Applied Biochemistry and Biotechnology (2019) 188:310–325
https://doi.org/10.1007/s12010-018-2905-1

One-Step Immobilization and Stabilization of a Recombinant *Enterococcus faecium* DBFIQ E36 L-Arabinose Isomerase for D-Tagatose Synthesis

Marylane de Sousa[1] · Vânia M. M. Melo[2] · Denise C. Hissa[2] · Ricardo M. Manzo[3] · Enrique J. Mammarella[3] · André Saraiva Leão Marcelo Antunes[4] · José L. García[5] · Benevides C. Pessela[6,7] · Luciana R. B. Gonçalves[1]

Received: 12 July 2018 / Accepted: 8 October 2018 /
Published online: 15 November 2018

Abstract
A recombinant L-arabinose isomerase from Enterococcus faecium DBFIQ E36 was immobilized onto multifunctional epoxide supports by chemical adsorption and onto a chelate-activated support via polyhistidine-tag, located on the N-terminal (N-His-L-AI) or on the C-terminal (C-His-L-AI) sequence, followed by covalent bonding between the enzyme and the support. The results were compared to reversible L-AI immobilization by adsorption onto charged agarose supports with improved stability. All the derivatives presented immobilization yields of above 75%. The ionic interaction established between agarose gels containing monoaminoethyl-*N*-aminoethyl structures (MANAE) and the enzyme was the most suitable strategy for L-AI immobilization in comparison to the chelate-activated agarose. In addition, the immobilized biocatalysts by ionic interaction in MANAE showed to be the most stable, retaining up to 100% of enzyme activity for 60 min at 60 °C and with K_m values of 28 and 218 mM for MANAE-N-His-L-AI and MANAE-C-His-L-AI, respectively.

Keywords L-Arabinose isomerase · Chelate-agarose · D-Tagatose · Enterococcus faecium · Immobilization · Enzyme activity

Introduction

D-Tagatose is a rare natural ketohexose that holds a GRAS status and shows great potential to serve as a replacement to D-sucrose in foods due to its similar flavor and sweetness, as well as to the lower caloric value, when compared to D-sucrose [1, 2]. These functional properties have

✉ Benevides C. Pessela
b.pessela@csic.es

✉ Luciana R. B. Gonçalves
lrg@ufc.br

Extended author information available on the last page of the article

stimulated the employment of D-tagatose as an anti-hyperglycemic, prebiotic, and cryoprotectant agent, and also as a drug for the treatment of type II diabetes [1–3].

Although tagatose can be chemically obtained from D-galactose [4], due to the several disadvantages inherent in this process, including by-product formation and chemical waste generation, the enzymatic route is preferred and L-arabinose isomerase (L-AI, EC 5.3.1.4) is the most commonly employed enzyme to this end [5]. There have been some attempts to produce tagatose in industrial quantities since 2002 but production is still modest and sells are concentrated in the USA and Europe. In 2012, Nutrilab NV, a subsidiary of the Belgian company Damhert, opened a facility near Rome (Italy) to manufacture 10,000 tons/year of tagatose. A two-step enzymatic process is the probable route for tagatose production, in which galactose is obtained by hydrolyzing whey lactose using β-galactosidase, and then galactose is isomerized by galactose isomerase, which is a genetically modified *Geobacillus stearothermophilus* arabinose isomerase [6]. However, this enzyme is not yet commercially available and hence, studies aiming to obtain this biocatalyst are necessary in order to expand dedicated industrial processes and achieve different markets, for instance in Latina America.

Although several immobilization strategies have been tested for L-AI from different bacteria [7–11], covalent immobilization strategies have showed the best results when compared to adsorption, entrapment, or encapsulation techniques. In this scenario, agarose is one of the most employed resins when aiming at enzyme immobilization, both at lab and industrial scales, due to their well-known physical and chemical properties such as robustness, reproducibility, and mechanical and chemical resistance [12–15]. Furthermore, the use of epoxy-activated supports, using epichlorohydrin as an activating agent, is of broad use due to the ability of easily obtaining supports with different activation degrees, to the variety of reaction conditions used and to the molecules (ligands and/or proteins) that can be immobilized onto these types of matrices [16, 17]. However, only a few studies concerning L-AI immobilization have employed both this matrix and epichlorohydrin for epoxy generation aiming to obtain insoluble L-AI biocatalysts [18].

In the present work, the use of different multifunctional activated epoxy supports for the immobilization of a recombinant *Enterococcus faecium* DBFIQ E36 L-arabinose isomerase is proposed. Enzyme immobilization onto these supports is conducted in two simultaneous steps, due to the presence of two different types of functional groups in the support. First, the enzyme is chemically adsorbed to the support by means of interaction between the histidines in the higher density region of the recombinant enzyme and the previously immobilized metal chelate present in the support, allowing an ideal orientation of the enzyme in relation to the support (IDA-Ni-agarose). Secondly, covalent bonds between the enzyme and the support are formed due to the interaction of the oxirane groups in the derivative with the exposed nucleophilic groups ($-NH_2$, -SH, -OH) of the enzyme, creating a covalent immobilization strategy for L-AI (IDA-Ni-glyoxyl-agarose) [12, 14, 17, 19–23]. Furthermore, this irreversible covalent immobilization strategy was compared to a reversible ionic adsorption of L-AI to MANAE-agarose supports. Immobilization parameters as well as thermal and pH stability and the kinetics constants of all biocatalysts were assessed and obtained.

Materials and Methods

Materials

Iminodiacetic acid (IDA), epichlorohydrin, sodium borohydride, 4B-CL and 6B-CL agarose, sodium (meta)periodate, monoaminoethyl-*N*-aminoethyl (MANAE), $CuSO_4$, $NiCl_2$, EDTA, ethylenediamine (EDA), bovine seroalbumin (BSA), carbazole, cysteine, sodium ampicillin, kanamycin sulfate, and sodium chloride were all from Sigma-Aldrich Co. (St. Louis, MI, USA). All the dehydrated culture media employed were purchased from Difco Laboratories (Detroit, MI, USA).

All experiments were performed in triplicates and the results are presented as the average of the values obtained, with standard deviations below 5%.

Obtaining the Recombinant L-Arabinose Isomerase

Recombinant crude extracts rich in *Enterococcus faecium* DBFIQ E36 L-arabinose isomerase (L-AI) were produced both from *E. coli* DH10B and *E. coli* BL21 (DE3) cells, and named N-His-L-AI and C-His-L-AI, respectively [24]. To this end, recombinant strains were grown in Terrific Broth (TB, Difco Laboratories, Detroit, MI, USA) added of 100 μg/mL ampicillin for *E. coli* DH10B and 50 μg/mL kanamycin for *E. coli* BL21, up to an optical density of 2.0 at 600 nm. Immediately after this step, 0.5% (*w*/*v*) L-arabinose and 1 mM IPTG were added as inducers to *E. coli* DH10B and *E. coli* BL21 (DE3), respectively, and incubated for 12 h at 30 °C for enzyme overproduction. The enzyme was then purified from the crude extracts through IMAC employing the methodology described by Sousa et al. [24].

In order to test different possibilities of host strains, promoters, and constructions, it was decided to develop the N- and the C-terminal His-tagged constructions of LAI in two different expression systems, since both systems and host strains worked properly and the production of both the N- and the C-terminal fusion proteins was achieved successfully and at good yields.

Enzyme Production

E. coli recombinant cells were usually cultured at 37 °C in LB media (Difco) containing ampicillin (100 μg mL^{-1}) for *E. coli* DH10B (pBAD-ARA) or kanamycin (50 μg mL^{-1}) for *E. coli* BL21 (DE3) (pET29-ARA). For enzyme production, the cultures were induced at a DO of 0.7–0.9 at 600 nm, containing 1 mM IPTG in the case of *E. coli* BL21 (DE3) (pET29-ARA) or 0.5% (*w*/*v*) L-arabinose in the case of *E. coli* DH10B (pBAD-ARA). The induced cells were incubated for 12 h at 37 °C under a 200-rpm orbital agitation speed. The cells were then harvested by centrifugation at 6000×*g* for 20 min at 4 °C, resuspended in 50 mM sodium phosphate buffer (pH 7.0), and disrupted by a French Press at 1100 lb/in^2. Following disruption, the cell debris was removed by centrifugation at 15,000×*g* for 20 min at 4 °C [24].

Preparation of Epoxy-Activated Agarose Supports

The 4% BCL agarose support was epoxy-activated using epichlorohydrin, as described by Bolivar et al. [16]. To this end, 100 g of filtered and vacuum-dried 4B-CL agarose (Sigma-Aldrich, St. Louis, MI, USA) was resuspended in 440 mL of distilled water and then, in the

following order, 160 mL of acetone, 32.8 g of NaOH, 2.0 g of $NaBH_4$ and 110 mL of epichlorohydrin were added. The suspension was gently stirred for 16 h at room temperature and then rinsed with distilled water in excess until it reached neutrality.

Preparation of Iminodiacetic Acid Supports

The preparation of IDA supports was carried out according to the methodology proposed by Pessela et al. and Armisén et al. [12, 19]. Briefly, 20 g of epoxy-activated agarose was suspended in 200 mL of 0.5 M IDA prepared in 0.1 M sodium carbonate/bicarbonate buffer. The pH of the solution was around 11 and it was gently stirred for 12 h at 25 °C. Subsequently, the IDA support was filtered, thoroughly rinsed with distilled water, and vacuum-dried immediately afterwards.

Preparation of Metal Chelate Supports

The preparation of metal chelate supports was done according to the methodology proposed by Pessela et al. [12]. Briefly, the IDA support was incubated in Milli-Q water containing either 5 mg/mL of $CuSO_4$ (support to solution ratio of 10 g of IDA support to 100 mL of $CuSO_4$ solution) or 50 mM sodium phosphate buffer (pH 6.0) containing 1.0 M NaCl and 5 mg/mL of $NiCl_2$ (support/solution/salt ratio of 10 g of IDA support to 100 mL of $NiCl_2$ solution and 0.1 g NaCl) and then, gently stirred for 2 h at 25 °C. Finally, the supports were washed thoroughly with distilled water and vacuum-dried.

Preparation of MANAE-Agarose Supports

The preparation of the MANAE-agarose support was performed according to the methodology proposed by Fernandez-Lafuente et al. with some modifications [25]. The epoxy-activated agarose support was oxidized with 1375 mL of a 100 mM sodium metaperiodate ($NaIO_4$) aqueous solution while the suspension was gently stirred for 90 min at room temperature. Then, the support was filtered, washed with 1 L of distilled water and vacuum-dried, thus producing the glyoxyl-agarose support [16]. Afterwards, 10 g of the glyoxyl-agarose derivative was suspended in 100 mL of 2% (*v*/*v*) ethylenediamine (EDA) solution at pH 8.5. After 2 h of gentle agitation, sodium borohydride ($NaBH_4$) was added to the suspension up to a final concentration of 10 mg/mL in solution and stirred for 2 h. Finally, the amino support was filtered and washed successively with 100 mL of 100 mM sodium acetate buffer (pH 5.0) added of 1 M NaCl, with 100 mL of 100 mM sodium carbonate/bicarbonate buffer (pH 10) added of 1 M NaCl and finally with 500 mL of distilled water [25].

Immobilization on Heterofunctional Supports

Immobilization onto the synthesized supports was performed by adding 1.0 g of IDA-Ni-epoxy agarose to 10 mL of a solution of recombinant L-arabinose isomerase prepared in 5 mM sodium phosphate buffer (pH 7.0) and incubated under agitation for 2 h at room temperature. Subsequently, the derivative was harvested, washed with distilled water, and vacuum-dried. The derivative was then added to a solution containing 100 mM carbonate/bicarbonate buffer (pH 10.0) with a mass/volume ratio of 1:10 to allow the establishment of the covalent bonds between the activated support and the target enzyme. For the formation of such bonds, the

reaction was allowed to run for 6 h. The support containing the covalently bound enzyme was previously washed with a 200 mM ethylenediamine tetraacetic acid (EDTA) solution for the removal of the metal bound to the agarose resin in order to prevent a possible reduction thereof, since such reaction can promote drawback interactions between the support and the His-tagged in the enzyme. The support reduction was performed with 1 mg/mL $NaBH_4$ solution for 15 min at 4 °C. Finally, the biocatalyst was washed with distilled water.

Immobilization on MANAE-Agarose Supports

MANAE-agarose (1.0 g) was added to 10 mL of a L-arabinose isomerase solution prepared in 5 mM sodium phosphate buffer (pH 7.0) with a mass/volume ratio of 1:10 and kept overnight (12 h) under continuous agitation. Then, the biocatalyst was harvested by filtration, washed with distilled water at room temperature, and stored at 4 °C until further use.

Protein Quantitation Assay

Protein concentration was determined according to Bradford et al. [26], using the Coomassie Blue G-250 dye. Bovine seroalbumin (BSA, Sigma) was used as the protein standard for building the calibration curve.

Enzyme Activity Assay

One hundred microliters of the immobilized L-arabinose isomerase suspension was placed in contact with 400 mM D-galactose (substrate), prepared in 50 mM sodium acetate buffer (pH 5.6) supplemented with 1 mM $MnCl_2$. The samples were incubated at 50 °C and the amount of D-tagatose was determined by measuring the absorbance at 560 nm via the cysteine-carbazole sulfuric acid method [27]. One unit (U) of L-AI activity was defined as the amount of enzyme required for the production of 1 μmol of D-tagatose per minute under the reaction conditions [28].

Polyacrylamide Gel Electrophoresis in Denaturing Conditions

In order to asses if the enzyme was covalently attached to the support, 0.07 g of each derivative was added to 100 μl of dissociation buffer containing 1% (*w*/*v*) of SDS and 2.5% of mercaptoethanol, and the suspension was boiled for 5 min. The non-covalently bound protein should desorb from the support to the supernatant. Afterwards, samples of the supernatant were analyzed by SDS-PAGE [29].

The determination of the protein profiling in the samples was carried out through a polyacrylamide gel electrophoresis employing sodium dodecyl sulfate as denaturing agent (SDS-PAGE). The gels were cast manually with a 6% T stacking zone and a 12.5% T separation zone, according to the method reported by Laemmli (1970) [30]. The SDS-PAGE was performed under constant voltage in a Bio-Rad equipment (model Mini-PROTEAN 3 Cell, USA), with a Bio-Rad PowerPack Basic and power supply, according to the following running conditions: 40 V until the dye tracker reached the separation zone and then 150 V for the remaining of the run. SDS-PAGE low-molecular-weight standards (14.4–97.0 kDa; GE Healthcare) were used for molecular weight estimation.

Immobilization Parameters

The immobilization of L-arabinose isomerase on several resins was assessed through the parameters defined by Silva et al. [31]. Briefly, immobilization yield (IY) is the percentage of enzyme activity from solution (free enzyme) that was immobilized (Eq. 1) and recovered activity (At_R) is the percentage of immobilized enzyme that remains active in the biocatalyst.

$$IY = \frac{At_i - At_f}{At_i} \times 100 \quad (1)$$

$$At_R = \frac{At_d}{At_i - At_f} \times 100 \quad (2)$$

where At_i and At_f are the initial and the final enzyme activity (U/mL) in the supernatant; At_d is the activity of the immobilized enzyme (U/g support).

Estimation of Kinetic Parameters of Insoluble Biocatalysts

The initial rates of conversion of D-galactose into D-tagatose were determined at 50 °C in 50 mM sodium acetate buffer (pH 5.6) with the addition of 1 mM $MnCl_2$. For this purpose, substrate concentrations from 0 to 800 mM of D-galactose were used and these reaction mixtures were incubated during 60 min. Assuming a Michaelis-Menten approach, the kinetic parameters (K_m and V_{max}) were calculated by the nonlinear fit of the initial rates obtained as a function of D-galactose concentration and using the Origin software (v8.1, OriginLab Corporation, Northampton, USA).

Temperature and pH Stability Profiles of Insoluble Biocatalysts

The thermal stability of L-AI was assessed by determining the remaining enzyme activity at 50 and 60 °C for 60 min. Samples were periodically withdrawn at different time points and immediately placed into a cold ice bath (0 °C). Then, the isomerase activity was measured through the production of D-tagatose, as described above. Relative activity curves as a function of time were built using Origin and half-lives ($t_{1/2}$) were determined according to a first-order enzyme deactivation model [32]. For the determination of the L-AI stability to pH, the recombinant enzyme was incubated in different buffer systems at room temperature for 2 h using a final concentration of 50 mM of sodium acetate buffer (pH 5.6), sodium phosphate buffer (pH 6.0, 7.0, and 8.0) and sodium bicarbonate buffer (pH 10.0), added of 1 mM $MnCl_2$ as a cofactor.

Results and Discussion

L-AI Immobilization on Different Agarose Supports

Recombinant L-AI enzymes have a polyhistidine (6×) tag located on their N-terminal sequence in N-His-L-AI and on the C-terminal, in the case of C-His-L-AI. These were immobilized on IDA-Ni-agarose supports by IMAC, on IDA-Ni-glyoxyl agarose by metal

affinity interaction followed by irreversible covalent attachment and finally on a MANAE-agarose support via reversible ionic adsorption between the enzyme and the support. The immobilization results are shown in Table 1. It was observed that all derivatives showed high immobilization yields, with values above 75%, but the results regarding the recovered activity were variable and depended on the derivative studied. The highest percentages of recovered activity were obtained with the IDA-Ni-agarose derivatives, being of 51.7% and 71.0% for N-His-L-AI and C-His-L-AI, respectively. The disparity is likely to have been due to the fact that the histidine-tag is located at different extremes of the enzyme. This might have caused distinct interactions between the enzyme and the support due to the different tridimensional reorganization of the enzyme structure.

MANAE-agarose biocatalysts showed higher immobilization yields compared to IDA-Ni agarose derivatives. However, a lower recovered activity was observed (Table 1). Enzymes covalently immobilized on the heterofunctional support IDA-Ni-glyoxyl showed a reduced recovered activity of 1.47% and 14.1% for N-His-L-AI and C-His-L-AI, respectively. The low activity obtained was due to the washing of the derivatives with EDTA in order to remove the metal chelated to the resin to avoid the metal reduction phenomenon. Generally, it is recommended that the metal chelated to the resin be eliminated before the reduction process, which generates glyoxyl groups for covalent immobilization of the enzyme onto the support through their free amino groups.

Subsequently, following on the results above, enzyme immobilization was repeated while avoiding the washing step with EDTA (Table 1). It was then observed that this practice had been considerably harmful to enzyme activity, as seen by the increased activity in the IDA-Ni-glyoxyl agarose derivative with the latter procedure. Moreover, it has been stated that EDTA is a strong inhibitor of L-AI mainly because isomerase has been described as a metalloenzyme [33–35].

Additionally, higher loadings of enzyme (approximately 24 U g^{-1}) were tested in the supports, for better visualization of the total recovered activity for each derivative. This modification resulted in an increase in the enzyme activity but, on the other hand, lower immobilization yields were observed in all evaluated supports. This behavior could be partially explained by the multimeric nature of the enzyme that, having a high molecular weight, may

Table 1 Immobilization parameters for both recombinant L-arabinose isomerases on heterofunctional IDA-Ni, IDA-Ni-glyoxyl, and MANAE agarose supports at low and high enzyme charges

		Low enzyme load		High enzyme load	
Enzyme	Supports	*IY (%)	At_R (%)	IY (%)	At_R (%)
N-His-L-AI	IDA-Ni	80.9	51.7	55.5	37.3
	IDA-Ni-glyoxyl	80.9	1.47	55.5	43.3
	MANAE	87.3	46.0	60.4	50.3
C-His-L-AI	IDA-Ni	75.1	71.0	56.5	81.7
	IDA-Ni-glyoxyl	75.1	14.1	56.5	55.9
	MANAE	88.6	49.0	42.0	100.0

Low enzyme load: offered enzyme: 15 U enzyme g^{-1} support; offered protein: 145 mg protein g^{-1} support. IDA-Ni-glyoxyl biocatalysts were filtered and washed with a 200 mM EDTA solution for 5 min for metal removal. High enzyme load: offered enzyme: 24 U enzyme g^{-1} support; offered protein: 290 mg protein g^{-1} support. IDA-Ni-glyoxyl biocatalysts were not washed with the 200 mM EDTA solution

*IY immobilization yield, At_R recovered activity

have caused blockages on the agarose surface pores and therefore, not allowing other enzyme molecules to have entered the interior of the support and becoming covalently or not-covalently attached to it. Also, the activation degree of the support may not have been high enough to allow the binding of the initially offered enzyme.

Under the reaction conditions tested, the best immobilization conditions were observed using the MANAE-agarose derivative, with 100% and 50.3% of recovered activity in the immobilization of C-His-L-AI and N-His-L-AI, respectively. However, the immobilization protocol still needs to be optimized aiming at achieving the best results while minimizing enzyme losses. Reversible immobilization on high-density ionic flexible supports has been the most adequate method for L-AI immobilization. This is probably due to some tridimensional distortions that the support imprints to the enzyme structure [12, 36]. From this point of view, the use of these flexible supports can be a very convenient protocol for the immobilization of several industrial enzymes, since the inactive form of the enzyme can be easily desorbed from the derivative and the resin can be reused, what becomes an economic advantage regarding their use in continuous enzymatic processes [36].

Regarding the parameters of immobilization, yield, and recovered activity (Table 1), it was also observed that the multipoint covalent immobilization strategy onto the IDA-Ni-glyoxyl agarose support did not result in an improved stability of the insoluble biocatalyst compared to the other adopted strategies. Therefore, due to the multimeric nature of the enzyme, for

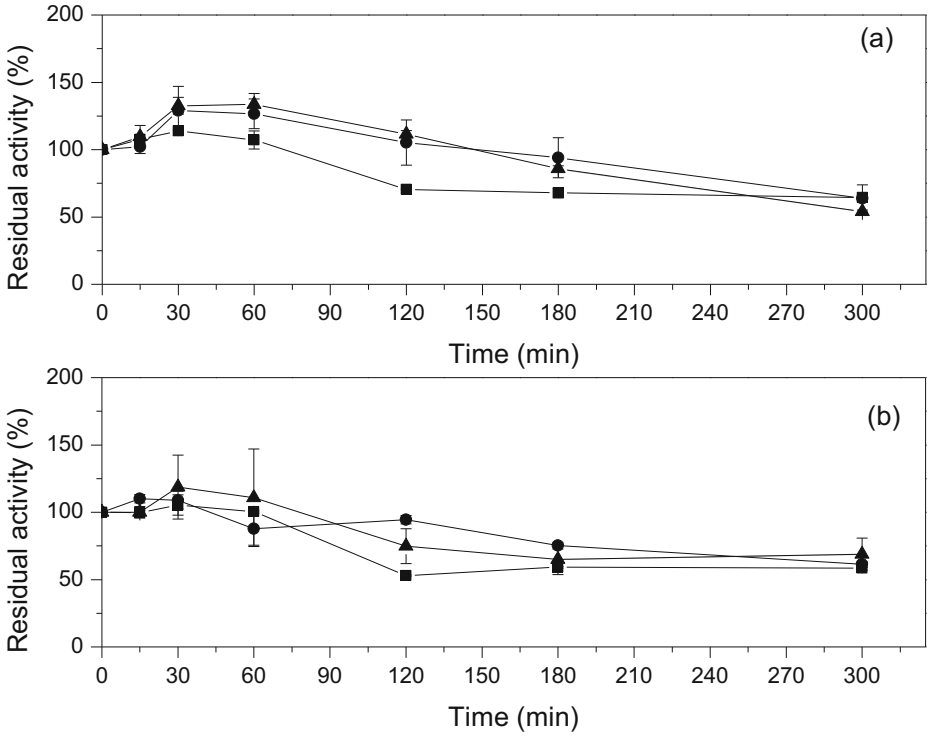

Fig. 1 Time course for thermal inactivation of the recombinant *E. faecium* DBFIQ E36 L-arabinose isomerases immobilized on different agarose supports at 50 °C. Enzymes: N-His-L-AI (**a**) and C-His-L-AI (**b**). Supports: IDA-Ni (black up-pointing triangle), IDA-Ni-glyoxyl (black circle), and MANAE (black square). The reaction conditions employed for inactivation were 5 mM sodium phosphate buffer (pH 7.0) at 50 °C

achieving stable and reusable derivatives, other immobilization and post-immobilization approaches must be tested [37].

Furthermore, the immobilization performed at pH 10.05 was not beneficial to the enzyme due to the low stability shown under this pH (data not shown). The high volume presented by the multimeric enzyme along with its low activation degree could have led to the generation of an insufficient amount of enzyme-support bonds. Also, to a lesser extent, a steric hindrance effect between enzyme units could also have contributed to the low stability shown by the derivative, causing a tridimensional rigidization of the enzyme structure [38].

After analyzing the immobilization data, the best performance for the recombinant L-AI from the host *E. coli* BL21, having the polyhistidine-tag at the C-terminal, can be confirmed in all immobilization assays due to the higher values of recovered activity, for all the biocatalysts evaluated.

Thermal Stability of the Obtained L-AI Biocatalysts

The stability of the obtained biocatalysts to temperature was assessed. Enzyme load was of 2 U mL^{-1} and derivatives were incubated at 50 and 60 °C for 300 min. At 50 °C, as seen in Fig. 1, all the derivatives showed similar thermal stability profiles and a relative activity above 60% after 300 min of reaction time and $t_{1/2} > 6$ h. It is important to mention, though, that the

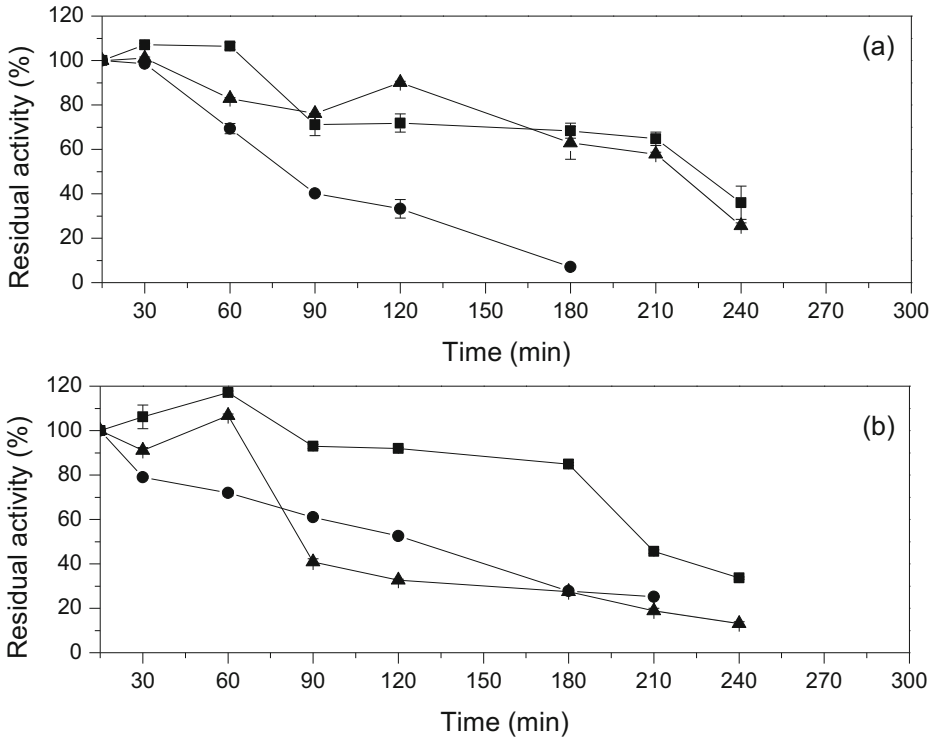

Fig. 2 Time course for thermal inactivation of the recombinant *E. faecium* DBFIQ E36 L-arabinose isomerases immobilized on different agarose supports at 60 °C. Enzymes: N-His-L-AI (**a**) and C-His-L-AI (**b**). Supports: IDA-Ni (black up-pointing triangle), IDA-Ni-glyoxyl (black circle), and MANAE (black square). The reaction conditions employed for inactivation were 5 mM sodium phosphate buffer (pH 7.0) at 60 °C

deactivation essays were conducted in the absence of substrate, in order to compare the different immobilized preparations and study stabilization by immobilization. When the biocatalyst is used for tagatose production (industrial application), the enzyme is protected by the substrate and inactivation is reduced. In a previous work, the soluble enzyme was capable of catalyze tagatose isomerization for more than 30 h [24].

At 60 °C, the L-AI MANAE-agarose derivatives were the most stable, retaining 100% of enzyme activity after 60 min of incubation (Fig. 2), and $t_{1/2}$ of 173 and 139 min, respectively, for N-His-L-AI-MANE and C-His-L-AI-MANE. Half-lives of IDA-Ni derivatives for N-His-L-AI and C-His-L-AI, respectively, were 139 and 77 min, whereas for the derivatives produced with IDA-Ni-glyoxyl supports were 43 and 99 min, respectively, for N-His-L-AI and C-His-L-AI.

All these facts (Figs. 1 and 2) serve to put in evidence that the immobilized enzyme retained its catalytic capability and thermostability when incubated at 50 °C and 60 °C, being higher for MANAE-agarose derivatives. Values of residual activity above 100% have also been reported by other authors [39–42], who attribute this effect on enzyme activity as a consequence of the additional stability provided to the enzyme by the carrier, leading to an apparent increase on thermal stability.

After analysis of the gathered data, MANAE-agarose turned out to be the best support for L-AI immobilization in terms of enzyme activity and stability. The enzyme presents several anionic patches on their surface that interact with the support and allow an oriented bonding between the enzyme and the support. This finally generates a stabilized tridimensional enzyme structure [38]. These results are in agreement with those reported by Silva et al. [43].

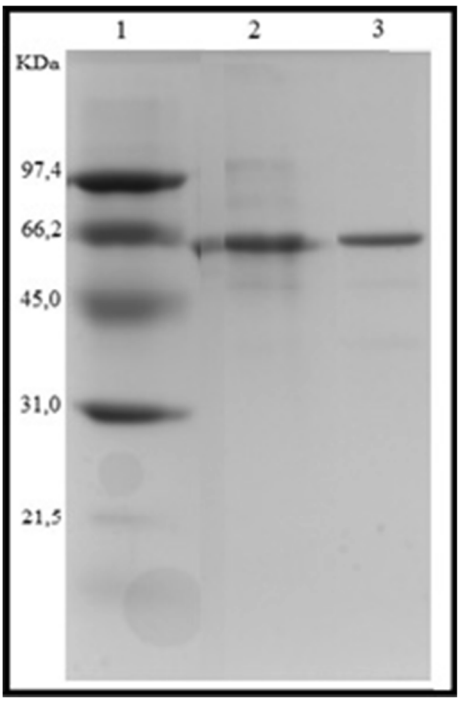

Fig. 3 SDS-PAGE analysis (12%) of the supernatants of different derivatives boiled under dissociating conditions. Lane 1—MW standards; lane 2—N-His-L-AI glyoxyl-agarose derivative; lane 3—C-His-L-AI glyoxyl-agarose derivative

L-AI immobilization onto IDA-Ni-glyoxyl support, in turn, showed a reduced thermal stability compared to the other derivatives. However, this derivative presented a higher stability when compared to the free enzyme (half-life less than 5 min at 60 °C) mainly due to the additional protection that the carrier brings to the enzyme. These could be owing to the fact that from the total enzyme activity units initially offered to support, only a minor amount was covalently attached to it, which led to a low stabilization factor of biocatalysts. This behavior was evidenced through an SDS-PAGE analysis from samples taken from the reaction mixture of the enzymatic assay of the L-AI-IDA-Ni-glyoxyl biocatalyst. As seen in Fig. 3, a 56-kDa protein band, which corresponds to L-arabinose isomerase, is clearly seen in lanes 2 and 3, which indicates that the quaternary structure of the enzyme was not properly stabilized, leading to enzyme loss during the assays with the biocatalyst.

Thus being, due to the multimeric nature of the enzyme, greater immobilization times and more drastic conditions, such as higher activation degrees, would be necessary for achieving active and highly stable glyoxyl derivatives. However, the recovered activity values would still not be as high as those in reversible enzyme-support interactions [44]. Blanco et al. observed that the lower the immobilization time, the closer the derivative stability was to that of the soluble enzyme [45].

Additionally, Fernandez-Lafuente et al. reported that quaternary-structure stabilization of multimeric enzymes with four or more enzyme units is a much more complex task than

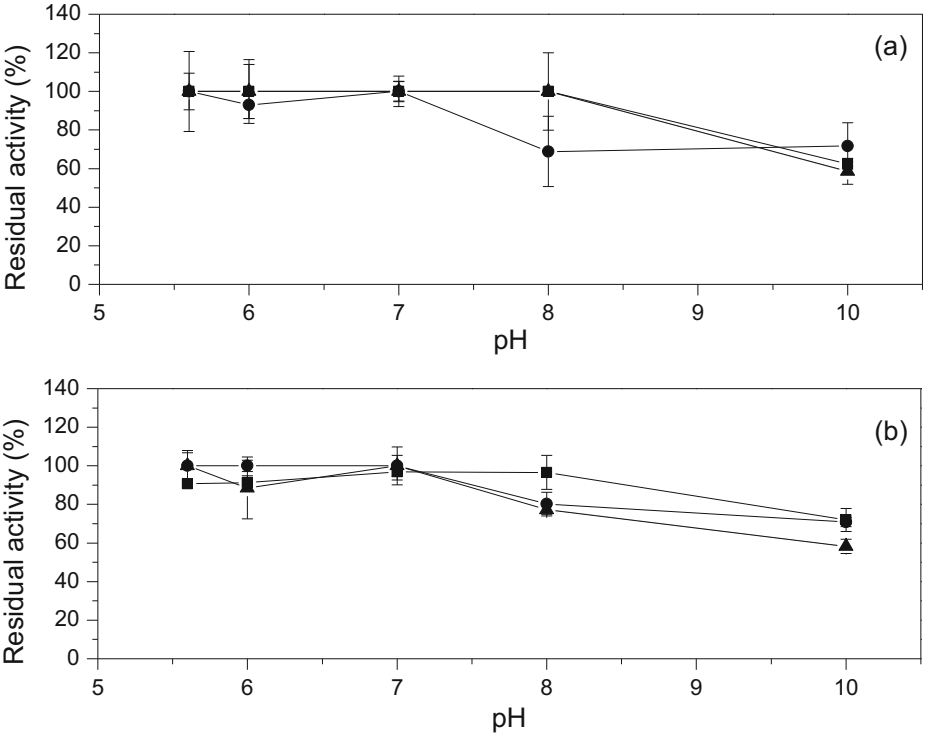

Fig. 4 Stability of the obtained biocatalysts to different pH values using both recombinant enzymes. Enzymes: N-His-L-AI (**a**) and C-His-L-AI (**b**). Supports: immobilized IDA-Ni (black up-pointing triangle), IDA-Ni-glyoxyl (black circle), and MANAE (black square). The derivatives were incubated at different pH values (5.6, 6.0, 7.0, 8.0, 10.0) using several buffering systems at a concentration of 50 mM for 2 h at room temperature

stabilizing smaller enzyme aggregates (dimers or trimers) due to geometric constraints [25]. The authors stated that it is impossible that all the enzyme subunits become covalently immobilized onto a planar support surface. Thus, they have proposed a dual strategy for stabilizing the protein complex where the subunits are first covalently immobilized and then stabilized with polyfunctional macromolecules, finally preventing the dissociation of the enzyme subunits and achieving more stable derivatives. However, diffusional limitations must be taken into account.

Effects of pH on the Stability of the Insoluble Biocatalysts

As seen in Fig. 4a, N-His-L-AI presented a similar behavior at pH 7.0 when it was immobilized onto IDA-Ni and MANAE agarose supports, with enzyme activity not having decayed after 2 h. However, at pH 10, the activity was diminished to 58% and 62%. For the IDA-Ni-glyoxyl agarose derivative, the residual activity at pH 8.0 and 10.0 was of 69% and 71%, respectively. In the case of C-His-L-AI derivatives, similar results were achieved in comparison to N-His-L-AI biocatalysts, as seen in Fig. 4b.

Kinetic Parameters of the Obtained Biocatalysts

In Fig. 5a, b, the variation of enzyme rate as function of the substrate concentration for all produced biocatalysts can be seen. A low enzyme concentration was employed (about 2 U mL^{-1}) in order to avoid possible diffusional constraints. The immobilized enzyme follows Michaelis-Menten kinetics and hence, apparent kinetic parameters V_{max}' and K_m' were determined (Table 2).

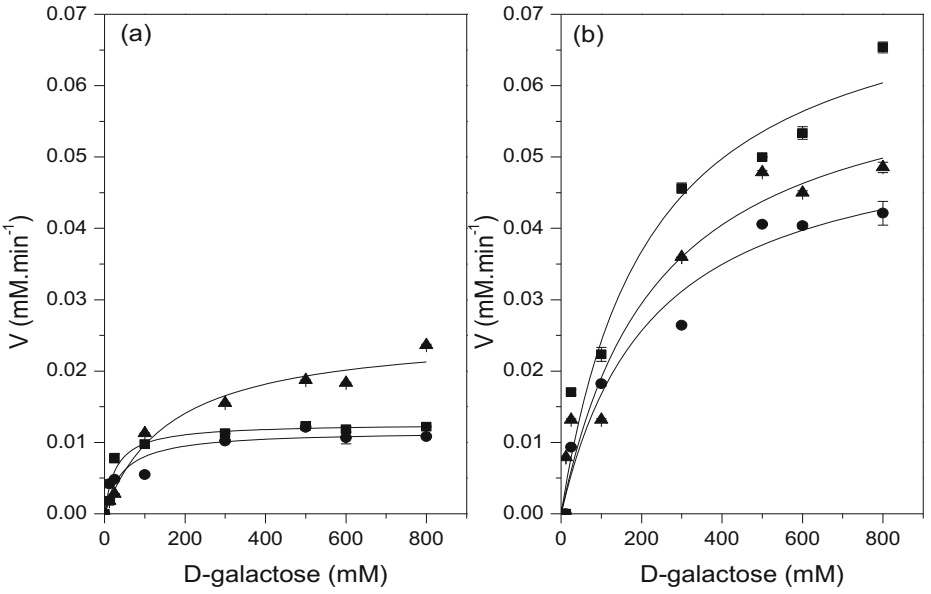

Fig. 5 Influence of D-galactose concentration on the initial rate of the isomerization reaction at 50 °C, pH 5.6. Enzymes: N-His-L-AI (**a**) and C-His-L-AI (**b**). Supports: IDA-Ni (black up-pointing triangle), IDA-Ni-glyoxyl (black circle), and MANAE (black square)

Table 2 Apparent kinetic parameters of the derivatives obtained for both recombinant enzymes using D-galactose as substrate at 50 °C and pH 5.6

Enzyme	Support	K_m (mM)	V_{max} (mM min^{-1})
N-His-L-AI	IDA-Ni	165 ± 49	0.026 ± 0.00
	IDA-Ni-glyoxyl	45 ± 19	0.011 ± 0.00
	MANAE	28 ± 9	0.013 ± 0.00
C-His-L-AI	IDA-Ni	239 ± 103	0.065 ± 0.01
	IDA-Ni-glyoxyl	227 ± 76	0.055 ± 0.01
	MANAE	218 ± 82	0.080 ± 0.01

Results are shown as the average of the triplicate values obtained

According to the results shown in Fig. 5, a decrease of almost 50% in the apparent V_{max} was evidenced for the L-AI expressed in DH10B in relation to BL21.

For N-His-L-AI derivatives, lower values for the Michaelis kinetic constant for IDA-Ni-glioxyl (45.0 mM), IDA-Ni (165 mM), and MANAE (28.0 mM) were observed when compared to the soluble recombinant enzyme, 252 mM [24]. The immobilization process may have promoted changes on the affinity of the enzyme to the substrate, reducing the K_m value.

Apparent kinetic parameters for C-His-L-AI derivatives could not be compared to the kinetic constants of the soluble enzyme because the latter presented a sigmoidal behavior whereas the former presented a hyperbolic nature [24], as shown in Fig. 5b. Cardoso et al. [38] stated that this is a clear example where enzyme immobilization brings new properties to the enzyme and even kinetic changes (K_m and V_{max}).

Conclusions

The different locations of the polyhistidine-tag on the enzyme structure (C- or N-terminal) showed an influence on the enzyme ability to be immobilized onto the supports. A better performance for C-His-L-AI was observed in all the supports and immobilization conditions tested, due to the higher values of the recovered activity obtained. Furthermore, the MANAE-agarose support yielded the best results for L-AI immobilization. The results revealed that, due to the multimeric nature of the enzyme, the enzyme immobilization was successfully performed, but achieving stable derivatives was not possible. To this end, innovative (e.g., covalent) immobilization strategies and different experimental conditions must be assessed for obtaining both highly active and stable enzyme derivatives. Additionally, further stabilization approaches must be studied due to the complex quaternary structure that the enzyme presents in order to improve the performance of the isomerization reaction, envisaging a feasible industrial bioprocess.

Funding Information This study received financial support from the Brazilian Research Agencies CAPES, CNPq, and FUNCAP. This work was partially sponsored by funds from the project Argentina–Brazil Bilateral Cooperation Program BR/12/06 MINCyT-CAPES 2012 (Buenos Aires, Argentina).

Compliance with Ethical Standards

Conflict of Interest The authors declare that there is no conflict of interest.

References

1. Hong, Y. H., Lee, D. W., Lee, S. J., Choe, E. A., Kim, S. B., Lee, Y. H., Cheigh, C. I., & Pyun, Y. R. (2007). Production of d-tagatose at high temperatures using immobilized escherichia coli cells expressing L-arabinose isomerase from *Thermotoga neapolitana*. *Biotechnology Letters, 29*(4), 569–574.
2. Levin, G. V., Zehner, L. R., Saunders, J. P., & Beadle, J. R. (1995). Sugar substitutes: their energy values, bulk characteristics, and potential health benefits. *The American Journal of Clinical Nutrition, 62*(5), 1161S–1168S.
3. Livesey, G., & Brown, J. C. (1996). D-Tagatose is a bulk sweetener with zero energy determined in rats. *The Journal of Nutrition, 126*(6), 1601–1609.
4. Beadle, J. R., Saunder, J. P., & Wajada, T. J. (1992). Process for manufacturing tagatose. US Patent 5,078,796.
5. Oh, D. K. (2007). Tagatose: properties, applications, and biotechnological processes. *Applied Microbiology and Biotechnology, 76*(1), 1–8.
6. Illanes, A., Guerrero, C., Vera, C. Wilson, L., Conejeros, R., Scott, F. (2016). *Lactose-derived prebiotics: a process perspective*. 1st Ed., San Diego: Academic Press. https://doi.org/10.1016/C2014-0-03393-X.
7. Oh, D. K., Kim, H. J., Ryu, S. A., Rho, H. J., & Ki, P. (2001). Development of an immobilization method of L-arabinose isomerase for industrial production of tagatose. *Biotechnology Letters, 23*(22), 1859–1862.
8. Kim, H. J., Ryu, S. A., Kim, P., & Oh, D. K. (2003). A feasible enzymatic process for D-tagatose production by an immobilized thermostable L-arabinose isomerase in a packed-bed bioreactor. *Biotechnology Progress, 19*(2), 400–404.
9. Ryu, S. A., Kim, C. S., Kim, H. J., Baek, D. H., & Oh, D. K. (2003). Continuous D-tagatose production by immobilized thermostable L-arabinose isomerase in a packed-bed bioreactor. *Biotechnology Progress, 19*(6), 1643–1647.
10. Jung, E. S., Kim, H. J., & Oh, D. K. (2005). Tagatose production by immobilized recombinant *Escherichia coli* cells containing *Geobacillus stearothermophilus* L-arabinose isomerase mutant in a packed-bed bioreactor. *Biotechnology Progress, 21*(4), 1335–1340.
11. Rhimi, M., Messaoud, E. B., Borgi, M. A., khadra, K. B., & Bejar, S. (2007). Co-expression of l-arabinose isomerase and d-glucose isomerase in E. coli and development of an efficient process producing simultaneously d-tagatose and d-fructose. *Enzyme and Microbial Technology, 40*(6), 1531–1537.
12. Pessela, B. C. C., Vian, A., Mateo, C., Lafuente, R., García, J. L., Guisan, J. M., & Carrascosa, A. V. (2003). Overproduction of Thermus sp. strain T2 B-galactosidase in Escherichia coli and preparation by using tailor-made metal chelate supports. *Applied and Environmental Microbiology, 69*(4), 1967–1972.
13. Guisán, J. M. (1988). Aldehyde-agarose gels as activated supports for immobilization-stabilization of enzyme. *Enzyme and Microbial Technology, 10*(6), 375–382.
14. Mateo, C., Grazú, V., Palomo, J. M., López-Gallego, F., Fernández-Lafuente, R., & Guisán, J. M. (2007). Immobilization of enzymes on heterofunctional epoxy supports. *Nature, 2*, 1022–1033.
15. Fernández-Lorente, G., Lopez-Gallego, F., Bolivar, J. M., Rocha-Martin, J., Perez, S. M., & Guisán, J. M. (2015). Immobilization of proteins on highly activated glyoxyl supports: dramatic increase of the enzyme stability via multipoint immobilization on pre-existing carriers. *Current Organic Chemistry, 19*(17), 1719–1731.
16. Bolívar, J. M., Rocha-Martin, J., Godoy, C., Rodrigues, R. C., & Guisán, J. M. (2010). Complete reactivation of immobilized derivatives of a trimeric glutamate dehydrogenase from Thermus thermophillus. *Process Biochemistry, 45*(1), 107–113.
17. Mateo, C., Bolivar, J. M., Godoy, C. A., Rocha-Martin, J., Pessela, B. C., Curiel, J. A., Munoz, R., Guisan, J. M., & Fernandez-Lorete, G. (2010). Improvement of enzyme properties with a two-step immobilization process on novel heterofunctional supports. *Biomacromolecules, 11*(11), 3112–3117.
18. Manzo, R. M., de Sousa, M., Fenoglio, C. L., Gonçalves, L. R. B., & Mammarella, E. J. (2015). Chemical improvement of chitosan modified beads for the immobilization of Enterococcus faecium DBFIQ E36 L arabinose isomerase through multipoint covalent attachment approach. *Journal of Industrial Microbiology & Biotechnology, 42*(10), 1325–1340.
19. Armisén, P., Mateo, C., Cortés, E., Barredo, J. L., Salto, F., Diez, B., Rodés, L., García, J. L., Fernández-Lafuente, R., & Guisan, J. M. (1999). Selective adsorption of poly-His tagged glutaryl acyiase on tailor-made metal chelate supports. *Journal of Chromatography. A, 848*(1-2), 61–70.
20. Hernaiz, M. J., & Crout, D. H. G. (2000). Immobilization-stabilization on Eupergit C of the, β-galatosidase from B. circulans and an β-galactosidase from A. oryzae. *Enzyme and Microbial Technology, 27*(1-2), 26–32.
21. Katchalski-Katzir, E., & Kraemer, D. (2000). Eupergit C, a carrier for immobilization of enzymes of industrial potential. *Journal of Molecular Catalysis B: Enzymatic, 10*(1-3), 157–176.
22. Mateo, C., Femández-Lorente, G., Benevides, C. C. P., Vían, A., Carrascosa, A. V., García, J. L., Fernández-Lafuente, R., & Guisan, J. M. (2001). Affinity chomatography of polyfiistidine tagged enzimes. New

dextran-coated immobilized metal ion affinity chromatography matrices for prevention of undesired multipoint adsorptions. *Journal of Chromatography A, 915*(1-2), 97–106.
23. Mateo, C., Femández-Lorente, G., Abian, O., Fernández-Lafuente, R., & Guisan, J. M. (2000a). Multifunctionai Epoxi Suports. A new tool to improve the covalent immobiiization of proteins. The promotion of physical adsorptions of proteins on the supports before theírcovalent linkage. *Biomacromoleculas, 1*(4), 739–745.
24. Sousa, M., Manzo, R. M., García, J. L., Mammarella, E. J., Gonçalves, L. R. B., & Pessela, B. C. (2017). Engineering the L-arabinose isomerase from *Enterococcus Faecium* for D-tagatose synthesis. *Molecules, 22*(12), 2164.
25. Fernández-Lafuente, R., Rossell, C. M., Rodríguez, V., Santana, C., Soler, G., Bastida, A., & Guisan, J. M. (1993). Preparation of activated supports containing low pK amino groups. A new tool or protein immobilization via the carboxyl coupling method. *Enzyme and Microbial Technology, 15*(7), 546–550.
26. Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry, 72*(1-2), 248–254.
27. Dische, Z., & Borenfreund, E. (1951). A new spectrophotometric method for the detection and determination of keto sugars and trioses. *The Journal of Biological Chemistry, 192*, 583–587.
28. Hung, X. G., Tseng, W. C., Liu, S. M., Tzou, W. S., & Fang, T. Y. (2014). Characterization of a thermophilic L-arabinose isomerase from Thermoanaerobacterium saccharolyticum NTOU1. *Biochemical Engineering Journal, 83*, 121–128.
29. Guisan, J. M. (2006). *Immobilization of enzymes and cells. Methods in biotechnology* (Vol. 22). Totowa: Humana Press.
30. Laemmli, U. K. (1970). Cleavage of structural protein during the assembly of the heat of bacteriophage T4. *Nature, 227*(5259), 680–685.
31. Silva, J. A., Macedo, G. P., Rodrigues, D. S., Giordano, R. L. C., & Gonçalves, L. R. B. (2012). Immobilization of *Candida antarctica* lipase B by covalente attachment on chitosan-based hydrogels using diferente support activation strategies. *Biochemical Engineering Journal, 60*, 16–24.
32. Illanes, A. (2008). *Enzyme biocatalysis: principles and applications*. Netherlands: Springer.
33. Cheng, L. F., Mu, W. M., Zhang, T., & Jiang, B. (2010). Na L-arabinose isomerase from Acidothermus cellulolytics ATCC 43068: cloning, expression, purification, and characterization. *Applied Microbiology and Biotechnology, 86*(4), 1089–1097.
34. Rhimi, M., Ilhammami, R., Bajic, G., Boudebbouze, S., Maguin, E., Haser, R., & Aghajari, N. (2010). The acid tolerant L-arabinose isomerase from the food grade Lactobacillus sakei 23 K is an attractive D-tagatose producer. *Bioresource Technology, 101*(23), 9171–9177.
35. Lee, S. J., Lee, D. W., Choe, E. A., Hong, Y. H., Kim, S. B., Kim, B. C., & Pyun, Y. R. (2005). Characterization of a thermoacidophilic L-arabinose isomerase from *Alicyclobacillus acidocaldarius*: role of Lys-269 in pH optimum. *Applied and Environmental Microbiology, 71*(12), 7888–7896.
36. Mateo, C., Abian, O., Lafuente, R., & Guisan, J. M. (2000b). Reversible enzyme immobilization via a very strong and nondistorting ionic adsorption on support–polyethylenimine composites. *Biotechnology and Bioengineering, 68*(1), 98–105.
37. Fernández-Lafuente, R., Rodríguez, V., Mateo, C., Penzol, G., Hernández-Justiz, O., Irazoqui, G., Villaríno, A., Ovsejevi, K., Francisco, B., & Guisan, J. M. (1999). Stabilization of multimeric enzymes via immobilization and post-immobilization techniques. *Journal of Molecular Catalysis B: Enzymatic, 7*(1-4), 181–189.
38. Cardoso, C. L., de Moraes, M. C., & Cass, Q. B. (2009). Imobilização de enzimas em suportes cromatográficos: uma ferramenta na busca por substâncias bioativas. *Quimica Nova, 32*(1), 175–187.
39. Pessela, B. C. C. (2002). Ingeniería de Biocatalizadores y Bioprocesos: β-Galactosidasa de *Thermus sp*., Cepa T2. PhD Thesis. Universidad Politécnica de Madrid, Espanha.
40. Pisan, F. M., Relia, C. A., Raya, C., Rozzo, R., Nucci, A., Gambacorta, M., Rosa, D., & Rossi, M. (1990). Thermostable p-galactosidase from archaebactrium Solfulobus solfatarius. Purification and properties. *European Journal of Biochemistry, 187*(2), 321–328.
41. Coolbear, T., Daniel, R. M., & Morgan, H. W. (1992). The enzymes from extreme thermophiles: bacterial source, thermostabilities and industrial relevance. *Advances in Biochemical Engineering / Biotechnology, 45*, 58–98.
42. da Silva, E. S., Gómez-Vallejo, V., & López-Gallego, F. (2015). Efficient nitrogen-13 radiochemistry catalyzed by a highly stable immobilized biocatalyst. *Catalysis Science & Technology, 5*(5), 2705–2713.

43. Silva, T. M., Pessela, B. C., Silva, J. C. R., Lima, M. S., Jorge, J. A., Guisán, J. M., & Polizeli, M. L. T. M. (2014). Immobilization and high stability of an extracellular β-glucosidase from Aspergillus japonicus by ionic interactions. *Journal of Molecular Catalysis B: Enzymatic, 104*, 95–100.
44. Mateo, C., Palomo, J. M., Fuentes, M., Betancor, L., Grazu, V., López-Gallego, F., Pessela, B. C. C., Hidalgo, A., Fernández-Lorente, G., Fernández-Lafuente, R., & Guisán, J. M. (2006). Glyoxyl agarose: a fully inert and hydrophilic support for immobilization and high stabilization of proteins. *Enzyme and Microbial Technology, 39*(2), 274–280.
45. Blanco, R. M., Calvete, J. J., & Guisan, J. M. (1989). Immobilization e stabilization of enzymes. Variables that control the intensity of the trypsin (amine)-agarose (aldehyde) multi-point covalent attachment. *Enzyme and Microbial Technology, 11*(6), 353–359.

Affiliations

Marylane de Sousa[1] · Vânia M. M. Melo[2] · Denise C. Hissa[2] · Ricardo M. Manzo[3] · Enrique J. Mammarella[3] · André Saraiva Leão Marcelo Antunes[4] · José L. García[5] · Benevides C. Pessela[6,7] · Luciana R. B. Gonçalves[1]

Marylane de Sousa
marylaneufc@gmail.com

Vânia M. M. Melo
vmmmelo@gmail.com

Denise C. Hissa
denisehissa@gmail.com

Ricardo M. Manzo
manzoricardo@yahoo.com.ar

Enrique J. Mammarella
ejoma@intec.unl.edu.ar

André Saraiva Leão Marcelo Antunes
aiococ@yahoo.com.br

José L. García
jlgarcia@cib.csic.es

[1] Department of Chemical Engineering, Federal University of Ceará, Campus do Pici, BL 709, Fortaleza, CE, Brazil

[2] Department of Biology, Federal University of Ceará, Campus do Pici, BL 909, Fortaleza, CE, Brazil

[3] Food and Biotechnology Engineering Group, Institute of Technological Development for the Chemical Industry, National University of the Litoral (UNL), National Council of Scientific and Technical Research (CONICET), RN 168 Km 472 "Paraje El Pozo" S / N, Santa Fe, Argentina

[4] Department of Infectious Diseases, King's College London, London WC2R 2LS, UK

[5] Center for Biological Research, CIB, Higher Council for Scientific Research, CSIC, C / Ramiro de Maeztu, 9, Madrid, Spain

[6] Department of Food Biotechnology and Microbiology, Institute of Research in Food Sciences, CIAL, Higher Council for Scientific Research, CSIC, C / Nicolás Cabrera 9, UAM Campus, Madrid, Spain

[7] Department of Engineering and Technology, Polytechnic Institute of Sciences and Technology, Av. Luanda Sul, Rua Lateral Via S10, Talatona, Luanda, Angola