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The β-galactosidase immobilization protocol determines its performance as catalysts in the kinetically controlled synthesis of lactulose

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ABSTRACT

In this paper, 3 different biocatalysts of β-galactosidase from *Kluyveromyces lactis* have been prepared by immobilization in chitosan activated with glutaraldehyde (Chi_Glu_Gal), glyoxyl agarose (Aga_Gly_Gal) and agarose coated with polyethylenimine (Aga_PEI_Gal). These biocatalysts have been used to catalyze the synthesis of lactulose from lactose and fructose. Aga-PEI-Gal only produces lactulose at 50 °C, and not at 25 or 37 °C, Aga_Gly_Gal was unable to produce lactulose at any of the assayed temperatures while Chi_Glu_Gal produced lactulose at all assayed temperatures, although a lower yield was obtained at 25 or 37 °C. The pre-incubation of this biocatalyst at 50 °C permitted to obtain similar yields at 25 or 37 °C than at 50 °C. The use of milk whey instead of pure lactose and fructose produced an improvement in the yields using Aga_PEI_Gal and a decrease using Chi_Glu_Gal. The operational stability also depends on the reaction medium and of biocatalyst. This study reveals how enzyme immobilization may greatly alter the performance of β-galactosidase in a kinetically controlled manner, and how medium composition influences this performance due to the kinetic properties of β-galactosidase.

1. Introduction

Enzymatic biocatalysts are receiving a growing interest due to the great efficiency of enzymes under mild conditions as well as their high selectivity and specificity [1,2]. This has converted them in ideal biocatalysts in the framework of green chemistry [3].

Among the many uses of enzymes in biocatalysis, kinetically controlled synthesis (KCS) may be highlighted [4]. These processes try to convert hydrolase enzymes in pseudo-transferases using an activated acid donor [5–7]. The activated acyl donor may be activated as an ester, amide or ether, depending on the enzyme. They are very popular in the production of peptides (e.g., using proteases) [6], β-lactamic antibiotics (using penicillin acylases) [7], biodiesel (using lipases), and others [8–11]. The compounds bearing glyosidic bonds are the activated substrates in the case of transglycosylations catalyzed by glycosidases (e.g., to produce galacto- or fructo-oligosaccharides) [12–14].

This synthetic strategy gives transient maximum yields, as they are over the equilibrium concentrations. Maximum yields are defined by 3 parameters that are characteristic of each biocatalyst: the synthesis rate of the target product/hydrolysis rate of the activated acyl donor ratio, the synthesis/hydrolysis rates of the target product (as it is over the equilibrium concentration and may be a substrate of the enzyme), and the saturation of the enzyme by the nucleophile [6,7,15,16]. This synthetic strategy is very popular because the use of an activated acyl donor can give high reaction rates and the reaction may be performed in aqueous medium, in opposition to the simpler thermodynamically controlled synthesis (TCS), that uses an unmodified acyl donor and drastic reaction conditions (low water activity systems) [4,17,18]. In TCS, the yields are only determined by the thermodynamics of the reaction. They do not depend on the biocatalysts (except when using multifunctional or chiral substrates, where the enzyme selectivity or specificity may alter the final mixture of products) [17,18].

The fact that the maximum yields are fully determined by the biocatalyst properties makes the selection of a proper enzyme a key point in the design of KCS reactions [15,19–22]. This permits that using an enzyme with very high synthesis/hydrolysis ratios and good affinity

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towards the nucleophile very high yields may be reached, while in a TCS, it is only mainly important that the enzyme presents good activity, stability and no-product inhibition by the product [6,7].

Enzymes tend to be used as industrial biocatalysts in immobilized forms to simplify their recovery and reuse [23–25]. Enzyme immobilization also improves, if properly performed, many enzyme features, like stability, activity, selectivity and specificity. It even decreases inhibitions or improves enzyme purity [26–31]. In the case of KCS, enzyme immobilization is another tool to tune the enzyme features, and that way, to permit the preparation of biocatalysts that can give higher maximum yields [4,20].

However, the fact that the maximum yields are fully determined by the biocatalyst properties can also have some additional implications not usually considered when selecting this synthetic strategy. Small changes in the enzyme structure during utilization can cause, despite the enzyme activity (determined as consumption of activated acyl donor) possibly being almost maintained, the synthetic properties of the biocatalyst to be significantly altered, as there are many parameters involved in the biocatalysts performance in kinetically controlled synthesis [4]. This has been scarcely studied and may have an impact higher than that expected in the implementation of these processes, and can give a new meaning to the "biocatalysts stability".

The study of the effect of the enzyme immobilization protocol and the operational stability of the biocatalyst in KCS has been the main objective of this paper. We have selected a very interesting model reaction, the lactulose production using lactose as activated acyl donor and fructose as nucleophile. Lactulose (4-O-β-D-galactopyranosyl-D-fructofuranose) is a sugar with prebiotic functions, such as stimulating the growth of beneficial microorganisms. It also acts in the treatment of chronic constipation and hepatic encephalopathy [32,33]. It is usually produced by lactose isomerization through chemical catalysis [34,35] or enzymatic synthesis catalyzed by different enzymes [36–40]. Its enzymatic production is more attractive than the chemical one because its milder experimental conditions and the prevention of side-reactions [41].

As catalyst, we have selected the multimeric β-galactosidase from *Kluyveromyces lactis* [42,43]. This enzyme, due to its multimeric character, may be especially tunable via immobilization [44,45]. In this paper, the enzyme has been immobilized following very different protocols, aiming to involve different mechanisms of enzyme immobilization, with the objective of checking if these different immobilization protocols may change the properties of the enzyme. It has been immobilized on agarose-glyoxyl support at neutral pH value [46]. This support only fixes the enzyme when several enzyme-support linkages have been established. At neutral pH value, only the terminal amino groups will be unprotonated, and therefore, they will be the only amino groups reactive versus the glyoxyl moieties on the support. That way, by immobilizing a multimeric enzyme on glyoxyl agarose at neutral pH values, the enzyme will be immobilized via the covalent attachment via the terminal amino groups of at least two enzyme subunits [47,48], leaving a fully inert support surface after immobilization. The enzyme has been also immobilized on agarose coated with PEI, where the enzyme will be immobilized in an ion polymeric bed via ion exchange, a large percentage of the enzyme will be in contact with the polymer, as the immobilization will be in a bed and not on a flat surface [49]. The possibility of these enzyme-PEI ionic interactions will remain along the use of the enzyme, and this may even fix partially or fully distorted enzyme structures [50,51].

Moreover, the enzyme has also been immobilized on chitosan activated with glutaraldehyde. Glutaraldehyde chemical reactivity is moderate, causing the direct covalent immobilization of an enzyme to be a quite slow process [49], therefore, using moderate ionic strength, the enzyme will be first ionically exchanged on the support and later on, some covalent bonds between the enzyme and the support may be established [52–54]. This way, the orientation of the enzyme may be similar to that using PEI coated supports, but it will have some differences: the enzyme is interacting with a flat surface and the interactions are ionic (the amino groups in the support), hydrophobic (the glutaraldehyde groups) and covalent (this groups will have a reduced mobility) [55,56].

This diversity of immobilization strategies gives good opportunities of preparing biocatalysts with different structural conformations and therefore, with different performance in KCS.

2. Materials and methods

2.1. Materials

Glycidol, 25% (*v*/v) glutaraldehyde, *o*-nitrophenyl-β-D-galactopyranoside (*o*NPG), polyethylenimine (PEI) (MW: 25,000 D) and β-galactosidase (E.C.3.2.1.23) from *Kluyveromyces lactis* (Lactozyme® 2600) were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO). Sodium acetate, acetic acid, boric acid, phosphoric acid, fructose, monobasic potassium phosphate, dibasic potassium phosphate, potassium hydroxide, sodium hydroxide, lactose and sodium tetraborate were obtained from Dinâmica Química Contemporânea Química LTDA (Indaiatuba, São Paulo, Brazil). Chitosan powder, with 85.2% deacetylation degree, was purchased from Polymar Ind. Ltda (Fortaleza, Ceara, Brazil), and the agarose beads were obtained from GE Healthcare (Chicago, Illinois, USA). Glucose concentration was determined using an enzymatic kit (Glucose monoreagent) supplied by Bioclin.

2.2. Supports preparation

Three different supports were evaluated in the immobilization of the β-galactosidase from *Kluyveromyces lactis*: chitosan activated with glutaraldehyde (Chi_Glu); agarose activated with glycidol and oxidized with periodate (glyoxyl agarose) (Aga_Gly) and agarose activated with glycidol, oxidized with periodate and coated with PEI (Aga_PEI).

2.2.1. Chitosan activated with glutaraldehyde (Chi_Glu)

Chitosan powder was dissolved in a 2% (*v*/v) acetic acid solution at a concentration of 2% *w*/*v* under stirring and mild heating. The chitosan solution was coagulated and precipitated with 0.5 M potassium hydroxide until homogenized. The chitosan particle size was not controlled and the appearance of the particles seems sandy. Then, the chitosan particles were activated with a 0.8% (*v*/v) glutaraldehyde solution for 30 min at 50 °C under stirring. The obtained support was washed with excess distilled water and with 100 mM potassium phosphate (pH 7.0) [57,58].

2.2.2. Glyoxyl agarose beads (Aga_Gly)

Glyoxyl agarose was obtained according to Guisán [59] with some modifications. Agarose beads were modified by etherification of the agarose anomeric hydroxyl groups in agarose with glycidol (glycidyl support), followed by oxidation with sodium metaperiodate ($NaIO_4$) to get glyoxyl groups. To prepare the glycidyl support, a solution of 1.7 M NaOH containing 0.75 M sodium borohydride ($NaBH_4$) was prepared, the support was added (equivalent to 1/3 (*w*/*v*) of the suspension) and 48% glycidol solution was added dropwise to this suspension to reach a concentration of 2 M, under gentle stirring and in an ice bath to prevent excessive heating. The suspension was kept under mechanical stirring in an open recipient for 18 h at 25 °C. The support was filtered under vacuum and washed with an excess of distilled water. After this step, the glycidyl groups formed on the support surface were oxidized to glyoxyl moieties by suspending them in distilled water at a ratio of 1/10 (*w*/*v*) and adding 300 μmoles of solid $NaIO_4$ per gram of support. The oxidation suspension was kept under gentle stirring for 2 h at 25 °C. The oxidized support was washed with distilled water in excess and stored under refrigeration.

2.2.3. Coating of agarose-glyoxyl beads with PEI (Aga_PEI)

Glyoxyl agarose beads were coated with PEI according to Pessela et al. [60] with some modifications. Aga_Gly support was suspended in 10% *w*/*v* PEI solution at pH 10 and gently stirred for 3 h at 25 °C. Then, solid sodium borohydride was added to reach a concentration of 10 mg/mL and the mixture was stirred for 2 h. The support was filtered and washed with 100 mM sodium acetate buffer (pH 4.0), 100 mM sodium borate buffer (pH 9.0) and finally with a large excess of distilled water in excess.

2.3. Immobilization of β-galactosidase from Kluyveromyces lactis on the different supports

The enzyme immobilization process was the same for all supports, using an enzymatic load of 10 mg of protein per g of support and carried out at pH 7.0 and 25 °C in 100 mM potassium phosphate buffer containing 0.1 mM $MnCl_2$ and 0.2 $MgCl_2$, under 120 rpm on a rotary shaker. The presence of Mg^{2+} and Mn^{2+} is effective for the performance of β-galactosidases, directly influencing their activity and stability [36]. In the case of glyoxyl, after 3 h, the pH was increased to 8 and solid sodium borohydride was added to reach a concentration of 2 mg/mL, left for 30 min under gentle stirring and finally washed with distilled water. In the other cases, after enzyme immobilization, the biocatalysts were washed with an excess of distilled water.

The calculated immobilization parameters were immobilization yield, offered activity and activity recovery according to Boudrant et al. [61].

2.4. Determination of the β-galactosidase activity

The hydrolytic activity of β-galactosidase was determined using 50 mM ortho-nitrophenyl-β-galactoside (*o*NPG) at 37 °C and pH 6.6. The product of the hydrolysis (*o*NP) was spectrophotometrically determined at 420 nm (ε under these conditions was 4.53 $M^{-1}.cm^{-1}$).

β-galactosidase activity was also determined using 5 mL of a solution of 5.0% (*w*/*v*) of lactose in 100 mM potassium phosphate buffer containing 0.1 mM $MnCl_2$ and 0.2 mM $MgCl_2$ at pH 7.0 and at 37 °C for 10 min. The glucose produced was quantified using the GOD-PAP method using an enzyme kit for glucose measurement. One unit of β-galactosidase was defined as the amount of enzyme capable of releasing 1 μmol of glucose per minute under the conditions described above. For the soluble enzyme, 80 μL of the soluble enzyme was added to the lactose solution. After 10 min, samples (150 μL) of lactose hydrolysis solution were taken and 150 μL of 0.1 M NaOH were added to stop the reaction. The activity of immobilized β-galactosidase was determined in a batch reactor maintained at 37 °C containing the substrate solution and using 0.1 g biocatalyst, under gentle mechanical agitation. Samples were taken from the supernatant and the produced glucose was quantified.

2.5. Biocatalysts thermal inactivation

The thermal inactivation of the biocatalysts was performed at 50 °C in 50 mM potassium phosphate containing 0.1 mM $MnCl_2$ at pH 6.6. Inactivation suspension samples were collected periodically and the enzymatic activity was determined using oNPG. The half-life time was calculated according to the model proposed by Sadana and Henley [62].

2.6. Determination of enzymatic lactose hydrolysis and lactulose production

To evaluate the efficiency of the obtained biocatalysts, simultaneous reactions of hydrolysis of lactose and production of lactulose were performed. The experiments of lactulose production were conducted in 25 mL Erlenmeyer flasks containing the synthetic solution of lactose and fructose, in a molar ratio of 1:2, with an initial total substrate concentration of 200 $g.L^{-1}$ in 50 mM phosphate buffer at pH 7.0 [38]. The enzymatic load used was 4 U/mL and the reaction was conducted under orbital shaking at 120 rpm and 25, 37 °C or 50 °C for 120 min. The 200 mg/mL lactose hydrolysis was studied under similar conditions but in the absence of fructose.

In some instances, Aga_PEI-Gal and Chi_Glu_Gal were previously incubated at 50 °C for 10 min.

Also, studies using cheese whey as substrate were performed. Whey powder was kindly provided by Alibra Ingredientes Ltda (Campinas, Brazil), and according to the manufacturer's technical specifications, the whey contained the following composition (per 100 g): carbohydrate 74 g; proteins 11 g; total fat 1 g; saturated fats 0.5 g; cholesterol 6.0 mg; calcium 796 mg, iron 0.90 mg and sodium 1080 mg, pH ranging from 6.0 to 7.0 and acidity (% lactic acid) of 2.5%. The reaction was conducted with fructose supplementation at the molar ratio of 1:2 (lactose: fructose), with an initial total substrate concentration of 200 $g.L^{-1}$ in 50 mM phosphate buffer at pH 7.0. The enzymatic load used was 4 U/mL and the reactions were conducted under the same conditions described in the study using lactose solution.

In all cases, reaction samples were collected periodically, using free enzyme the reactions were stopped by heating at 100 °C for 2 min, and they were analyzed to determine the concentrations of the different carbohydrates.

2.7. Analytical methods

Protein concentrations were determined by Bradford method [63]. The standard used was bovine serum albumin (BSA), purchased from Sigma-Aldrich Co. (St. Louis, MO).

Carbohydrates concentrations (lactose, lactulose, glucose, fructose and galactose) were determined by High-Performance Liquid Chromatography (HPLC) (Thermo Finnigan Surveyor HPLC System, Thermo Fisher Scientific, San Jose, CA, USA), using a refractive index detector and Supelco 610-H column at 30 °C. The used eluent was 0.1% (*v*/*v*) H_3PO_4 at a flow rate of 0.5 $mL.min^{-1}$ [38]. The injection volume was 20 μL.

2.8. Statistical analysis

The results were analyzed by analysis of variance (ANOVA), using Tukey test, and the minimum significance difference intervals were judged according to the *p* value, determined at a significance level of 5% ($p < 0.05$).

3. Results and discussion

3.1. β-Galactosidase biocatalysts preparation

A previous study [38], conducted in our research group, reported the production of lactulose using the free enzyme β-galactosidase from *K. lactis*. In that study, the optimal conditions for lactulose synthesis were presented (lactose: fructose ratio, substrate concentration, temperature and pH) from the free enzyme. In turn, in this current study, the increased performance of this reaction was investigated from the synthesis of a biocatalyst formulated from different immobilization strategies. Fig. 1 shows the immobilization courses of the enzyme in the different supports (A: agarose-glyoxyl; B: agarose-PEI; C: chitosan-glutaraldehyde). Immobilization on agarose-glyoxyl permitted to immobilize around 40% of the enzyme in the first control (around 5 mg protein/g of support), without immobilizing more enzyme if the immobilization was permitted to proceed further. Using the agarose-glyoxyl support coated with PEI, 61% of the enzyme was immobilized in a similar time (around 7 mg protein/g of support). Anionic exchange permitted a higher immobilization yield. This may be due to the fact that the immobilization on agarose-glyoxyl at pH 7 involved the terminal amino groups of the enzyme, and perhaps they can be blocked in

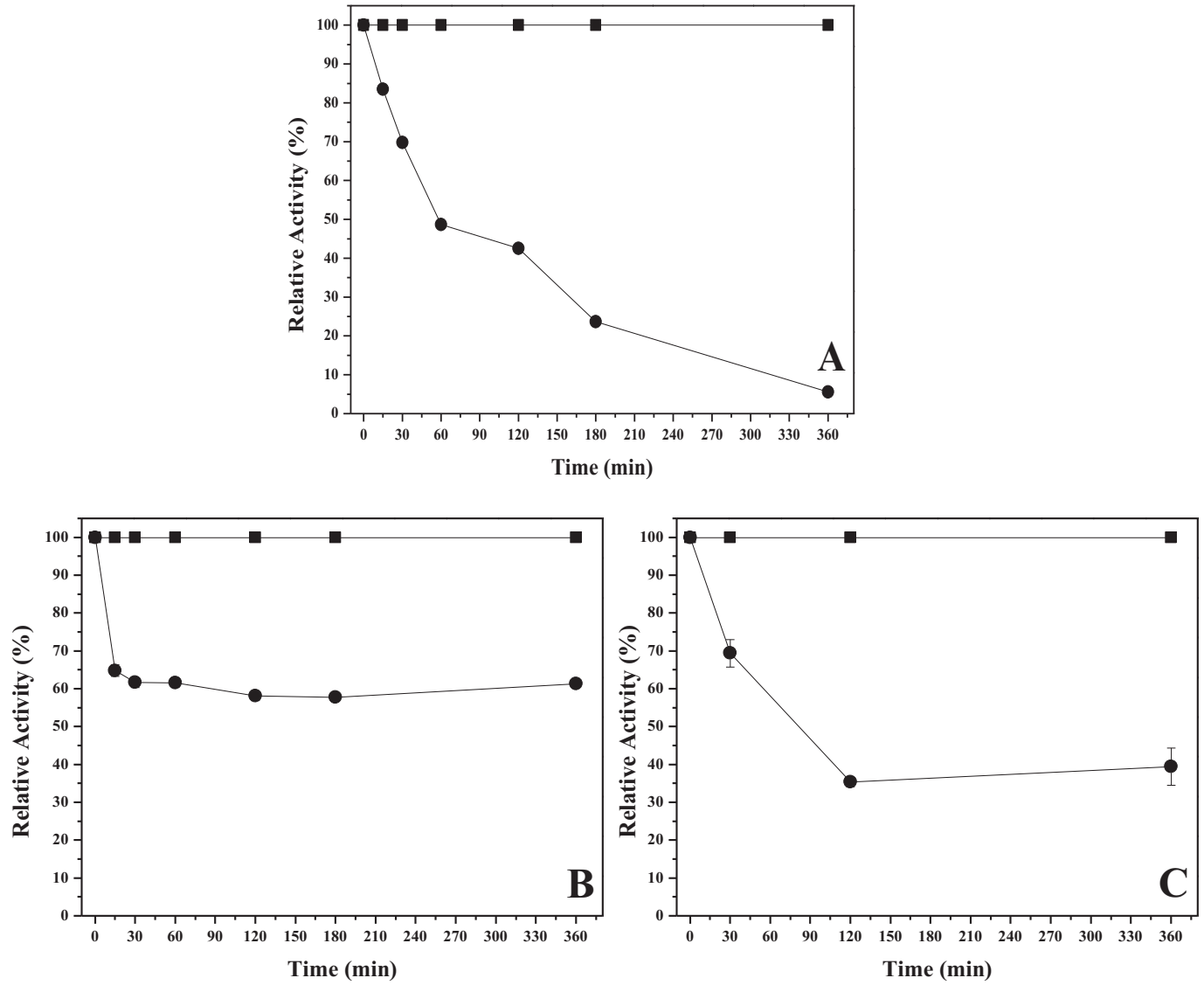


Fig. 1. Immobilization course of β-galactosidase from *Kluyveromyces lactis* at pH 7.0 and 25 °C in 100 mM potassium phosphate buffer containing 0.1 mM $MnCl_2$ and 0.2 mM $MgCl_2$, under 120 rpm stirring. β-galactosidase immobilized on: (A) chitosan_glutaraldehyde; (B) agarose-glyoxyl, (C) agarose-glyoxyl-PEI. (■) control; (●) supernatant.

some of the enzyme molecules by the sugar chains, making it impossible to immobilize those enzyme molecules in glyoxyl-agarose. In this protocol, it is necessary that the terminal amino groups of the enzyme may be available to interact with the support. Any steric hindrance of these groups may avoid enzyme immobilization [37]. The immobilization on chitosan activated with glutaraldehyde permitted to immobilize around 98% of the enzyme (around 8 mg protein/g of support), also in a very rapid fashion. Table 1 shows a drop in the activity after immobilization, but this can be associated to strong diffusional problems of oNPG.

The activity using lactose as substrate of the different preparations was 193.1 ± 18.9 U/g, 168.2 ± 22.3 U/g and 252.5 ± 45.7 U/g for Chi_Glu, Aga_Gly and Aga_PEI, respectively.

The stability of the different biocatalysts was also checked (Fig. 2 and Table 2). The stability of the covalently immobilized preparations was similar (slightly higher for the chitosan-glutaraldehyde preparations), while the enzyme immobilized in PEI was much less stable.

3.2. Performance of different biocatalysts for lactulose production

The different biocatalysts were used in the reaction of transglycosylation using lactose and fructose as substrates (Fig. 3). Using the enzyme immobilized on Aga_PEI a maximum of 11 g/L of lactulose was produced, when 17 g/L of lactose remained in the medium. In the process carried out with the enzyme immobilized on

Table 1
Immobilization parameters obtained in the process carried out at pH 7.0 and 25 °C in 100 mM potassium phosphate buffer with 0.1 mM $MnCl_2$ and 0.2 mM $MgCl_2$, under 120 rpm for 6 h using oNPG as substrate. β-galactosidase immobilized on: chitosan activated with glutaraldehyde (Chi_Glu); glycidol activated agarose (Aga_Gly); agarose activated with glycidol and coated with PEI (Aga_PEI). Different letters represent statistical differences at a 95% confidence level ($p < 0.05$).

Support	Immobilization Yield (%)	Offered activity (U/g)	Activity recovery (%)	Biocatalyst activity (U/g)
Chi_Glu	97.50 ± 0.2^c	1111.7 ± 62.5^a	3.59 ± 1.0^a	39.95 ± 9.54^b
Aga_Gly	38.59 ± 1.8^b	1129.3 ± 21.3^a	3.62 ± 0.3^a	40.88 ± 1.83^b
Aga_ PEI	60.54 ± 4.88^a	1093.6 ± 32.2^b	2.30 ± 0.2^b	25.19 ± 1.49^a

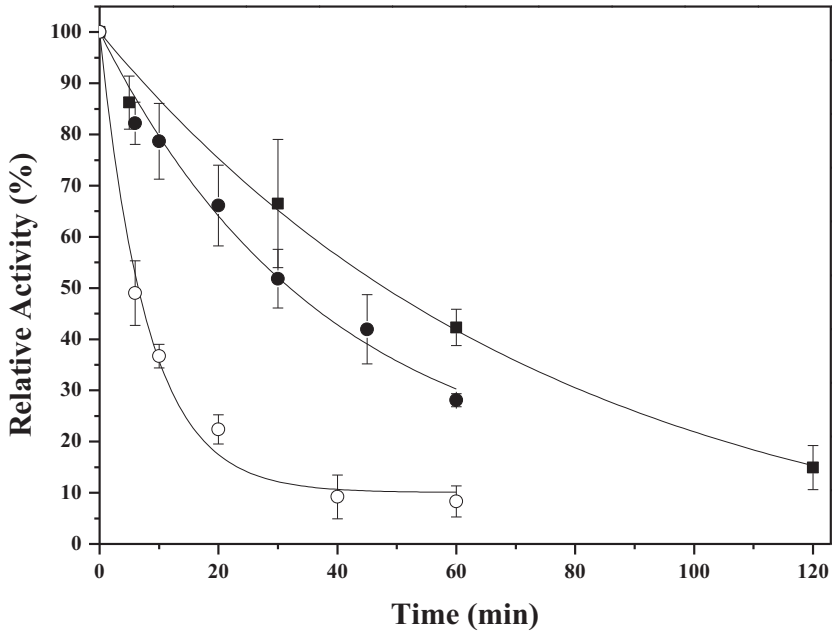


Fig. 2. Inactivation courses of the different β-galactosidase from *Kluyveromyces lactis* biocatalysts at 50 °C, pH 6.6 in 50 mM potassium phosphate buffer, containing 0.1 mM $MnCl_2$. (■) Chi_Glu_Gal; (●) Aga_Gly_Gal; (○) Aga_PEI_Gal. The curves represent the Sadana and Henley model.

Chi_Glu, only a maximum of 7 g/L lactulose was produced, reaching the maximum value when 23 g/L of lactose remained in the reaction medium. The reaction was extremely rapid and the lactulose was hydrolyzed, in a sharper way using glutaraldehyde preparations than using the PEI biocatalysts. This suggests that one of the main differences between both biocatalysts is the preference of the enzyme to hydrolyze lactulose or lactose (that is the second ratio that determines the maximum yield in a KCS) [4–7].

However, when studying the glyoxyl biocatalysts, the previous very significant differences between the other two biocatalysts appear to be almost negligible: the enzyme immobilized on glyoxyl agarose was unable to produce detectable amounts of lactulose. The lower activity observed in the hydrolysis of lactose already suggested that this biocatalyst presented a distorted enzyme conformation, but this complete disappearance of the transglycosylase activity was unexpected, suggesting a very strong tuning of the properties of the enzyme via immobilization. We have not found such strong modulation by immobilization results in literature, exemplifying in a very "dramatic way" how enzyme immobilization may alter the capacities of an enzyme as biocatalysts in kinetically controlled synthesis.

Similar to the results obtained using pure lactose, the enzyme immobilized on agarose-glyoxyl was not able to produce lactulose (results not shown) when cheese whey and fructose were used as feedstock (Fig. 4), while the enzymes immobilized on agarose-PEI or chitosan-glutaraldehyde produced lactulose using this substrate. Although the reaction was faster using cheese whey than using the synthetic medium, the amount of lactulose decreased to only 2.5 g/L lactulose using the chitosan-glutaraldehyde preparation when around 15 g/L of lactose remained in the medium, but this concentration remained around a similar value after several minutes, suggesting a slow hydrolysis of the product. In opposition, using the enzyme adsorbed on PEI, maximum yields are higher than using the synthetic medium (14 g/L of lactulose when 12 g/L of lactose remained in the supernatant).

When whey was used as a source of lactose, other components were presented in the reactional medium, for instance, ions (such as Ca^{2+}) and proteins, which may strongly affect the immobilized enzyme performance. These effects may be very different depending on the immobilization method, as reported in the literature for other enzymes [64–66], and also this effect agrees with our hypothesis that each immobilized enzyme has different properties, that are translatable to a different behavior in kinetically controlled synthesis and a different response to changes in the medium.

Freitas et al. [36] investigated the influence of ions in the enzymatic activity of beta-galactosidase and reported that Ca^{2+} and Zn^{2+} promoted inhibitory effects on β-galactosidase. The inhibitory effect is higher in the presence of Zn^{2+}. Mg^{2+} and Mn^{2+} (up to 1 mg/L), on the other hand, caused an activation of β-galactosidase. It is important to point out that whey contains a high amount of Ca^{2+}, which may have influenced enzyme activity and explains the lower yields obtained when using Chi-Glu-Gal. Aga_PEI_Gal, due to the coating with PEI, may have a positive charge on its surface. Therefore, a partition effect may cause a reduced calcium concentration in the environment near the enzyme, when compared to Chi_Glu_Gal. The primary amino groups on the surface of chitosan would have a similar partition effect, if it was not for the reaction with glutaraldehyde. In order to enhance lactulose production and yields, reaction medium and biocatalyst engineering should be conducted simultaneously.

Table 2
Deactivation parameters and half-life time of biocatalysts at pH 6.6 and 50 °C obtained using the Sadana and Henley model. Different letters represent statistical differences at a 95% confidence level ($p < 0.05$).

Biocatalyst	K_d (min^{-1})	α	R^2	$t_{1/2}$ (min)
Chi_Glu_Gal	0.0131	0.0010	0.99	47.4 ± 7.5[a]
Aga_Gly_Gal	0.0263	0.1211	0.99	32.0 ± 4.4[a]
Aga_PEI_Gal	0.1249	0.1005	0.99	6.5 ± 0.8[b]

3.3. Effect of temperature on the biocatalysts performance

When the reaction was performed at 25 °C or 37 °C, no detectable lactulose was detected using Aga_PEI_Gal biocatalyst, although lactose was consumed (results not shown). That is, it looks that the enzyme conformation at these temperatures did not favor the transferase properties of the immobilized enzymes. It seems that at 50 °C, an enzyme conformation that was active for this reaction was acquired, and that this form did not exist at 25 °C or 37 °C.

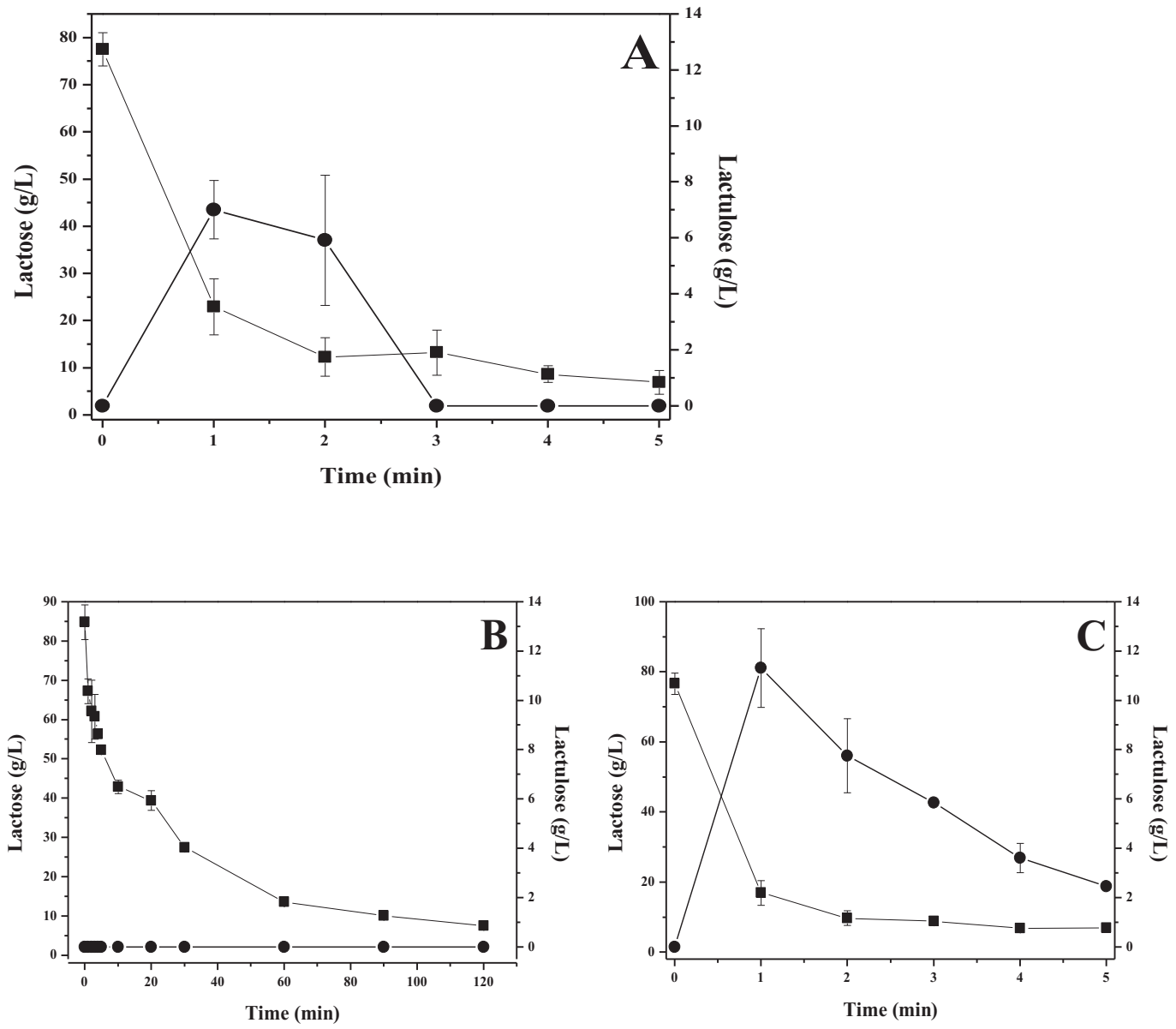


Fig. 3. Lactose hydrolysis and lactulose production by the β-galactosidase enzyme from *K. lactis* immobilized in: (A) chitosan_glutaraldehyde; (B) agarose-glyoxyl; (C) agarose_PEI. Reaction conditions: mass ratio of 1:2 lactose/fructose, total substrate concentration of 200 g/L, 50 °C, pH 7, 4 U/mL and 120 rpm. Concentrations of lactose (■) and lactulose (●).

We decide to check if this conformational change induced at 50 °C could be maintained and the immobilized enzyme, after incubating at 50 °C, could still have some activity in lactulose production at 25 °C or 37 °C. When the relatively unstable biocatalyst Aga_PEI_Gal was submitted to this process, lactulose production was not detected neither at 25 °C nor 37 °C, although lactose could be hydrolyzed (20% of hydrolysis, much lower than when performed at 50 °C, where around 90% of lactose was hydrolyzed).

When the Chi_Glu_Gal biocatalyst was directly used at 25 or 37 °C (Fig. 5A), the yields of lactulose are significant but much lower than at 50 °C (around 40% lower). However, after pre-incubating the biocatalyst at 50 °C for 10 min, no significant difference was observed in lactose hydrolysis conducted at 25 °C, 37 °C or 50 °C (Fig. 5B) by the Chi_Glu_Gal biocatalyst. The biocatalysts hydrolysis yields in the first measure at 25 °C, 37 °C and 50 °C were 93.16%, 93.18% and 94.18%, respectively. Curiously, after heating, the maximum lactulose concentration was slightly higher at 25 °C (6.7 g/L), when compared to those obtained at 37 °C (6.2 g/L) and 50 °C (6.2 g/L). The change of the enzyme features after incubation at 50 °C, the continuous exposition at 50 °C may have a detrimental effect on enzyme properties (as shown by the lack of operational stability, as it will be presented in the topic 3.4), while if after incubation the enzyme is used at 25 °C, the changes are maintained in a better way. In any case, the differences are not so significant as when studying other parameters.

These results further reinforced the hypothesis that a conformational change produced at 50 °C was positive for the use of these biocatalyst in KCS of lactulose, the higher stability of these biocatalysts permitted to appreciate this effect, while in the PEI adsorbed enzyme its low stability prevented detection this positive effect.

3.4. Operational stability of the biocatalysts

The operational stability of Aga_PEI_Gal when utilized under the reaction conditions (50 °C, 200 g/L substrate, pH 7, 4 U/mL and 120 rpm) using lactose and fructose medium is shown in Fig. 6. Focusing on lactose consumption (Fig. 6A), the biocatalysts activity remained almost unaltered after 9 reaction cycles, with a decrease in the activity in the 10th cycle. However, if we focus our attention on the lactulose

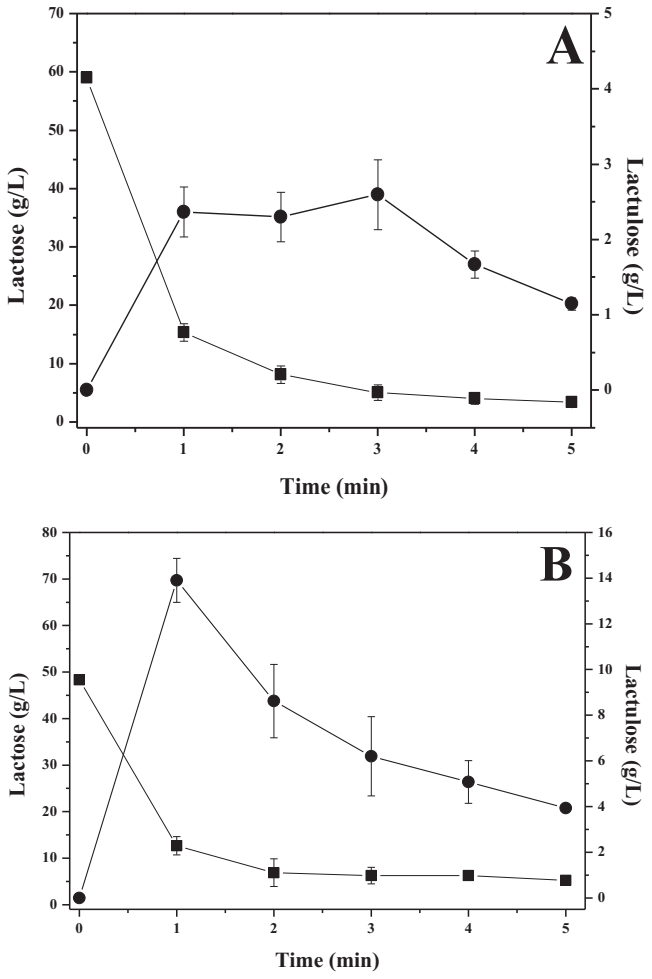


Fig. 4. Lactose hydrolysis and lactulose production using whey as a substrate for: (A) Chi_Glu_Gal biocatalyst; (B) Aga_PEI_Gal biocatalyst. Reaction conditions: mass ratio of 1:2 lactose/fructose, total substrate concentration of 200 g/L, 50 °C, pH 7, 4 U/mL and 120 rpm. Concentrations of lactose (■) and (●) lactulose.

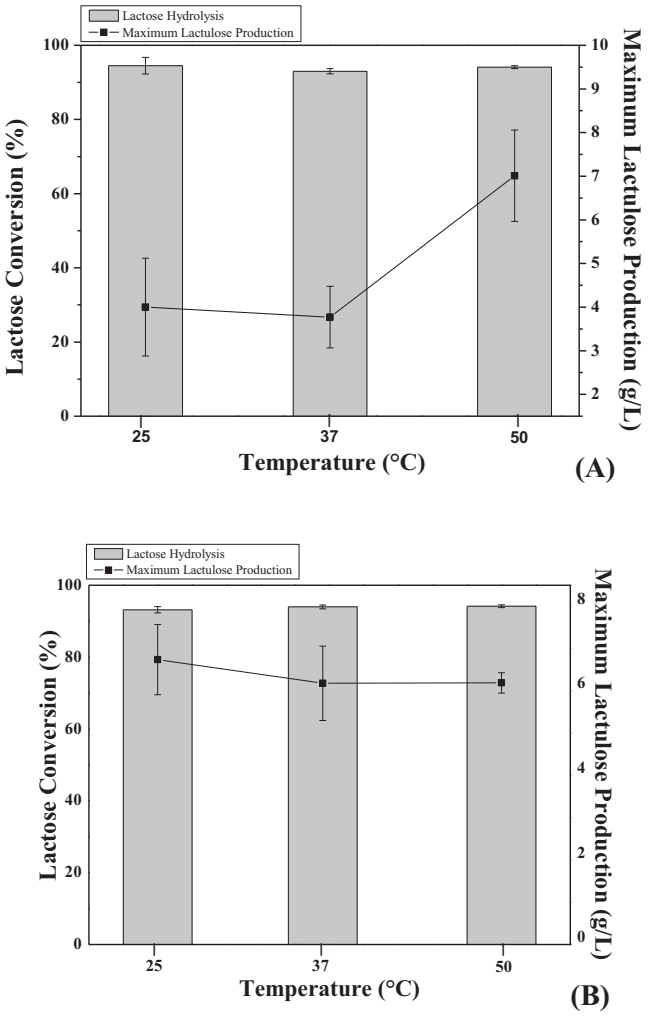


Fig. 5. Influence of temperature in the reaction after the incubation of the Chi_Glu_Gal biocatalyst at 50 °C for 10 min on the lactose hydrolysis and lactulose production: (A) Reaction without incubation and (B) reaction with incubation. Reaction conditions: mass ratio of 1:2 lactose/fructose; total substrate concentration of 200 g/L, 4 U/mL, 50 mM phosphate buffer pH 7.0 and 120 rpm.

production (Fig. 6B), the lactulose concentration dropped just in the second cycle (from 11 g/L to 5 g/L), and lactulose became undetectable in the third cycle. This is another unexpected result, as the activity versus lactose was almost fully maintained in all studied reaction cycles and suggests that at 50 °C the poor stability of the biocatalysts permitted some unfavorable change in the enzyme conformation that led to the full loss of the transglycosylation activity. There are some reports in the literature showing that the transglycosylation activity of other enzymes is also influenced by the temperature, but not using specifically the enzyme employed in this paper. These authors [67,68] report that the activity of transglycosylation increases is greater at temperatures in the range of 50 to 65 °C, but the enzyme does not show good stability in this temperature range. Therefore, this drastic effect of temperature in lactulose production with this specific enzyme has been firstly reported in this study.

The composition of the whey, perhaps the presence of some salts or some proteins, may strongly affect the immobilized enzyme performance in this kind of reactions, and these effects may be very different depending on the immobilization method, as mentioned in the discussion of the results in topic 3.2. There are studies in the literature that show that the immobilization method affects enzyme properties (specifically enzymatic stability) differently when they are immobilized by different protocols and evaluating different reaction media [64–66].

Using the enzyme immobilized on glutaraldehyde-chitosan, the lactose hydrolysis was maintained for 10 cycles (Fig. 7A) for the three evaluated temperatures. However, the produced lactulose observed varied along the different reaction cycles, slightly increasing or decreasing along the cycles. Only at 50 °C a clear decrease in the yield is appreciated in the 10th cycle (Fig. 7B). This did not correspond to changes in the global activity versus lactose (Fig. 7A), showing that the effects of small changes in enzyme structure that have not significant effects on enzyme hydrolytic activity may have great effects on the performance of a biocatalysts in KCS.

Fig. 8 shows an operational stability of Aga_PEI_Gal and Chi_Glu_Gal biocatalysts using cheese whey as feedstock in the reaction at 50 °C and it was observed a behavior of the immobilized enzymes fully different to that found above. Now, the enzyme activity, considering lactose consumption, decreased slowly in each cycle for both preparations, more clearly using the chitosan-glutaraldehyde preparation, but still maintained around 50–60% in the 10th cycle. This biocatalyst fully lost the lactulose production activity after 5 cycles.

However, using the enzyme immobilized on Aga_PEI, the lactulose production is almost maintained along the 10 cycles even though this was a less stable enzyme preparation. This may be explained by the presence of different compounds in the whey, which could stabilize the enzyme immobilized on PEI. That way, the components of the whey that were positive for the enzyme immobilized on agarose-PEI, were negative for the enzyme immobilized in glutaraldehyde-chitosan.

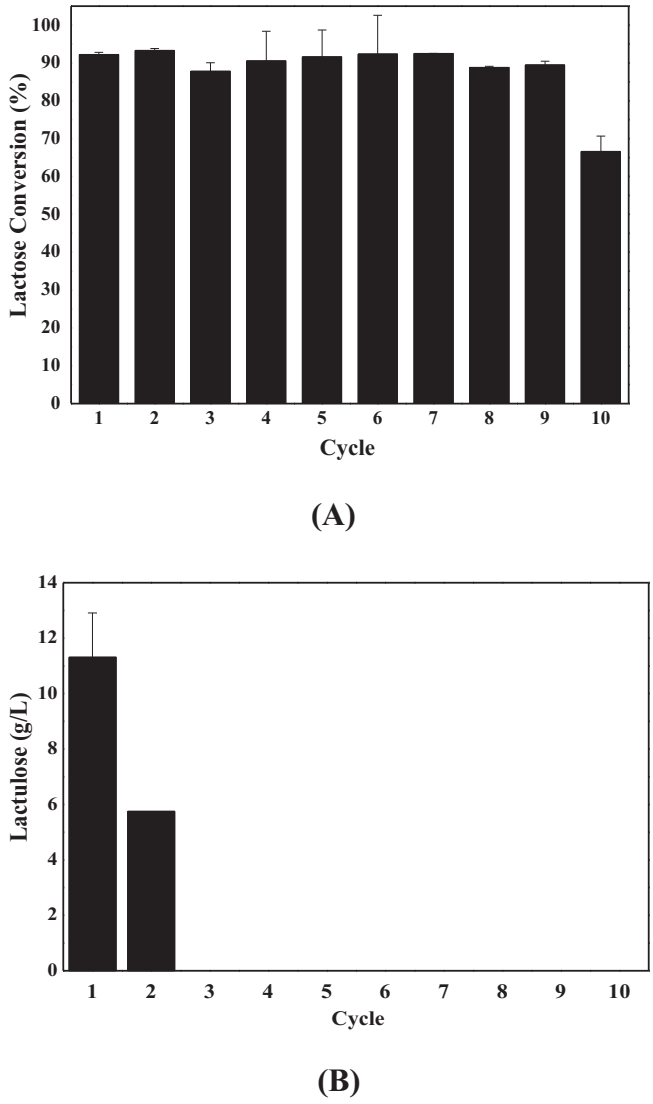


Fig. 6. Lactose conversion (A) and lactulose concentrations (B) obtained during the study of the operational stability of Aga_PEI_Gal in catalysis of synthetic lactose solution. Reaction conditions: mass ratio of 1:2 lactose/fructose, total substrate concentration of 200 g/L, 50 °C, 50 mM phosphate buffer pH 7, 4 U/mL and 120 rpm.

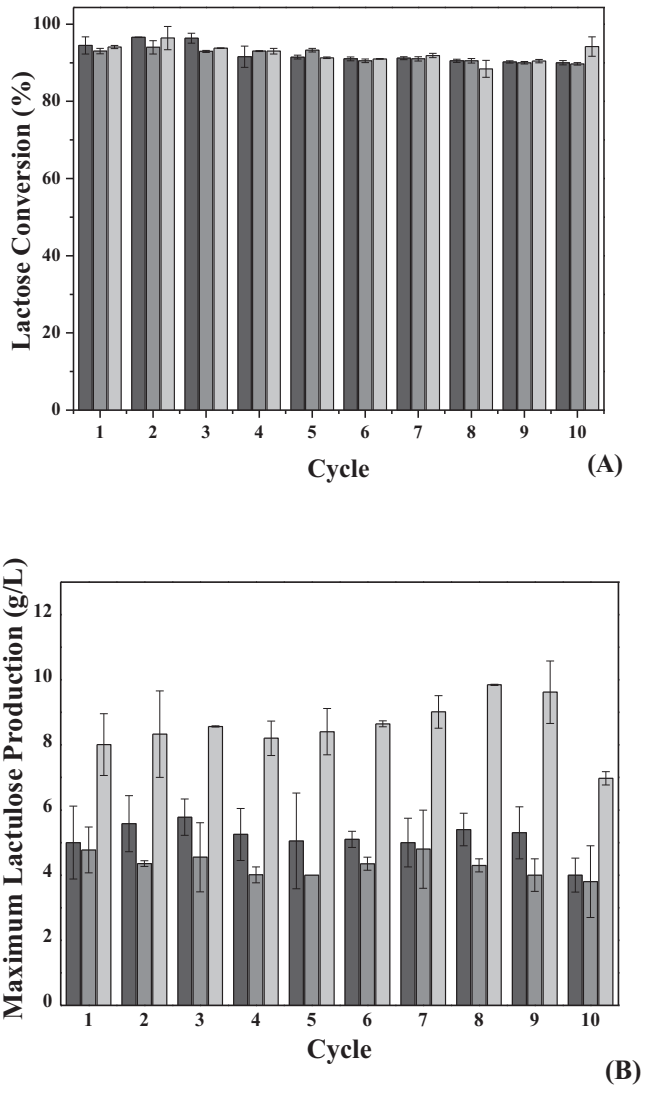


Fig. 7. Operational stability of Chi_Glu_Gal biocatalyst at 25 °C (dark gray bar, ■), 37 °C (gray bar, ■) and 50 °C (light gray bar, ■) without adaptation at 50 °C. (A) Evaluation of lactose hydrolysis percentage and (B) lactulose production, both per cycle. Reaction conditions: mass ratio of 1:2 lactose/fructose; total substrate concentration of 200 g/L; 4 U/mL, 50 °C, phosphate buffer pH 7.0 and 50 mM and 120 rpm.

4. Conclusions

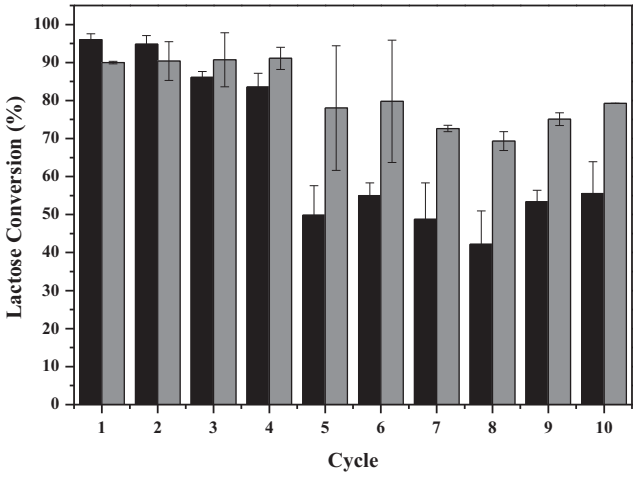
This paper shows that the immobilization of the β-galactosidase from *Kluyveromyces lactis* greatly alters the enzyme features in kinetically controlled synthesis. Using just 3 immobilization protocols, which follow very different mechanism, the prepared biocatalysts presented very different features: the enzyme immobilized on glyoxyl is fully inactive in the lactulose production at all assayed temperatures, the enzyme immobilized in PEI only is able to produce lactulose at 50 °C and only the enzyme immobilized via glutaraldehyde is active at 25, 37 and 50 °C in this reaction.

The pre-incubation of the biocatalysts at 50 °C permits to improve the results on lactulose production using the glutaraldehyde-biocatalysts, while having only a negative effect on enzyme activity using the enzyme absorbed on PEI.

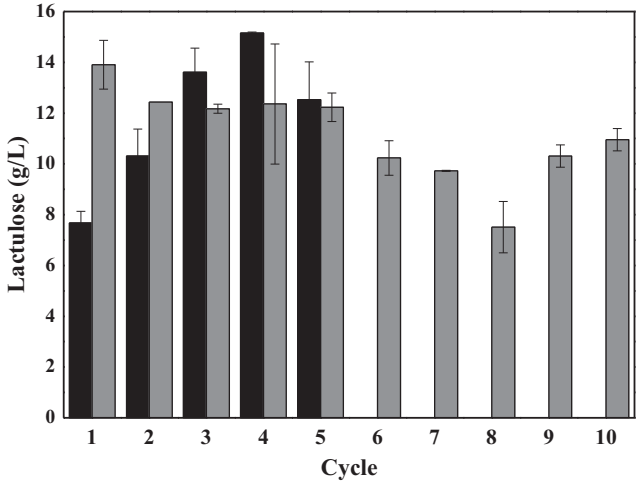
Variations in lactulose yields were observed when whey was used as a substrate in reactions with immobilized enzymes. The yield decreased in the biocatalysts in which glutaraldehyde was present. However, there was an increase in lactulose yield in biocatalysts that had PEI.

The operational stability analysis shows that very small changes in the enzyme, unable to alter the hydrolytic activity, may be enough to fully eliminate the transglycosylation activity of the enzyme. Thus, using lactose and fructose mixtures and high temperatures, the lactose consumption activity of both preparations was maintained, while the lactulose activity of the enzyme immobilized on PEI was gone after only two reaction cycles. The enzyme immobilized on glutaraldehyde maintained some transglycosidase activity throughout all the reaction cycles. But the situation reverses using whey, now the glutaraldehyde biocatalyst lost the lactulose production activity after 5 cycles, while the PEI biocatalysts maintained some activity throughout the 10 cycles. This suggested a dissimilar effect of the interactions of the whey components with the enzyme immobilized following different strategies. Only using mild reaction conditions, the biocatalyst could maintain results along time.

That way, the design of biocatalysts to be used in KCS and the process itself, must consider that is necessary to determine the operational stability in the target reaction and using the reaction medium to really know the enzyme performance in the reaction.



(A)



(B)

Fig. 8. Lactose conversion (A) and lactulose concentrations (B) obtained during the study of the operational stability of biocatalysts in catalysis of cheese whey solution. Reaction conditions: mass ratio of 1:2 lactose/fructose, total substrate concentration of 200 g/L, 50 °C, 50 mM phospahet buffer pH 7, 4 U/mL and 120 rpm. Black bars: Chi_Glu_Gal biocatalyst; Gray bars: Aga_PEI_Gal biocatalyst.

CRediT authorship contribution statement

In order to qualify for authorship of the submitted manuscript, the listed authors had substantial intellectual contributions both to the research and to its preparation. The authors involved in activities related to the following categories:

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2. Data acquisition and analysis/interpretation: **Carlos Alberto Chaves Girão Neto**, **Natan Câmara Gomes e Silva**, **Thaís de Oliveira Costa**, **Tiago Lima de Albuquerque**, **Roberto Fernandez-Lafuente**, **Maria Valderez Ponte Rocha**

3. Drafting of the article: **Carlos Alberto Chaves Girão Neto**, **Tiago Lima de Albuquerque**, **Roberto Fernandez-Lafuente**, **Maria Valderez Ponte Rocha**

4. Collaboration in the preparation/revision of the submitted manuscript: **Luciana Rocha Barros Gonçalves**, **Roberto Fernandez-Lafuente**, **Maria Valderez Ponte Rocha**

5. Participation in reaching the approval for the publishable manuscript version: **Roberto Fernandez-Lafuente**, **Maria Valderez Ponte Rocha**

6. Obtaining of funding: **Luciana Rocha Barros Gonçalves**, **Maria Valderez Ponte Rocha**

7. Final approval of the article: **Maria Valderez Ponte Rocha**

8. Ensuring a proper explanation to possible questions that could be raised regarding accuracy and scientific integrity of the submitted manuscript: **Maria Valderez Ponte Rocha**

Declaration of competing interest

The authors declare no conflict of interest. The authors report no commercial or proprietary interest in any product or concept discussed in this article.

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