonds, after the enzyme immobilization to cellulose by the CBD tag.

Determination kinetic characteristics of the β-galactosidase-CBD reaction helps increase the enzymatic efficiency in the lactose hydrolysis processes. Thus, enzyme catalysis in ONPG, lactose, and skim milk solutions were studied, and the results are shown in Table 2. Free Gal, Fe-ALK-Gal, and MP-MCC-Gal in the synthetic chromogenic substrate (ONPG) hydrolysis reaction showed no significant difference ($p < 0.05$)

Table 2
Kinetic parameters, using different substrate solutions, of free and immobilized β-galactosidases on magnetic cellulose supports.

	K_M (mM)	V_{max} (mM/min)	k_{cat} (min^{-1})
ONPG			
Free Gal	11.04 ± 2.22 [a]	42.83 ± 2.88[b]	3.95 ± 0.54 [a]
Fe-ALK-Gal	11.13 ± 2.10 [a]	43.04 ± 3.35[b]	3.31 ± 0.29 [a]
MP-MCC-Gal	15.18 ± 1.82 [a]	53.63 ± 3.40 [a]	3.55 ± 0.19 [a]
Lactose			
Free Gal	72.40 ± 1.34[b]	640.70 ± 4.16 [a]	8.85 ± 0.15 [a]
Fe-ALK-Gal	72.92 ± 0.88[b]	534.90 ± 2.63[b]	7.34 ± 0.06[b]
MP-MCC-Gal	82.22 ± 0.45 [a]	612.13 ± 2.86 [a]	7.44 ± 0.02[b]
Milk			
Free Gal	259.57 ± 5.35 [a]	593.40 ± 6.22 [a]	2.29 ± 0.03[c]
Fe-ALK-Gal	147.83 ± 2.18[c]	521.20 ± 4.97 [a]	3.53 ± 0.02 [a]
MP-MCC-Gal	209.50 ± 5.82[b]	541.00 ± 4.06 [a]	2.58 ± 0.02[b]

in the Michaelis constant (K_M) and the catalytic constant (k_{cat}). Regarding V_{max}, the MP-MCC-Gal derivative showed the best results ($p < 0.05$), approximately 1.3 × higher than free Gal and Fe-ALK-Gal. In the lactose hydrolysis reaction (5% w/v solution), the MP-MCC-Gal derivative showed the lowest affinity between β-galactosidase and the substrate ($p < 0.05$). This effect may have been caused by changes in the microenvironment of the active site after the enzyme bound to MP-MCC (Ricardi et al., 2021). In contrast, the lowest maximum speed was observed for the Fe-ALK-Gal derivative. This variation of the kinetic parameters K_M and V_{max}, resulted in higher values of k_{cat} for free Gal.

Regarding the lactose hydrolysis reactions in skim milk (5% w/v of lactose), Fe-ALK-Gal and MP-MCC-Gal derivatives showed affinity values between β-galactosidase and the substrate 1.8 and 1.2 × higher ($p < 0.05$) than free Gal, respectively. As a consequence of higher K_M values, the recombinant β-galactosidase immobilized on Fe-ALK and MP-MCC supports showed a significantly higher catalytic constant ($p <$

0.05), indicating that more lactose molecules in milk are converted into glucose and galactose when the enzyme is bound to magnetic cellulose particles. K_M values in the hydrolysis processes in lactose solution and milk increased approximately 3.5, 2.0, and 2.6 × for free Gal, Fe-ALK-Gal, and MP-MCC-Gal, respectively. This variation is probably related to harder interaction between lactose in milk and the active site of enzymes due to the other components present in this food matrix (Neto et al., 2021).

Regarding V_{max} values in the hydrolysis reactions of the three evaluated substrates, β-galactosidase-CBD under targeted immobilization on magnetic cellulose supports showed similar or superior values compared to free Gal, indicating that the use of nanometric supports helps disperse the derivative in the reaction medium. In contrast, Gennari et al. (2018), Ricardi et al. (2021), and Wahba (2016) reported a reduction in the V_{max} of the hydrolysis reaction catalyzed by immobilized β-galactosidase due to precipitation of the support, limiting enzyme diffusion in the reaction medium.

3.4. Determining the effect of the presence of galactose and ions on the activity of free and immobilized β-galactosidases

Considering that galactose, one of the products of the lactose hydrolysis reaction, acts as a competitive inhibitor for the active site of β-galactosidase (Husain et al., 2011), the effect of this monosaccharide was evaluated on free and immobilized enzyme activity (Fig. 2B). The activity of soluble β-galactosidase-CBD was primarily affected in the presence of galactose at all concentrations evaluated. The immobilization of β-galactosidase-CBD on Fe-ALK and MP-MCC supports reduced this inhibitory effect, showing relative activity values 20% higher compared to free Gal, at the concentration of 5% of galactose. Overall, immobilized enzymes showed a lower inhibition by the product than the tested commercial *K. lactis* β-galactosidase. According to Mateo et al. (2007), the conformational changes in the enzyme, due to its binding to the support, reduce its contact with the inhibitory compound. In addition, the microenvironment created with CBD may have contributed to reduce the inhibitory effect of galactose.

β-Galactosidase is an enzyme primarily used in the hydrolysis of lactose from milk and dairy products, which have several mineral salts in their composition, such as Na^+, K^+, Mg^{2+}, and Ca^{2+}. Thus, evaluating the effect of these ions on enzymatic reactions enables an improvement in the catalytic efficiency. Therefore, the enzymatic activities of free and immobilized β-galactosidases after 30 min of contact with the enzymes were evaluated in the presence of the ions Na^+, K^+, Mg^{2+}, Ca^{2+}, Cl^-, $(SO_4)^{2-}$ and $(PO_4)^{3-}$. Na^+, K^+, Mg^{2+}, Cl^-, $(SO_4)^{2-}$ and $(PO_4)^{3-}$ increased the activity of free Gal and derivatives (Fe-ALK-Gal and MP-MCC- Gal). The addition of Mg^{2+} in the biocatalysis provided the most significant activating effect, increasing the activity of β-galactosidases by up to 24%. The Ca^{2+} ion, as already reported by Liu et al. (2021), showed a repressive effect on the hydrolysis reaction catalyzed by β-galactosidases, regardless of the enzymatic concentrations. The differences observed between the ions' effects may be related to their interactions with the enzyme's positively or negatively charged regions, which can attract or repulse peptide chains, changing the conformation of the active site of the enzymes (Karimi Alavijeh et al., 2020).

3.5. Determining thermal, storage and operational stability

The stability of free and immobilized β-galactosidases at high temperatures was evaluated to investigate the enzyme applicability on an industrial scale (see supplementary materials). The targeted immobilization process of β-galactosidase-CBD on magnetic cellulose supports (Fe-ALK and MP-MCC) reduced the thermal inactivation constant, increased half-life values, and increased thermal stability by up to 7×, evidenced by the stabilization factor. The derivative MP-MCC-Gal showed greater stability with increasing temperature, indicating that the interactions of β-galactosidase-CBD with the support MP-MCC provided a more significant protective effect due to the increased resistance to enzyme splits during heat treatment (Banjanac et al., 2016). Estevinho et al. (2018) immobilized β-galactosidase on bacterial cellulose membranes containing a carbohydrate-binding module (CBM). They found that structural changes induced by the enzyme's bond to cellulose and the different microenvironments formed from this interaction contribute to enzymatic stability at high temperatures. In turn, CBD - despite its different structure from CBM - can also present protecting characteristics that increase the thermal resistance of β-galactosidase-CBD.

Considering the importance of storage stability in applying immobilized enzymes to large-scale bioprocesses, the enzymatic activities of free and immobilized β-galactosidases were determined over 90 days at 4 °C. Although free Gal showed high stability, maintaining approximately 70% and 40% of its initial activity after 45 and 90 days, respectively, targeted immobilization improved this property. Fe-ALK-Gal and MP-MCC-Gal derivatives maintained approximately 70% and 60% of their initial activities, respectively, after 90 days of storage. These results indicate that catalytic activity and enzyme binding to supports were probably not affected under the storage conditions. Therefore, the derivatives Fe-ALK-Gal and MP-MCC-Gal are promising for application in lactose hydrolysis bioprocesses.

The oriented immobilized β-galactosidases (Fe-ALK-Gal and MP-MCC-Gal) were used in sequential batch reactions for the hydrolysis of lactose in milk (Fig. 2C). Fe-ALK-Gal derivative showed greater operational stability up to the 10th reuse cycle. From this batch, the relative hydrolysis percentages were similar between both enzymatic preparations, with a hydrolytic efficiency of immobilized β-galactosidase of 50% after 15 cycles. Further reductions can be attributed to enzymatic inactivation, mass loss of the immobilized enzyme due to washings during the reuse processes, and detachment of the enzyme from the support (Eskandarloo and Abbaspourrad, 2018; Estevinho et al., 2018). Estevinho et al. (2018) evaluated the reuse potential of a β-galactosidase immobilized on cellulose membranes and found an approximate enzyme activity rate of 30% after 8 cycles.

The derivatives obtained in this study (Fe-ALK-Gal and MP-MCC-Gal) showed greater operational stability. The observed differences may be related to the cellulose forms used in both works, membranes (Estevinho et al., 2018), and magnetic particles in the present investigation.

3.6. Cytotoxic effect of supports and free and immobilized β-galactosidases

The cytotoxicity of the supports (Fe-ALK and MP-MCC) and the derivatives and free Gal at concentrations of 10 and 20 U/ml were evaluated in HepG2 and Vero cell cultures using the MTT and NR assays (Fig. 3). The supports developed for β-galactosidase-CBD immobilization did not change the viability of both cells in both methods evaluated. Cellulose magnetic particles did not show significant apoptotic or necrotic effects ($p < 0.05$) on cell cultures, probably due to the chemical characteristics of the materials used to obtain the cellulose magnetic particles (Chaabane et al., 2020; Gennari et al., 2020). In addition, β-galactosidase in its free form and in the derivatives also did not show toxicity. Therefore, the derivatives produced in this study can be considered non-toxic, allowing their application in different industries. Magnetic nanoparticles for applications in nanobiotechnology, nanomedicine, and enzyme immobilization also did not show toxicity, being considered biocompatible materials (Chaabane et al., 2020; Shafi et al., 2021).

4. Conclusions

This study presents a low-cost, sustainable, and efficient one-step process to immobilize and purify recombinant β-galactosidase on magnetic cellulose particles using the biospecific affinity of the CBD tag. The recombinant β-galactosidase, linked to both magnetic cellulose

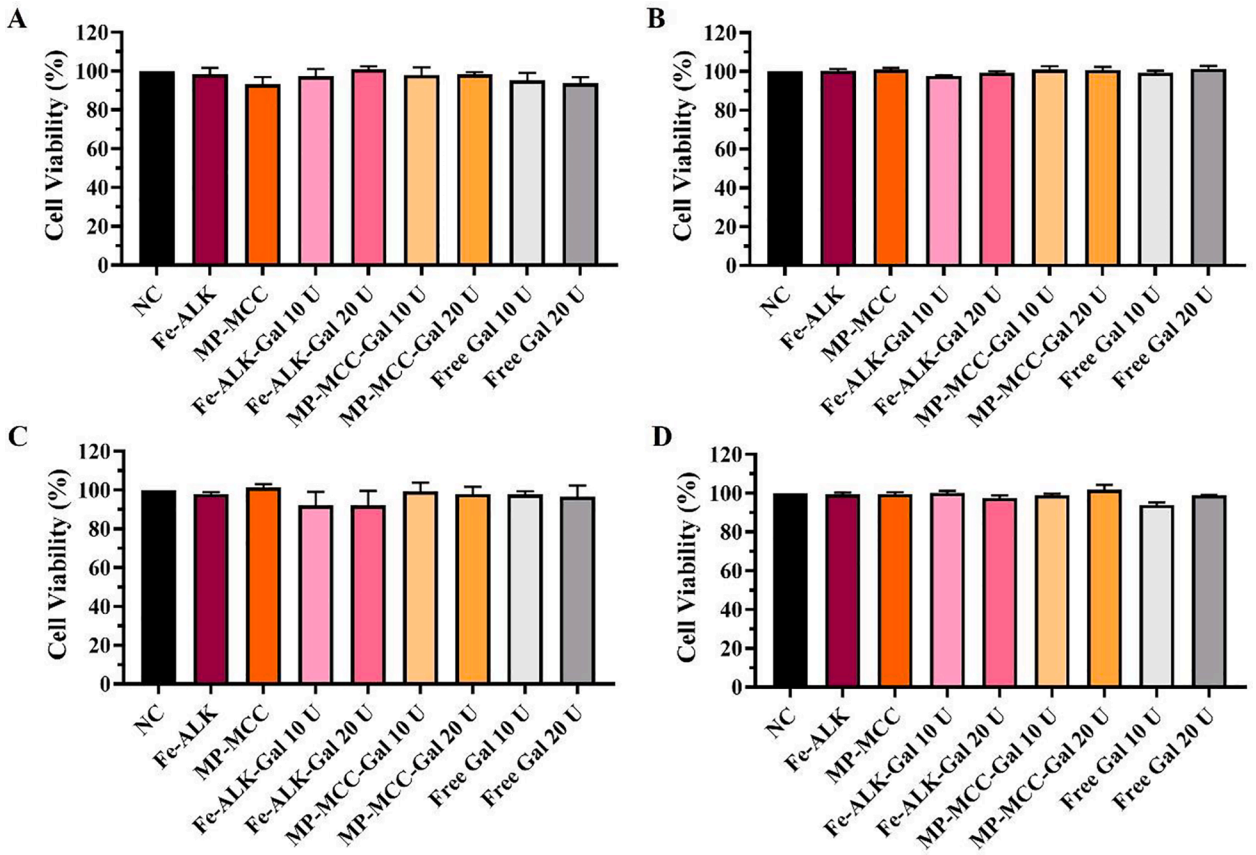

Fig. 3. Cytotoxic evaluation using MTT and NR assays from HepG2 and Vero cells treated with the produced supports, derivatives, and enzyme preparations. (A) HepG2 – MTT; (B) Vero – MTT; (C) HepG2 – NR, and (D) Vero – NR. NC: Negative control.

supports, presented catalytic, cytotoxic, and operational characteristics that allow its applicability in the food and pharmaceutical industry, considering the feasibility of separating the enzyme from the reaction medium and the low cost of obtaining the biocatalyst.

CRediT authorship contribution statement

Adriano Gennari: Conceptualization, Investigation, Methodology, Formal analysis, Writing – original draft, Writing – review & editing. **Renate Simon:** Investigation, Methodology. **Nathalia Denise de Moura Sperotto:** Methodology, Formal analysis, Writing – original draft, Writing – review & editing. **Cristiano Valim Bizarro:** Formal analysis, Writing – original draft. **Luiz Augusto Basso:** Formal analysis, Writing – original draft. **Pablo Machado:** Formal analysis, Writing – original draft. **Edilson Valmir Benvenutti:** Methodology, Formal analysis, Writing – original draft, Writing – review & editing. **Alexandre Da Cas Viegas:** Methodology, Formal analysis, Writing – original draft, Writing – review & editing. **Sabrina Nicolodi:** Methodology, Formal analysis, Writing – original draft, Writing – review & editing. **Gaby Renard:** Conceptualization, Methodology, Formal analysis, Writing – original draft, Writing – review & editing. **Jocelei Maria Chies:** Resources, Supervision. **Giandra Volpato:** Conceptualization, Formal analysis, Resources, Supervision, Writing – original draft, Writing – review & editing. **Claucia Fernanda Volken de Souza:** Conceptualization, Investigation, Methodology, Formal analysis, Resources, Supervision, Writing – original draft, Writing – review & editing.

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.biortech.2021.126497.

References

Peprah Addai, F., Wang, T., Kosiba, A.A., Lin, F., Zhen, R., Chen, D., Gu, J., Shi, H., Zhou, Y., 2020. Integration of elastin-like polypeptide fusion system into the expression and purification of *Lactobacillus sp.* B164 β-galactosidase for lactose hydrolysis. Bioresour. Technol. 311, 123513. https://doi.org/10.1016/j.biortech.2020.123513.

Karimi Alavijeh, M., Meyer, A.S., Gras, S., Kentish, S.E., 2020. The role of cations in regulating reaction pathways driven by *Bacillus circulans* β-galactosidase. Chem. Eng. J. 395, 125067. https://doi.org/10.1016/j.cej.2020.125067.

Alptekin, Ö., Tükel, S.S., Yıldırım, D., Alagöz, D., 2010. Immobilization of catalase onto Eupergit C and its characterization. J. Mol. Catal. B Enzym. 64 (3-4), 177–183.

Bahrami, A., Hejazi, P., 2013. Electrostatic immobilization of pectinase on negatively charged AOT-Fe_3O_4 nanoparticles. J. Mol. Catal. B. Enzym. 93, 1–7.

Banjanac, K., Carević, M., Ćorović, M., Milivojević, A., Prlainović, N., Marinković, A., Bezbradica, D., 2016. Novel β-galactosidase nanobiocatalyst systems for application in the synthesis of bioactive galactosides. RSC Adv. 6 (99), 97216–97225.

Cao, S.-L., Xu, H., Lai, L.-H., Gu, W.-M., Xu, P., Xiong, J., Yin, H., Li, X.-H., Ma, Y.-Z., Zhou, J., Zong, M.-H., Lou, W.-Y., 2017. Magnetic ZIF-8/cellulose/Fe_3O_4 nanocomposite: preparation, characterization, and enzyme immobilization. Bioresour. Bioprocess. 4, 56. https://doi.org/10.1186/s40643-017-0186-0.

Chaabane, L., Chahdoura, H., Mehdaoui, R., Snoussi, M., Beyou, E., Lahcini, M., V Baouab, M.H., 2020. Functionalization of developed bacterial cellulose with magnetite nanoparticles for nanobiotechnology and nanomedicine applications. Carbohydr. Polym. 247, 116707. https://doi.org/10.1016/j.carbpol.2020.116707.

Chen, K.-J., Wu, Y.-T., Lee, C.-K., 2019. Cellulose binding domain fusion enhanced soluble expression of fructosyl peptide oxidase and its simultaneous purification and immobilization. Int. J. Biol. Macromol. 133, 980–986.

De Andrade, B.C., Gennari, A., Renard, G., Nervis, B.D.R., Benvenutti, E.V., Costa, T.M. H., Nicolodi, S., da Silveira, N.P., Chies, J.M., Volpato, G., Volken de Souza, C.F., 2021. 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, 159–169. https://doi.org/10.1016/j.ijbiomac.2021.06.060.

de Freitas, M.de.F.M., Hortêncio, L.C., de Albuquerque, T.L., Rocha, M.V.P., Gonçalves, L.R.B., 2020. Simultaneous hydrolysis of cheese whey and lactulose production catalyzed by β-galactosidase from Kluyveromyces lactis NRRL Y1564. Bioprocess Biosyst. Eng. 43 (4), 711–722. https://doi.org/10.1007/s00449-019-02270-y.

Eskandarloo, H., Abbaspourrad, A., 2018. Production of galacto-oligosaccharides from whey permeate using β-galactosidase immobilized on functionalized glass beads. Food Chem. 251, 115–124.

Estevinho, B.N., Samaniego, N., Talens-Perales, D., Fabra, M.José., López-Rubio, A., Polaina, J., Marín-Navarro, J., 2018. Development of enzymatically-active bacterial cellulose membranes through stable immobilization of an engineered β-galactosidase. International Journal of Biological Macromolecules. Elsevier B.V. 115, 476–482.

Fan, Y., Miozzi, J., Stimple, S., Han, T.-C., Wood, D., 2018. Column-free purification methods for recombinant proteins using self-cleaving aggregating tags. Polymers (Basel). 10 (5), 468. https://doi.org/10.3390/polym10050468.

Fang, Y.M., Lin, D.Q., Yao, S.J., 2018. Review on biomimetic affinity chromatography with short peptide ligands and its application to protein purification. J. Chromatogr. A 1571, 1–15. https://doi.org/10.1016/j.chroma.2018.07.082.

Gennari, A., Führ, A.J., Volpato, G., Volken de Souza, C.F., 2020. Magnetic cellulose: Versatile support for enzyme immobilization - A review. Carbohydr. Polym. 246, 116646. https://doi.org/10.1016/j.carbpol.2020.116646.

Gennari, A., Mobayed, F.H., Catto, A.L., Benvenutti, E.V., Volpato, G., Volken de Souza, C.F., 2019a. *Kluyveromyces lactis* β-galactosidase immobilized on collagen: catalytic stability on batch and packed-bed reactor hydrolysis. React. Kinet. Mech. Catal. 127 (2), 583–599. https://doi.org/10.1007/s11144-019-01598-6.

Gennari, A., Mobayed, F.H., Da Rolt Nervis, B., Benvenutti, E.V., Nicolodi, S., da Silveira, N.P., Volpato, G., Volken de Souza, C.F., 2019b. Immobilization of β-Galactosidases on Magnetic Nanocellulose: Textural, Morphological, Magnetic, and Catalytic Properties. Biomacromolecules 20 (6), 2315–2326.

Gennari, A., Mobayed, F.H., Volpato, G., de Souza, C.F.V., 2018. 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, 303–310. https://doi.org/10.1016/j.ijbiomac.2017.12.088.

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

Guerrero, C., Valdivia, F., Ubilla, C., Ramírez, N., Gómez, M., Aburto, C., Vera, C., Illanes, A., 2019. Continuous enzymatic synthesis of lactulose in packed-bed reactor with immobilized Aspergillus oryzae B-galactosidase. Bioresour. Technol. 278, 296–302. https://doi.org/10.1016/j.biortech.2018.12.018.

Hassan, A., Sorour, N.M., El-Baz, A., Shetaia, Y., 2019. Simple synthesis of bacterial cellulose/magnetite nanoparticles composite for the removal of antimony from aqueous solution. Int. J. Environ. Sci. Technol. 16 (3), 1433–1448.

Huang, X.-J., Chen, P.-C., Huang, F., Ou, Y., Chen, M.-R., Xu, Z.-K., 2011. Immobilization of *Candida rugosa* lipase on electrospun cellulose nanofiber membrane. J. Mol. Catal. B Enzym. 70 (3-4), 95–100. https://doi.org/10.1016/j.molcatb.2011.02.010.

Huang, Z.X., Cao, S.L., Xu, P., Wu, H., Zong, M.H., Lou, W.Y., 2018. Preparation of a novel nanobiocatalyst by immobilizing penicillin acylase onto magnetic nanocrystalline cellulose and its use for efficient synthesis of cefaclor. Chem. Eng. J. 346, 361–368. https://doi.org/10.1016/j.cej.2018.04.026.

Husain, Q., Ansari, S.A., Alam, F., Azam, A., 2011. Immobilization of *Aspergillus oryzae* β galactosidase on zinc oxide nanoparticles via simple adsorption mechanism. Int. J. Biol. Macromol. 49 (1), 37–43.

John, S.P., Mathew, J., 2019. Superparamagnetism of Mg0.5Zn0.5Fe2O4 nanoparticles: Dependence of pH in the sol-gel auto-combustion method. AIP Conf. Proc. 2162, 1–7. https://doi.org/10.1063/1.5130276.

Jordan, J., Kumar, C.S.S.R., Theegala, C., 2011. Preparation and characterization of cellulase-bound magnetite nanoparticles. J. Mol. Catal. B Enzym. 68 (2), 139–146. https://doi.org/10.1016/j.molcatb.2010.09.010.

Liu, Y., Wu, Z., Zeng, X., Weng, P., Zhang, X., Wang, C., 2021. A novel cold-adapted phospho-beta-galactosidase from *Bacillus velezensis* and its potential application for lactose hydrolysis in milk. Int. J. Biol. Macromol. 166, 760–770.

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

Mateo, C., Palomo, J.M., Fernandez-Lorente, G., Guisan, J.M., Fernandez-Lafuente, R., 2007. Improvement of enzyme activity, stability and selectivity via immobilization techniques. Enzyme Microb. Technol. 40 (6), 1451–1463.

van Meerloo, J., Kaspers, G.J.L., Cloos, J., 2011. Cell Sensitivity Assays: The MTT Assay. Cancer Cell Culture: Methods and Protocols. 237–245.

Neto, C.A.C.Girão., Silva, N.Câmara.G.e., de Oliveira Costa, T., de Albuquerque, T.L., Gonçalves, L.R.B., Fernandez-Lafuente, R., Rocha, M.V.P., 2021. The β-galactosidase immobilization protocol determines its performance as catalysts in the kinetically controlled synthesis of lactulose. Int. J. Biol. Macromol. 176, 468–478.

Netto, C.G.C.M., Toma, H.E., Andrade, L.H., 2013. Superparamagnetic nanoparticles as versatile carriers and supporting materials for enzymes. J. Mol. Catal. B Enzym. 85–86, 71–92. https://doi.org/10.1016/j.molcatb.2012.08.010.

Repetto, G., del Peso, A., Zurita, J.L., 2008. Neutral red uptake assay for the estimation of cell viability/ cytotoxicity. Nat. Protoc. 3 (7), 1125–1131.

Rezaei, S., Shahverdi, A.R., Faramarzi, M.A., 2017. Isolation, one-step affinity purification, and characterization of a polyextremotolerant laccase from the halophilic bacterium *Aquisalibacillus elongatus* and its application in the delignification of sugar beet pulp. Bioresour. Technol. 230, 67–75.

Ricardi, Natália.C., Arenas, L.T., Benvenutti, E.V., Hinrichs, R., Flores, Elí.E.E., Hertz, P. F., Costa, T.M.H., 2021. High performance biocatalyst based on β-D-galactosidase immobilized on mesoporous silica/titania/chitosan material. Food Chem. 359, 129890. https://doi.org/10.1016/j.foodchem.2021.129890.

Shafi, A., Ahmed, F., Husain, Q., 2021. β-Galactosidase mediated synthesized nanosupport for the immobilization of same enzyme: Its stability and application in the hydrolysis of lactose. Int. J. Biol. Macromol. 184, 57–67.

Sheldon, R.A., van Pelt, S., 2013. Enzyme immobilisation in biocatalysis: why, what and how. Chem. Soc. Rev. 42 (15), 6223–6235. https://doi.org/10.1039/C3CS60075K.

Wahba, M.I., 2016. Treated calcium pectinate beads for the covalent immobilization of β-D-galactosidase. Int. J. Biol. Macromol. 91, 877–886.

You, S., Zhang, J., Yin, Q., Qi, W., Su, R., He, Z., 2017. Development of a novel integrated process for co-production of B-galactosidase and ethanol using lactose as substrate. Bioresour. Technol. 230, 15–23.

Yu, S.-Q., Liu, Y.-H., 2012. Expression and one-step purification of a β-galactosidase by fusion with elastin-like polypetides. Process Biochem. 47 (7), 1108–1114.

Zhang, L., Sun, Y., Wang, Y., Wang, X., Zhuang, H., Chen, C., Wang, P., 2019. Easy-cleaning surfaces functionalized with an interface-binding recombinant lipase. Colloids Surfaces B Biointerfaces 184, 110433. https://doi.org/10.1016/j.colsurfb.2019.110433.