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Review

β-Galactosidase from *Kluyveromyces lactis*: Characterization, production, immobilization and applications - A review

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ABSTRACT

A review on the enzyme β-galactosidase from *Kluyveromyces lactis* is presented, from the perspective of its structure and mechanisms of action, the main catalyzed reactions, the key factors influencing its activity, and selectivity, as well as the main techniques used for improving the biocatalyst functionality. Particular attention was given to the discussion of hydrolysis, transglycosylation, and galactosylation reactions, which are commonly mediated by this enzyme. In addition, the products generated from these processes were highlighted. Finally, biocatalyst improvement techniques are also discussed, such as enzyme immobilization and protein engineering. On these topics, the most recent immobilization strategies are presented, emphasizing processes that not only allow the recovery of the biocatalyst but also deliver enzymes that show better resistance to high temperatures, chemicals, and inhibitors. In addition, genetic engineering techniques to improve the catalytic properties of the β-galactosidases were reported. This review gathers information to allow the development of biocatalysts based on the β-galactosidase enzyme from *K. lactis*, aiming to improve existing bioprocesses or develop new ones.

1. Introduction

β-Galactosidases (β-D-galactohydrolase, EC 3.2.1.23) catalyze different reactions of industrial interest [1,2]. These enzymes may be obtained from several sources: plants [3,4], animals [5,6], and microorganisms such as fungi [7,8], bacteria [9,10], and yeasts [11]. β-Galactosidases from *Kluyveromyces lactis* (Kl-β-gal) are among the most reported in the literature [12–14], owing to the numerous applications they have enabled in the environmental, food, and biotechnological industries. In the food industry, this enzyme is essential in the manufacture of lactose-free dairy products since there is a vast and increasing number of people who cannot digest the carbohydrate [15]. Due to the diversity of lactose-free products developed in the last decades, the commercialization of β-galactosidases has great market potential. The most prominent company concerning the production of Kl-β-gal is Novozymes, under the trade name "Maxilac" [6].

Apart from the extensive use of β-galactosidases in dairy to obtain lactose-free products, they have also been employed in the treatment of commercially discarded whey [16]. Studies have proven the great potential of Kl-β-gal for lactose hydrolysis in dairy industries, with reported yields of above 99.5% [17]. This has enabled the employment of lactose hydrolysis in the manufacture of products targeted at many lactose-intolerant consumers worldwide, as well as in the conversion of lactose into more commercially useful sugars. Kl-β-gal is also able to produce 6′galactobiose (Gal-(1,6)-β-D-Gal), allolactose (Gal-(1,6)-β-D-Glc) and the trisaccharide 6′galactosyllactose (Gal-(1,6)-β-D-Gal-(1–4)-D-Glc) in large quantities by transglycosylation, and it shows a remarkably high hydrolytic activity when using lactose as a substrate [18].

Besides that, several works [2,19,20] have reported on galactooligosaccharides (GOS) production by Kl-β-gal. GOS are a group of substances containing two to eight monosaccharide units, with one of them being a terminal glucose and the other, galactose [21]. Lactulose

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also can be used as a substrate for the synthesis of GOS during transgalactosylation, which creates innovative opportunities for obtaining novel complex oligosaccharides with new structures and improved prebiotic properties. Enzymatic transgalactosylation of lactulose has been studied with different sources of β-galactosidase, including *Kluyveromyces lactis* [22].

In this context, this review addresses the main characteristics and the application potential of Kl-β-gal, with a particular focus on the dairy industry. Furthermore, several other relevant aspects, such as the structure, production, characterization, immobilization, and chemical and genetic modifications of this enzyme are also discussed.

2. Structure and catalytic mechanism

2.1. General structure

Based on their amino acid sequence, β-galactosidases are classified into four groups in the glycosyl hydrolases family (GH 1, GH 2, GH 35, and GH 42), with Kl-β-gal specifically belonging to the GH 2 group [23]. The enzyme comprises a tetramer of four identical polypeptide chains, each consisting of 1023 amino acids that interconnect to form five well-defined structural domains [24]. However, only one of these domains shows catalytic function, and its active site is formed mainly by residues from the third domain.

The Lac4 Kl-β-gal gene was sequenced in 1992 by Poch et al. [25], who confirmed that this yeast could ferment lactose [25,26]. The enzyme is formed by oligomers consisting of individual monomers with a molecular weight of 119 kDa that can form active dimeric or tetrameric structures.

This enzyme has two different active forms, attributed to the presence of dimers and tetramers in its structure. This oligomerization pattern was observed both in the crystal [12] and in the electrophoresis results [13]. In addition, this reinforces the hypothesis that the Kl-β-gal structural tetramer originates from a "dimerization of dimers". Another important point to note is the fact that the energy required to dissociate the homo-oligomer in two dimers is low (ΔGint = 6 kcal/mol) when compared to the energy required to dissociate the subunits of each dimer (ΔGint = 20 kcal/mol) [27]. That is, the KL-β-Gal tetramer is a set of dimers, with higher dissociation energy calculated for the dimers than for their assembly, which, according to Pereira-Rodríguez et al. [27], could explain the fact that, in solution, the dimeric and tetrameric forms of the enzyme are in equilibrium.

Rico-Díaz et al. [28] reported that Kl-β-gal dimers are unstable and can dissociate into monomeric forms. The thiol reagents used by the authors (2-mercaptoethanol and dithiothreitol (DTT)) caused the balance to shift completely towards the dimer form. It was also observed that the native PAGE dimer band was sharp and distinct in the gel. This indicates that little or no dissociation took place during the runs in native PAGE. Dithiothreitol had a similar effect to that of 2-mercaptoethanol, but the concentration of DTT necessary to cause the formation of the dimer was lower due to the higher equilibrium constant for the reduction of DTT and to the lower amount of disulfide present in DTT. The control groups used in these experiments showed that reagents with structures similar to mercaptoethanol and DTT that do not contain sulfhydryl groups (e.g., ethanol and glycerol) had no effect on the monomer-dimer balance, indicating that the reduction of the —SH groups is highly relevant.

Fig. 1 shows how the chains of monomers A–C and B–D form two dimers, leading to the structure known as "dimer of dimers". The tetramer stabilization is due to the assembly of these dimers, which occurs essentially through the interaction between monomers A and B. On the other hand, there are also some interactions between monomers A and D and monomers B and C. The surface area under the identical interfaces formed by monomers A–C and B–D is 2521 Å^2 (Surface 1). The interface responsible for the greatest contact between the dimers that stabilize the tetramer encompasses a surface area of 2438 Å^2 (Surface 2)

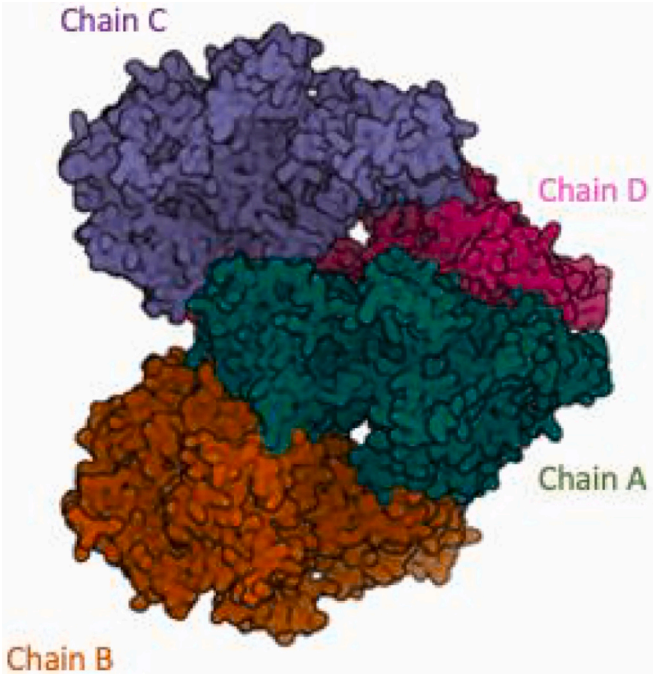

Fig. 1. Structure of Kl-β-gal: surface representation, showing the 4 monomers that comprises the enzyme [250] PBD: 3OBA.

between monomers A and B. Other interfaces that contribute to the stabilization of the tetramer include the monomers A and D, and the monomers B and C, with a surface area of 350 Å^2 (Surface 3). The interactions between the monomers of the surfaces described are largely non-polar (approx. 75% on surface 1 and approx. 65% on surfaces 2 and 3) and, with the formation of the tetramer, the total external surface area is reduced by approximately 11% [27].

The location of the catalytic site in Kl-β-gal was also reported in the literature [27]. Despite the different enzyme sources and structures, the active site was pinpointed between domains 1, 3, and 5 of the monomers. In the case of the first study, the dimerization of the dimers was shown to cause the catalytic sites to move slightly downwards since they are located opposite each other at the interfaces of the dimers. This cavity-shaped arrangement, together with a fold in domain 3 of each monomer, creates a tunnel-like structure (approx. 10 Å wide) to enable access to the enzymatic active site. Similarly, in the second study, the catalytic site of the enzyme was located at the bottom of a funnel-like structure that starts at the top of domain 3 and that is complemented by parts of the chain originating in domains 1 and 5 [27,29].

2.2. Reactions and mechanisms of action of Kl-β-gal

β-Galactosidase possesses hydrolytic and transgalactosylation activities [30,31]. The hydrolytic potential of Kl-β-gal is vastly exploited by the dairy industry to produce compounds with low or no lactose content [32]. Furthermore, several studies [33–36] have also demonstrated its potential for manufacturing galactooligosaccharides (GOS) via its transgalactosylation activity. Both activities can occur simultaneously, but the latter is prevalent when the lactose concentration is high.

In the hydrolysis reactions catalyzed by β-galactosidase, the formation of an enzyme–galactosyl complex occurs upon a simultaneous liberation of glucose and a transfer of the enzyme–galactosyl complex to an acceptor that contains a hydroxyl group [37]. Glucose and galactose are formed by a hydrolytic reaction from lactose, and galactose is obtained as a product if the acceptor in the reaction is the water [37–39].

β-Galactosidases belong to the subclass of glycosidase, and as such, they also catalyze reactions called reverse hydrolysis, which is an

equilibrium or thermodynamically controlled process in which a free monosaccharide is combined with a nucleophile, excluding a water molecule [40,41]. According to Maksimainen [42], β-galactosidases mainly use this reverse hydrolysis mechanism instead of transglycosylation for the synthesis of GOS, and the reaction is controlled mainly thermodynamically. In the case of lactose hydrolysis, as opposed to reverse hydrolysis, a kinetically controlled mechanism occurs. The reaction includes an intermediate step in which a covalent bond is formed between galactose C1 and Glu307. The intermediate product can be released either by a water molecule, leading to complete hydrolysis, or by an acceptor molecule such as glucose (which leads to the formation of the lactose substrate), another oligosaccharide, or alcohol. Due to the release of water molecules, a condensation reaction occurs. However, it is important to keep in mind that the hydrolysis of lactose is an endothermic reaction, and the chemical balance strongly favors the formation of monosaccharides. Due to the inherent entropy in this reaction, the state of final equilibrium also contributes to the generation of small amounts of GOS [42]. Also, according to this author [42], methods to synthesize GOS include the use of extremely high concentrations of lactose at very high temperatures, favoring the formation of endothermic products (oligosaccharides) and implying that the reaction is strongly thermodynamically controlled.

The lactose present in the medium can also act as an acceptor, and, under these circumstances, GOS are formed instead via a transgalactosylation reaction [37–39,43,44]. This reaction is classified as a kinetically-controlled synthesis (KCS) [45]. Similar to the GOS formation process, in a KCS reaction, after the maximum rates are reached, the lactulose concentration decreases due to the subsequent hydrolysis and formation of fructosyl-galactooligosaccharides (fGOS) [30,46]. The kinetically-controlled transglycosylation reaction employs an activated glycoside to tackle a leaving group, as well as to reduce the water activity [47]. Kasche [48] was one of the first scientists to discuss this reaction system, reporting that during an enzymatic KCS, a nucleophile attacks the substrate-enzyme complex and initiates the production of the desired product. At the same time, water also acts as a nucleophile, and competitive hydrolysis of the enzyme-substrate complex occurs. The rates of lactulose synthesis, for example, being a KCS, could, in theory, be increased by the continuous removal of the final product. To evaluate this theory, Boon et al. [49] demonstrated that production yields could, in fact, be increased by the continuous removal of oligosaccharides from the reaction mixture. These studies used methods based on carbon adsorption to continuously remove the product from the medium and observed that by using this strategy, there was an increase of 23% in relation to a process where there was no product removal.

Schuster-Wolff-Bühring [50] reported that both the tertiary structure and the amino acids present in the active site could affect the selectivity of galactosidase. The authors also report that selectivity can also be affected by the donor (lactose) and acceptor molecules (lactose, in the case of GOS, or fructose, in the case of lactulose) in transgalactosylated galactose. Comparatively, reverse hydrolysis is controlled thermodynamically, but the reaction occurs more slowly [51].

Enzyme source is the main factor that can favor or disfavor the reactions of hydrolysis and transgalactosylation. For example, β-galactosidases from *Aspergillus oryzae* and *Bacillus circulans* have more intense transgalactosylation activity, while those from *K. lactis* and *K. fragilis* tend to favor hydrolysis [26,36,52]. In the case of Kl-β-gal, its high hydrolytic performance may be associated with an exclusive insertion in loop 420-443 of its catalytic site. Such insertion acts as a specific link for lactose, increasing the specificity of the enzyme for this substrate [27]. In more detail, the mentioned insert projects and bonds to the aglycone portion of the coupled lactose, which would lead to a high affinity of Kl-β-gal for this substrate which would strongly increase the hydrolytic activity of the enzyme [18]. Such insertion acts as a specific link for lactose, increasing the specificity of the enzyme with this substrate [27]. Besides, the enzyme's natural affinity for donors (in general, lactose) and/or for acceptors (fructose, lactulose, and lactose) will depend on the source of the enzyme. Also, the balance between hydrolytic and transgalactosylation activities can be altered through changes in the concentration of the galactosyl donor [30]. And the selection of this compound is a key aspect, as it is often the limiting substrate in transgalactosylation reactions [53].

β-Galactosidases catalyze reactions with β-D-galacto-pyranosides that contain glycosidic oxygen bonds [54], but they can also react with other substrates containing other types of glycosidic bonds, such as nitrogen [55] or fluorine [56]. However, a reduction in catalytic efficiency is usually observed in the latter groups [57]. Also, these functional groups must be present in the correct conformation for the enzyme to be able to catalyze the aforementioned reactions. Reverse reactions, as in the case of reverse hydrolysis, occur in the presence of high concentrations of substrates that contain those functional groups in different orientations and/or in their absence, as is the case with D-galactopyranose, L-arabinopyranose, and D-fucopyranose. In the presence of these compounds, the specificity of the enzyme changes and reverse reactions are favored [57].

Rutkiewicz et al. [58] reported that the active site cavity has an acidic character throughout, which facilitates the binding of the saccharide substrate, usually lactose. This type of active site cavity is observed in β-galactosidases with transglycosylation activities (like those obtained from *Arthrobacter* sp. 32cB, *Escherichia coli*, and *K. lactis*) and it facilitates the attachment of the galactosyl group to other acceptors, such as galactose, fructose, or salicin. Also, monomers' architectures are clearly similar in these β-galactosidases, which enabled the determination of the catalytic residues E441 and E517. Kl-β-gal enzymes are classified as homodimers and homotetramers, in which each subunit has a molecular mass of approximately 119 kDa [59]. Juers et al. [57] also reported that the critical elements of the active site also included amino acids from other locations of the same polypeptide chain, as well as from other chains in the tetramer.

Several reports [60–63] showed that lactose and D-galactose could stabilize the overall structure of the enzyme by coupling the substrate to its active site. The binding of reaction products and other ligands to a protein is a straightforward way to stabilize its conformation, and this is a widely-used strategy to stabilize proteins and enzymes during various operations [64].

2.3. Galactosylation and transgalactosylation

As previously emphasized, lactose hydrolysis can trigger GOS production by transgalactosylation [19,30,38]. This path is usually taken when the galactosyl acceptor is another saccharide (e.g., glucose, galactose, lactose, or a smaller GOS molecule). If the galactosyl acceptor is water, then a hydrolysis reaction occurs instead, leading to an undesired hydrolytic degradation of GOS and lactose and a consequent production and release of galactose and glucose units into the medium [65,66]. Guerrero et al. [30], for example, reported on the effect of donor and acceptor concentrations on the hydrolysis and transgalactosylation activities of β-galactosidase. The authors stated that the balance between both activities could be adjusted by changing the concentration of the galactosyl-donor, i.e., galactose is the limiting substrate for transgalactosylation, which means that this reaction can be favored by varying the saccharide concentration.

Zhu et al. [36], for instance, studied GOS production using goat's milk treated with two commercial β-galactosidases from *K. lactis* and *A. oryzae*. The authors observed that the Kl-β-gal was more efficient than its *A. oryzae* counterpart to produce 6-galactobiose, allolactose, and 6-galactosyllactose. Guerrero et al. [30], in turn, evaluated three different β-galactosidases, from *A. oryzae*, *B. circulans*, and *K. lactis*. The authors observed that the *A. oryzae* and *B. circulans* β-galactosidases exhibited greater transgalactosylation activity compared to their *K. lactis* β-galactosidase. However, Kl-β-gal showed higher hydrolytic activity.

Other authors [67] also evaluated the GOS synthesis during the

lactose hydrolysis of goat milk using Kl-β-gal; the maximum GOS production was 2.91 g/kg of lactose after 20 min of reaction. However, the hydrolysis level was higher than 92.3%, showing that hydrolytic activity prevailed over transgalactosylation in this process [67]. Another important factor that favors the simultaneous lactose hydrolysis and GOS production is that the compounds formed by transgalactosylation reactions can act as a substrate for reverse hydrolysis due to the high affinity of this enzyme towards the carbohydrates present in the reaction medium [19]. Kl-β-gal has a tendency to favor the lactose hydrolysis reaction and promote the reverse hydrolysis of the obtained oligosaccharides [35,68].

β-Galactosidases can also act on other carbohydrates, such as fructose or sucrose, leading to different transgalactosylated compounds. This triggers the synthesis of other molecules, such as lactulose [30]. Lactulose is a synthetic disaccharide composed of fructose and galactose, whose chemical nomenclature is 4-O-β-D-galactopyranosyl-D-fructofuranose. Lactulose has shown therapeutic and health-promoting properties by stimulating the growth of microorganisms whose action is beneficial for the body, and they can also be employed in the treatment of chronic constipation and hepatic encephalopathy [69,70].

The enzymatic synthesis of lactulose occurs through either a molecular rearrangement of lactose or the formation of a β-glycosidic bridge between galactose and fructose [68,71]. The lactulose production catalyzed by β-galactosidase from lactose requires fructose as a second substrate and, in general, yields lower conversion levels. This process is also a KCS, where lactose acts as an activated acyl donor, and fructose, as a nucleophile [49,72]. In KCS reactions, the maximum yields are not usually the final yields, as the product may be the substrate of the enzyme. Thus, the yield is determined by the ratio of lactose conversion and lactulose synthesis, i.e., final yields are predetermined by intrinsic characteristics such as the properties and source of the enzyme [73–76].

There are reports in the literature that confirm that the concentration of lactulose produced will be at its maximum when fructose, as a galactosyl acceptor, is present at a higher concentration than water during the hydrolysis of lactose [34,77]. The determination of lactulose yields is done by assessing the availability of fructose and the possibility of continuous removal of lactulose during the reaction. Fig. 2 presents a schematic with the main reactions catalyzed by β-galactosidase and highlighting the reactions of hydrolysis and transgalactosylation.

2.4. Other β-galactosidase applications

An alternative application that presents high technological potential is the use of Kl-β-gal to treat residual cheese whey permeates in ethanol production. Sampaio et al. [78] investigated the activity of Kl-β-gal, and the impact of enzyme pretreatment on cheese whey fermentation, in their original and concentrated forms, in terms of ethanol production. The authors also looked into the potential of reducing the release of the waste generated in the process into the environment. Although the enzyme was not applied directly to the system, since the yeast fermented the substrate, the results obtained in this study were still satisfactory. The highest concentration of ethanol was 15.0 $g{\cdot}L^{-1}$, and the lactose yield and productivity were 0.47 $g{\cdot}g^{-1}$ and 0.31 $g{\cdot}L^{-1}{\cdot}h^{-1}$, respectively.

The Kl-β-gal application for the synthesis of ascorbic acid galactosides, which are molecules that show numerous beneficial properties, by transgalactosylation using lactose as the donor of the galactosyl portion, has been studied [79]. It was reported that 12.7 g/L of ascorbic acid was formed in the most favorable condition, which were: 50% (*w/v*) sodium ascorbate, and 28,600 U of enzyme per 100 g of lactose at pH 7.0. The addition of Mg^{2+} or K^{+} ions to the reaction medium caused an increase in the content of the final product (up to 17.2 g/L), while Na^{+} or Mn^{2+} had an adverse impact on yield.

Other studies show the use of Kl-β-gal in strategies for simultaneous reactions. Jin et al. [1] evaluated the simultaneous hydrolysis and cofermentation of whey lactose with wheat for ethanol production using β-galactosidases from *K. lactis* and *A. oryzae* and the yeast *Saccharomyces cerevisiae*. However, in this scenario, it was found that β-galactosidase from *A. oryzae* was more effective for lactose hydrolysis during cofermentation, and the whey permeate supplement promoted ethanol production. The simultaneous hydrolysis of whey and lactulose production was recently investigated by De Freitas et al. [80] using the biocatalyst obtained from immobilization of Kl-β-gal in chitosan-glutaraldehyde; the authors reached a lactulose production of 17.32 $g{\cdot}L^{-1}$.

The application of Kl-β-gal in the conversion of lactose into new products with high added value has become a research priority

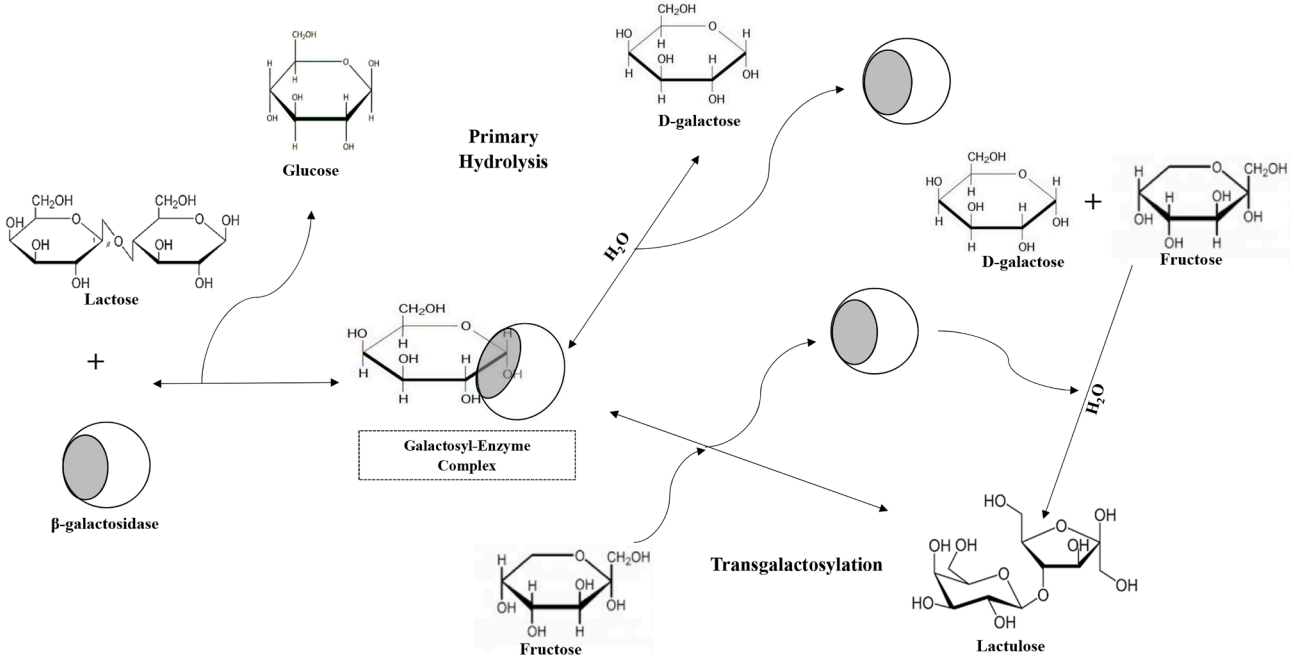

Fig. 2. Transgalactosylation products in the presence of fructose catalyzed by β-galactosidase.
*Adapted from Sitanggang et al. [243].

[81,82,83]. Jayamuthunagai et al. [83] developed a two-step process for the enzymatic production of D-tagatose from aqueous lactose. D-Tagatose, a prebiotic carbohydrate, is an isomer of D-galactose and it is commercially produced via enzymatic isomerization of galactose by L-arabinose isomerase (L-AI) [84,85]. The hydrolysis of lactose catalyzed by β-galactosidase is first performed to produce glucose and galactose. Subsequently, the galactose formed is isomerized to D-tagatose by the enzyme L-arabinose isomerase. Fig. 3 depicts a diagram with these reactions.

Padilla et al. [85] described a strategy to increase the diversity of potentially prebiotic carbohydrates using β-galactosidases from *K. lactis* and *K. marxianus* to transgalactosylate lactose from cheese whey permeate to obtain a mixture with a higher content of oligosaccharides. Isomerization of the transgalactosylated product was carried out using sodium aluminate as a catalyst. This strategy allowed to achieve a mixture (50 g/100 g total carbohydrates) composed of tagatose, lactulose, GOS, and prebiotic oligosaccharides (OsLu). The procedure proposed (transgalactosylation and isomerization of cheese whey permeate) yielded 322 g prebiotics/kg whey permeate.

Table 1 summarizes different reactions catalyzed by Kl-β-gal, as well as the optimal conditions for generating each bioproduct.

3. Factors influencing β-galactosidase activity

3.1. Reaction medium components and presence of inhibitors

Besides enzyme source, substrate concentration and the composition of the reaction medium are key factors that affect the activity of Kl-β-gal [86].

Lactose hydrolysis is a key industrial process, especially in the food industry, that generates galactose and glucose as reaction products [87,88]. However, some authors have highlighted that this reaction can be hindered by product inhibition, such as glucose and galactose, which reduces the reaction rates of both hydrolysis and transgalactosylation. In some cases, this phenomenon was even reported to completely halt the reaction, preventing it from being fully carried out [88–91].

Some kinetic models for lactose hydrolysis [92] have demonstrated that glucose is the first molecule to be released from the active site, leaving an enzyme-galactosyl complex for subsequent hydrolysis. In the second step, the enzyme-galactosyl complex is transferred to an acceptor containing a hydroxyl group. In diluted solutions, lactose itself may be more competitive to be an acceptor. On the other hand, in a high lactose solution, lactose molecules are more likely to act as acceptors, binding to the enzyme-galactose complex to form trisaccharides.

Klein et al. [60] investigated the effect of substrate and products (D-glucose and D-galactose) on enzyme stability. According to the results published by these authors, when only D-glucose (200 $g{\cdot}L^{-1}$) was present, the chitosan-immobilized β-galactosidase showed about 50% of its initial activity after 11 min of incubation; also, the stability of the enzyme did not increase significantly compared to its inactivation under non-reactive conditions (4.8 min). Conversely, when the inactivation was performed in the presence of D-galactose (200 $g{\cdot}L^{-1}$), the residual activity of β-D-galactosidase was about 47% after 30 min of incubation which was greater than that obtained with D-glucose in the same concentration. For Kl-β-gal, D-glucose is known to be a noncompetitive inhibitor [93], i.e., lactose and D-glucose will bind independently and in different locations of the enzyme. Some inhibitors affect only one enzyme or a group of closely related enzymes. Alcohols, for example, can change the polarity of the solvent and, consequently, affect the ionization of the main residues of the active site, such as Glu-482 and Glu-551 [59].

Gosling et al. [65] report that Kl-β-gal was inhibited by glucose and galactose, but only the latter reduced the final concentration of the GOS synthesized by the enzyme. Galactose is a competitive inhibitor [35] more effective in inhibiting lactose hydrolysis than glucose. When it is formed, a complex of galactosyl is also produced, competing for the site of the active enzyme, and reducing its availability to catalyze reactions [91], making complete lactose hydrolysis difficult [84,94]. This may limit its use in industrial processes. However, structural changes in the enzyme's active site can reduce the inhibitory effect of galactose, consequently increasing its catalytic performance [94].

Alterations in the active site structure reduce the competitive inhibition effect of galactose, thus enhancing its catalytic properties for industrial applications [88,89,94]. Some strategies have been studied, such as protein engineering involving the affinity of the enzyme-substrate bond, which may result in greater catalytic efficiency. Andrade et al. [88], for example, applied computational techniques to reduce the inhibition effects by galactose in the enzyme's active site, hindering complete lactose hydrolysis. Therefore, an alternative to circumvent this inhibition was to implement mutations in the enzyme's active site. The single mutations showed better results, with the technique enabling a stronger effect on the binding energy of galactose than on lactose. Moreover, enzyme immobilization strategies can also decrease the levels of inhibitory effects and alter important kinetic properties [90,95].

Additionally, the negative effects of tannic acid on the catalytic

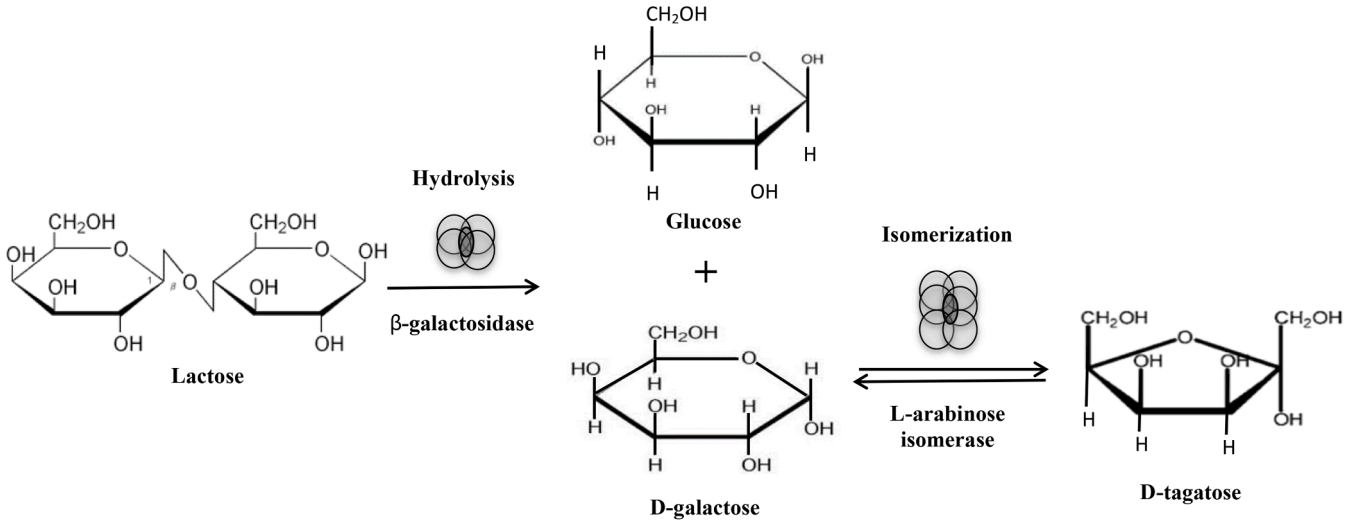

Fig. 3. Use of β-galactosidase enzyme in the process of D-tagatose production.

Table 1
Reaction conditions for the use of β-galactosidase from *Kluyveromyces lactis* (Kl-β-gal) as a biocatalyst in different processes.

Microorganism source/ commercial enzyme	Microorganisms expressed	Obtained product	Immobilization support	Optimal reaction conditions			Reference
				pH	Temperature (°C)	Ions added	
K. lactis NRRL Y1564	–	Lactulose	Chitosan-glutaraldehyde	7.0	50	Mn^{2+}	De Freitas et al. [80]
Kl-β-gal GODO–YNL2 (DuPont™)	–	GOS	Free enzyme	4.4	37	–	Kaczyński et al. [67]
Lactomax Pure (Prozyn Company)	–	Lactose hydrolysis	Immobead 150 using	7.0	40	–	Gennari t al. [228]
Lactozyme 2600 L (Novozymes)	–	GOS	Free enzyme	7.0	37	Mn^{2+} and Na^{+}	Wojciechowska et al. [79]
Lactozym (Sigma)		Lactulose	Chitosan-glutaraldehyde	7.0	50	Mg^{2+}	Albuquerque et al. [77]
Lactozym 2600 L (Novozymes)		GOS	Polysiloxane – polyvinyl alcohol (POS-PVA) and	4.5	60	–	González-Cataño et al. [21]
Kl-β-gal from Novozymes	–	Galactosyl mannitol derivative	Free enzyme	6.5	37	–	Klewicki et al. [113]
Lactozym Pure 2600 L (Novozymes)	–	Ascorbic acid galactoside	Free enzyme	7.0	37	Mg^{2+}/K^{+}	Wojciechowska et al. [79]
Lactozym Pure 6500 L (Novozymes)	–	GOS	Free enzyme	7.0	40	Mg^{2+}	González-Delgado et al. [74]
K. lactis GODO-YNL2	*Escherichia coli*	Lactose hydrolysis	Free enzyme	6.8		Mg^{2+}	Erich et al. [17]
K. lactis and CECT 13121		GOS	Free enzyme	6.5	50	–	Padilla et al. [85]
Maxilact 5000 (DSM)	–	Lactulose	Free enzyme	7.5	40	Mg^{2+}	Hua et al. [244]
Lactozyme Pure 6500 L (Novozymes)	–	Fructosyl-galacto-oligosaccharides	Free enzyme	4.5	40	–	Guerrero et al. [245]
Kl-β-gal (not specified)	*Arxula adeninivorans*	β-D-galactopyranoside	Free enzyme	6.5	35	–	Rauter et al. [246]
Maxilact® 5000 (DSM)		GOS	Free enzyme	6.8	38	–	Shen et al. [247]
Lactozym 3000 L HP G (Novozymes)	–	GOS	Free enzyme	6.8	40	–	Rodriguez-Colinas et al. [248]
K. lactis ATCC 8585	*Escherichia coli*	Not applied	Free enzyme	7.0	40	Mn^{2+}	Kim et al. [249]

activity of Kl-β-gal were evaluated by kinetic analysis and correlated with changes in the structure of the enzyme [96]. From the results of this work, it is clear that changes in conformation and enzyme activity must be taken into account when dairy products are to be consumed along with tannin-rich foods.

Albuquerque et al. [77] evaluated the influence of the lactose/fructose ratio on lactulose synthesis using Kl-β-gal. The different lactose/fructose ratios tested were: (1) 40%: 5%, (2) 35%: 10%, (3) 30%: 15%, (4) 22.5%: 22.5%, (5) 15%: 30% and (6) 10%: 35% *w*/w. The maximum lactulose production (10.34 g/L) was achieved using 15% (*w/v*) lactose and 30% (w/v) fructose. In the reaction conducted using a lactose/fructose ratio of 10%:35% w/v, the hydrolysis of lactose was increased; however, the lactulose concentration was reduced to approximately 8 g/L. Also, the lactulose concentration increased along with the increase in the concentration of fructose. Similar studies [34,71,97] have reported results that show a similar trend, in which there is a need for a high initial lactose concentration to enable lactulose synthesis.

The presence of polyols can also influence the activity of the Kl-β-gal. Athès and Combes [98] reported that the presence of different polyols containing 3-6 carbon atoms (such as glycerol (C3), erythritol (C4), xylitol (C5), and sorbitol (C6)), and these polyols showed a positive effect on enzyme activity at high pressures. The polyol with the greatest influence was xylitol, as a stabilizing factor of 70 (at 1 M) and more than 10,000 (at 2 M).

There are also studies that analyze the physicochemical properties of deep eutectic solvents and their usefulness as an element of the reaction medium for Kl-β-gal. Hoppe et al. [99] evaluated the influence of choline chloride, choline acetate, and hydrogen bonds donors, such as glycerol, ethylene glycol, urea, thiourea, and levulinic acid, in the activity of Kl-β-gal. The results showed that the reaction medium with an adequate concentration of eutectic solvents based on choline acetate had a beneficial effect on the enzyme activity. The addition of 5% (*v*/v) of the developed choline acetate/glycerol eutectic solvents increased the enzyme activity by almost 3-fold. The authors also report that the addition of polyols appears to have a major impact on the stability and activity of the enzyme, regardless of the ionic liquid used.

3.2. Temperature and pH

Temperature and pH are the main influencing factors in the activity of Kl-β-gal [86]. The temperature effect on kinetic parameters can be properly described by the Arrhenius equation, except for the inhibition parameter, which follows the Van't Hoff equation. Many studies have reported the effect of temperature on this enzyme. Lima et al. [100] investigated the production of Kl-β-gal strain NRRL Y1564, with the obtained catalyst being immobilized on chitosan and chemically characterized. The optimal temperatures for the hydrolytic activity of the soluble and immobilized β-gal were 50 °C and 37 °C, respectively.

Zhou and Chen [101] studied the temperature effect on enzyme activity and stability of catalysts obtained from the immobilization of the Kl-β-gal on graphite surfaces, evaluating temperatures between 30 °C and 50 °C. The maximum activity of the biocatalyst was obtained at 50 °C, and the results showed that the enzyme immobilization increased its thermal stability. Increasing the temperature to an ideal point can improve the diffusion in the medium, thus facilitating the contact of the substrate with the immobilized enzyme.

Kl-β-gal is an enzyme traditionally used in industrial processes to produce lactose-free dairy products, such as milk and yogurt. In general, all commercial β-galactosidases basically have the same performance in lactose hydrolysis; however, depending on the enzyme, different products will be obtained. On the other hand, as highlighted in a recent review [102], β-galactosidases from *A. oryzae* (Ao-β-gal) are less used due to their optimal pH and temperature. This difference between the enzyme's performances can also be useful depending on the process to be applied for the hydrolysis of lactose, that is, in batch processes, milder in terms of temperature, the use of Kl-β-gal could be adopted. For example, the study conducted by Bosso et al. [103] shows that the optimal temperature to maintain high reaction rates of Kl-β-gal during lactose hydrolysis is approximately 40 °C, while for Ao-β-gal, this is 55 °C.

The GOS synthesis by Kl-β-gal shows a slightly higher yield when it occurs at higher temperatures, which would be an additional advantage when operating at high initial lactose concentrations and, consequently, at high temperatures. The lower stability of native genes, compared to

genetically modified ones, limits their catalytic activity in applications that require high temperatures, such as in the GOS production [61]. Thus, genetic engineering strategies have been applied aiming at the recombination of *K. lactis* genes for the synthesis of β-galactosidase by microorganisms that show greater thermal resistance [104].

Rico-Díaz et al. [28] designed variants of two Kl-β-gal by rational mutagenesis based on the structure of the enzyme, introducing disulfide bonds at the monomer-monomer and dimer-dimer interfaces. These two mutants improved thermostability, measured by the residual activity after incubation at 45 °C, and also increased its half-life compared to the native enzyme under the same conditions. These improvements did not affect its affinity for the substrates, but they increased the value of V_{max}. It was confirmed experimentally that in both mutants, the improvements correlated positively with an increase in the proportion of dimeric and tetrameric species, which were the active forms of the enzyme in question.

Among several factors evaluated in the production of GOS, Martínez-Villaluenga et al. [18] analyzed the effects of pH (5.5, 6.5 and 7.5) and temperature (40, 50 and 60 °C) on the synthesis of di- and trisaccharides by Kl-β-gal, Lactozym 3000 L HP G. They observed that the synthesis of these two types of oligosaccharides showed different optimal conditions. For the production of disaccharides, such as galactobiose and allolactose, the temperature of 50 °C and pH 6.5 delivered better yields. In turn, for the synthesis of trisaccharides, such as 6′-galactosyl lactose, 40 °C and pH 7.5 were the pinpointed optimal conditions. This study showed that, at pH 6.5, the rapid degradation of 6′-galactosyl lactose occurred under shorter reaction times, and this increased the formation of disaccharides. At pH 5.5, these led to enzymatic inactivation, and this was identified as the ideal pH range for the production of GOS, with a pH between 6.5 and 7.5 [105].

Other study [22] similarly evaluated the influence of pH, temperature, time, and concentration of lactulose and enzyme on the formation of 6′-galactosyl-lactulose and 1-galactosyl-lactulose using the commercial preparation of the enzyme Lactozym 3000 L HP-G. The authors found that the optimum temperature and pH in the process were 50 °C and 6.5, respectively.

The structure-activity relationship as a function of the pH of Kl-β-gal is reported in the literature [106]. In the far UV (195–240 nm), CD ellipticity can be used to determine changes in the secondary structure of proteins over time, since structural elements (α-helix, β-sheet, and random coils) have different spectra [107]. So, Tello-Solís et al. [106] used circular dichroism spectroscopy to study the changes in the ellipticity of lactase at 215 nm as well as enzyme activity as a function of pH. They observed that the pH of maximum enzyme activity (pH 7.5) was not the same that provided the highest secondary structure content (pH 7.0), which corresponds to 100% ellipticity. They attributed this behavior to favorable conditions for the interaction between the enzyme and the substrate, promoted by the changes produced in the secondary structure under this pH. At pH 6.5–7.0, a slight decrease in ellipticity percentage was observed, suggesting only a little structural change. Activity, on the other hand, decreased significantly, probably due to variations in critical residues. Last, at pH 8.5, a loss of activity was observed because, at this pH, aggregation of β-galactosidase is observed.

3.3. Ions

β-Galactosidase inactivation is mainly associated with the dissociation of its subunits, and ionic bonds present between subunits, through multivalent cations or anions, are an important factor to maintain a stable enzyme structure [27,108].

One of the first studies that reported the effect of the ionic environment on the stability of Kl-β-gal was carried out in 1994 [109]. Several ions such as Mn^{2+}, Mg^{2+}, Zn^{2+}, Cu^{2+}, and Ca^{2+}were evaluated for their influence on the activity of this enzyme. It was shown that the nature and concentration of cations present in a specific substrate at a given temperature are essential for the success of Kl-β-gal applications. With the reported results, it was observed that only Mn^{2+} (0.1 to 0.2 $mmol{\cdot}L^{-1}$) and Mg^{2+} (2.5 to 5 $mmol{\cdot}L^{-1}$) protected the enzyme against thermal deactivation, while Zn^{2+} (10^{-3} $mmol{\cdot}L^{-1}$) and Cu^{2+} (10^{-4} $mmol{\cdot}L^{-1}$) completely deactivated the enzyme. The cation Ca^{2+} had a negative effect on the activity of the enzyme, and this information is especially relevant given the importance of the application of Kl-β-gal in the hydrolysis of dairy products, which naturally contain a high content of this ion [109].

It is also known that some β-galactosidases show dependence on cations to be fully active. In some cases, the enzyme requires the presence of Na^+ or K^+ and Mg^{2+} [110,111]. According to Dickson et al. [112], some divalent cations have different effects on the activity of the enzyme. For example, while the presence of Ca^{2+} or Zn^{2+} causes a slight inhibition on the activity of the Kl-β-gal, the presence of Mg^{2+} promotes an activity increase. On the other hand, monovalent cations are necessary to stabilize enzymes while maintaining an enzymatic conformation that favors maximum activity. This process can also be related to a reduction in the flexibility of the polypeptide structure, which leads to higher thermal stability of β-galactosidases in the presence of some ions [109].

Several studies [27,57,59] have concluded that the active site induces the formation of bonds to one Mg^{2+} ion and two Na^+ ions. Additionally, Mg^{2+} ions can play an important role in stabilizing key structures during interactions between subunits. This mechanism is aided by Mg^{2+} ions and requires two residues of glutamic acid, with one of them being protonated (Glu-482 in *K. lactis*). It then acts as an acid and donates its proton to the glycosidic oxygen of lactose. The second, on the other hand, is deprotonated (Glu-551 in *K. lactis*), acts as a nucleophile, and reacts with a protonated lactose molecule. It then forms a covalent bond with the galactosyl residue and releases glucose. In the final step of this mechanism, a water molecule hydrolyses the enzyme galactose and restores the protonation states of the two glutamic acid residues.

Several cations in concentration levels normally present in the natural substrates of Kl-β-gal, such as milk, also affect the activity and stability of this enzyme. For example, Voget et al. [109] studied the effect of Na^+, K^+ and NH_4^+ on the stability and activity of Kl-β-gal. They observed that in the absence of such cations, the Kl-β-gal could become unstable. On the other hand, the presence of cations provided higher stability in the following order: $Na^+ > NH_4^+ > K^+$. Noteworthy, Cd^+ was the cation the promoted the highest enzyme stabilization levels. This stabilization also depends on the substrate that is used, i.e., when the substrate was lactose, the stabilization effects of the different cations obeyed the following order: $Na^+ > NH_4^+ > K^+$. On the other hand, when ONPG was used, K^+ or Na^+ provided the best stabilization.

De Freitas et al. [80] investigated the influence of the $MnCl_2.4H_2O$, $MgCl_2.6H_2O$, $CaCl_2.4H_2O$, and $ZnSO_4.6H_2O$ ions, at different concentrations, in the enzymatic activity of Kl-β-gal. The authors observed that the effects of these ions depended on their concentrations; Ca^{2+} ions (>1 mg/L) and Zn^{2+} (>0.011 mg/L) showed an inhibitory effect on Kl-β-gal, with Zn^{2+} presenting a more accentuated effect. Conversely, Mg^{2+} and Mn^{2+} (up to 1 mg/L) caused the activation of Kl-β-gal. Therefore, the inhibitory effect of Ca^{2+} may disfavor the lactose hydrolysis when it is present in concentrations above 10 mg/L, which is the case with dairy products that are rich in Ca^{2+} (240 mg/L) [80].

Albuquerque et al. [77] evaluated the effect of cations (Mg^{2+}, Mn^{2+}, Ca^{2+} and Zn^{2+}) on the lactulose production by Kl-β-gal, and they reported that a maximum lactulose production of 22.6 g/L was obtained with the addition of 0.01% *w/v* Mg^{2+}; in turn, the addition of Ca^{2+} or Zn^{2+} decreased the production of this prebiotic.

Klewicki et al. [113] evaluated the synthesis of galactosyl mannitol derivative (gal-mannitol) using Kl-β-gal. These authors studied the influence of the concentration of the reactive mixture (23–48 g/100 mL), pH (6.5–9.0), presence of NaCl (0.05–0.25 $mol{\cdot}L^{-1}$), and enzyme dosage (2850–28,500 LAU/100 g lactose) in the synthesis reactions. In the reaction conducted using an enzyme load in the range of 2850 to 11,400

LAU/100 g of lactose, the obtainment of gal-mannitol reached 21.8% of total saccharides; higher loads intensified the decomposition of the product. An increase in the concentration of the reactive mixture had a positive impact on the production of gal-mannitol. There was a relatively low increase in the amount of the product (by about 5%) after the pH increased from 6.5 to 9.0. The use of NaCl at a concentration of 0.25 $mol{\cdot}L^{-1}$ showed better results and delivered an increase of 12.8% in the maximum content of gal-mannitol in total sugar.

3.4. High pressure

High pressure is considered a denaturation factor, and hence, it has been tested in several studies on protein behavior. This technique was applied to adjust the activity and stability of various enzymes and, therefore, it shows potential applications in enzymology. There are old reports about Kl-β-gal performance under high-pressure conditions. For example, Athès and Combes [98] concluded that the use of polyols, such as xylitol, allows for better stabilization of β-galactosidases against pressure deactivation more than salts do, increasing the enzyme half-life by factors between 1 and 10,000. These authors also noted that NaBr and KBr are essentially destabilizing compounds, leading to almost null protective effects.

4. Biocatalyst improvement

4.1. Immobilization of Kl-β-gal

Enzyme immobilization was developed to solve issues inherent in the practices of enzyme recovery and reuse [114,115]. However, researchers soon decided also to study the possibility of linking this process to the improvement of some other inherent enzyme limitations. The results obtained were very successful, and currently, enzyme immobilization is employed not only in the production of heterogeneous biocatalysts but aiming to maintaining and improving enzyme activity, widening the range of operation conditions, guaranteeing enzyme stability (by multipoint or multi-subunit immobilization), increasing the resistance to chemicals, obtaining biocatalysts that are less sensitive to inhibitions, altering their selectivity or specificity, and even to improve enzyme purity [116–129]. Therefore, enzyme immobilization has become a critical step in the development of industrially efficient biocatalysts.

In the specific case of the Kl-β-gal, one of the main objectives of immobilization is to reduce the enzyme inactivation by subunit dissociation, mitigating the risk of the loss of oligomeric structures [130]. Moreover, it could also be interesting to reduce the inhibition caused by formed products, as mentioned previously [125].

Kl-β-gal immobilization also presents some specific obstacles. Firstly, successful use is limited to reaction conditions under which the enzyme is stable, i.e., where the multimeric structure is resistant enough. Using preexisting porous supports, the large size of the enzyme will condition the pore diameter of the matrix pores, and this will affect the final loading capacity and the mechanical resistance of the support itself [131]. These are no longer relevant issues when using other enzyme immobilization strategies, such as the production of enzyme aggregates [132–136], or when using preexisting non-porous nanomaterials such as magnetic nanoparticles [137–139].

Another common strategy to immobilize this enzyme is the use of whole cells [140,141], which can or cannot be immobilized later. Under these instances, the risks of enzyme inactivation during handling are much lower since the enzyme is trapped in a small volume, and the risk of enzyme dissociation is diminished [130]. This practice can also lead to better enzyme stabilization via interactions with cellular components. However, the risks associated with the release of the cell components by cell breakage will still exist, which can reduce the loading of the enzyme.

Also, multifunctional biocatalysts are widely applicable in reaction systems, but for the success of these methodologies, enzymatic immobilization is necessary. In some cases, the immobilization of two enzymes on the same support is possible and an efficient way to increase the performance of multiple reactions. Henriques et al. [129] studied the co-immobilization of lipase from *Thermomyces lanuginosus* (TLL), and Kl-β-gal in magnetic nanoparticles (Fe_3O_4) supports. The choice of these two enzymes is due to their high industrial applicability and the differences in their molecular size and surface structural groups. In this study, the enzyme derivatives showed better thermal stability than the enzymes in their free form (50 °C for TLL and 30 °C for Kl-β-Gal), and their use was also enabled under a wider range of pH and temperature: up to 50 °C for β-Gal and up to 70 °C after the crosslinking step.

Next, we review some articles available in the literature regarding these practices applied to this specific enzyme.

4.1.1. Immobilization of K. lactis whole cells containing β-galactosidase

Whole cell immobilization is a technology that aims at minimizing the cost of enzyme extraction while intending to protect the cells from external environmental action [142].

Considering the problems associated with the handling of the Kl-β-gal, this was the first method used to produce heterogeneous biocatalysts with this enzyme. In the first published work, the production of the enzyme in *K. lactis* was optimized, and the cells were permeabilized using Triton (the effects of which were observed in SEM micrographs) [143]. The permeabilized cells were immobilized in polyurethane foam, followed by its utilization in a continuous plug-flow reactor, which confirmed a half-life of around 3 days. Later, another research group studied the neutralization of anionic groups in the surface of the yeast with several poly-cations to produce walls with positive zeta potential [144]. Chitosan was selected for this purpose, and the modified cells were immobilized on polycarbonate, glass, or polystyrene; also, the possibility of modifying the support with this cationic polymer was analyzed. When the support was coated with chitosan, a monolayer of cells was obtained. In case the cells were treated with chitosan, a heterogeneous layer was identified, mainly due to floc formation. The cells were permeabilized with $CHCl_3$/ethanol and treated with glutaraldehyde to enable covalent bonds within the support. The enzymatic activity was well maintained after the treatments, and their application to hydrolyze lactose in a continuous micro-reactor at 30 °C showed a half-life of 25 days [144]. Later, the same research group focused on the use of insulation-glass fibers as a support to immobilize the chitosan-modified cells [145]. The fibre was fully coated with the cells, and the permeabilization of the immobilized cells with diverse solvents led to the obtainment of a useful β-galactosidase biocatalyst. The main issue with this approach was the formation of cell multilayers that caused some diffusional limitations, but these were circumvented by the optimization of cell deposition [146].

In another research, *Kluyveromyces lactis* was used to produce β-galactosidase from ultrafiltered cheese whey, and later, the cells were immobilized by trapping in alginate beads [147]. Higher activity was obtained using manganese alginate beads instead of the more popular calcium alginate beads. Storage in 5 mM DTT or 50% glycerol allowed for the reuse of the biocatalyst in 5 reaction cycles without any activity decrease [147]. Calcium alginate was also used in another study on whole cell immobilization, where a 2^3 full factorial experimental design was performed to optimize the resulting activity [148]. The activity increased in a bimodal trend with time, and it was affected by the dimensions and the structure of the gel. Moreover, the cell permeabilization increased the enzyme activity and prevented the initiation of ethanol fermentation by the cell, allowing a lactose hydrolysis yield of 99.5% in milk whey at 30 °C after 30 h of hydrolysis [148].

Another article shows the covalent immobilization via glutaraldehyde of β-galactosidase-rich *Kluyveromyces lactis* cells on corn grits [149]. Permeabilization of immobilized cells with ethanol increased the β-galactosidase activity by 240-fold and enabled the hydrolysis of over 90% milk whey lactose in a packed-bed bioreactor at 37 °C. Also, permeabilized dead cells of a *K. lactis* were immobilized into a cellulose-

gelatin matrix using glutaraldehyde as a crosslinker, and the system retained 30% of the initial enzymatic activity after cell trapping [150].

Finally, in one instance, cells from several microorganisms were co-immobilized. In a very interesting study, the β-galactosidase confined in dead cells of *K. lactis* permeabilized in organic solvents was utilized to improve the production of 2, 3-butanediol by *Klebsiella oxytoca* from lactose [145]. Both cells were co-immobilized by adhesion to chitosan-glass wool, enabling a 3-fold faster fermentation of lactose to 2, 3-butanediol.

Although successful protocols had been reported using these biocatalysts, the protocols of whole-cell immobilization for this enzyme were abandoned at the beginning of this century, and researchers focused their attention on the immobilization of the cell-free enzyme.

4.1.2. Immobilization of the cell-free enzyme

4.1.2.1. Immobilization by enzyme entrapment. Enzyme immobilization by entrapment is still not a very widespread enzyme immobilization strategy [151–153]. This is due to the high risks of enzyme leakage if the pores are not small enough or to the high diffusional limitations, in case the pores are too small to avoid leakage. In fact, some authors propose the trapping of previously immobilized enzymes [154–158]

The large size of the tetrameric and glycosylated β-galactosidase can enable the entrapment of the enzyme to diverse matrices. However, its immobilization via this strategy may stabilize the enzyme by preventing subunit dissociations [130]. For example, the enzyme has been trapped into LentiKats®, a polyvinyl alcohol hydrogel containing lens-shaped capsules [159,160]. The expressed activity of the immobilized biocatalyst was 20%, which was enabled by the use of Na_2SOM_4, which was the hardening agent used in the LentiKat preparation [161]. The change of this reagent by K_2SO_4 prevented this problem, and the expressed activity of the immobilized enzyme was near 100%. After 10 repeated batches at 30 °C, the biocatalyst retained 80% of its initial activity [161].

Alginate is the most used polymer for immobilizing enzymes by trapping [162]. Our β-galactosidase, for example, was trapped in sodium alginate and utilized for hydrolysis of acid whey in a fluidized bed reactor [163]. In another research, the enzyme was trapped in sodium alginate and l-carrageenan [164]. The complexes were more stable when alginate was used. The activity of the trapped β-galactosidase was higher than that of the free enzyme at pH 4.0 [164]. In some instances, mixtures of two polymers were used. In one article, lactose from cheese whey was hydrolyzed using calcium alginate spheres and gelatin, with glutaraldehyde and concanavalin A being used to prevent enzyme [165]. The encapsulation of the enzyme-concanavalin A complex to further enlarge enzyme size in alginate-gelatin spheres, without treatment with glutaraldehyde, improved the rate of lactose hydrolysis of the biocatalysts, with a conversion of 72%. Later, alginate and chitosan were used to immobilize the β-galactosidases from *K. lactis* and *A. oryzae*, which yielded better results than those obtained by using chitosan [166]. The optimal pH shifted towards more acidic values upon enzyme immobilization, and the optimal temperature was increased by 20 °C for the Kl-β-gal.

Katrolia et al. [166] evaluated the influence of temperature on the lactose hydrolysis properties of Kl-β-gal (Maxilact and Lactozym). The enzymes were immobilized by trapping in barium alginate and chitosan macrospheres. The ideal temperature of the Maxilact enzyme immobilized in chitosan increased to 60 °C, which is significantly higher than that of free Maxilact (40 °C) and of other immobilized forms. It was also noted that the immobilized enzyme achieved 100% lactose hydrolysis within 2 h of reaction time and that these biocatalysts showed excellent reuse levels (above 95% lactose in milk after five cycles) [166]. The enzyme immobilized in sodium alginate was also used in the hydrolysis of rennet "coalho" cheese whey [167]. Immobilization increased the enzyme stability, and as such, the range of operation conditions (both temperature and pH) was also increased, likely by preventing enzyme dissociation.

4.1.2.2. Immobilization onto preexisting solids. Immobilization onto preexisting solids has a few advantages: the mechanical properties of the support may be selected as a function of the final reactor, the particle size and pore volume can be strictly controlled, among others. We can further classify this type of supports regarding their macromolecular structure into nanomaterials, membranes, and particulate supports.

4.1.2.2.1. Immobilization onto nanomaterials. The use of non-porous nanomaterials as immobilization matrices have become popular in recent years [137,168]. While they show a few advantages, there are also some inherent drawbacks in these elements when compared to porous materials [121,169]. All immobilized enzyme molecules are located on the support surface, and this prevents the occurrence of diffusional limitations. This can also enable the use of biocatalysts to hydrolyze very large substrates and even to modify solids. However, this approach is known to generate undesirable effects, among which is the fact that the enzyme is not protected from interactions with external interfaces [154] and can consequently interact with molecules immobilized onto other particles (e.g., allowing proteolysis) [121,169]. Moreover, the loading capacity of nanomaterials depends on their particle size: the smaller the diameter of the particles, the higher their loading capacity. However, if the diameter of the nanoparticle is too small, the geometric congruence with the enzyme decreases, and so do the possibilities for multipoint covalent immobilization [170]. Moreover, the current cost of the porous matrices is much lower than that of nanoparticles or nanotubes.

Nevertheless, some examples of immobilization of Kl-β-gal in nanomaterials may be found in the literature. First, the enzyme was covalently immobilized in functionalized silicon dioxide nanoparticles [171]. Immobilization increased the optimal temperature of the enzyme and thermostability by 5 °C but almost doubled the K_m value while fairly maintaining V_{max}.

Immobilized concanavalin A was utilized in some instances to immobilize glycosylated proteins via the strong interaction between this protein and sugar chains [172]. Concanavalin A layered with Al_2O_3 nanoparticles has then been successfully utilized to immobilize Kl-β-gal [95]. Enzyme immobilization reduced the inhibitory effect of galactose and maintained 85% of its initial activity after six reuses.

In another article, nano-crystalline cellulose through acid or alkaline treatments was produced and attached to magnetic nanoparticles, and this composite was used to immobilize β-galactosidases from *K. lactis* or *A. oryzae* [173]. The biocatalysts were characterized and applied in the hydrolysis of lactose in cheese whey, permeate or milk for 30× and it reached hydrolysis higher than 50% in batch process. The continuous process in a fixed-bed reactor using an *A. oryzae* biocatalyst was possible to hydrolyze over 50% of lactose in both milk and whey after 24 h of reaction.

In another interesting work, a Kl-β-gal and a lipase from *Thermomyces lanuginosus* were co-immobilized [129]. The lipase was immobilized via interfacial activation, and then, it was aminated using ethylenediamine and 1-ethyl-3-(dimethylaminopropyl) carbodiimide. Subsequently, the β-galactosidase was ionically immobilized onto the aminated lipase and treated with glutaraldehyde or aldehyde-dextran to produce a stable biocatalyst. Thus, both enzymes were proven to be more stable than their free counterparts in thermal inactivation experiments [129].

Kl-β-gal was also immobilized onto functionalized multi-walled carbon nanotubes activated with glutaraldehyde [174]. The optimal temperature increased 10 °C upon immobilization, and galactose inhibition was reduced.

4.1.2.2.2. Immobilization in membranes. Membranes are often used to immobilize enzymes, as they may be of interest if the final utilization of the enzyme is in a membrane reactor, for example [175,176]. Thus, some examples of the use of membranes to immobilize the Kl-β-gal can be found in the literature. First, plain and plasma-modified cellulose

acetate membranes were utilized to adsorb β-galactosidase and used for analyzing the impact on galacto-oligosaccharide (GOS) productivity [177]. Oxygen plasma activation gave better immobilization results, with the immobilization yield increasing by 42%, but the yields were still not satisfactory. The enzyme was also covalently immobilized onto ethylendiamine plasma-treated matrix. However, the expressed activity decreased along with GOS yield. Thiolated membrane surfaces delivered the highest immobilization yields and highest enzyme activities [177]. Another work shows the use of 2′,4′-dinitrophenyl 2-deoxy-2-fluoro-β-D-galactopyranoside as a β-galactosidase active site titration agent to determine the number of active enzymes immobilized onto a mixed matrix membrane [56]. Although the protocol was valid for the use of particulate resins on immobilization supports (as we discuss in the next section of this review), it did not work for the mixed matrix membrane due to the strong partial adsorption of 2′,4′-dinitrophenolate by the membrane. This pointed to the relevance of interactions between the leaving group used and of the inhibitor with the support.

4.1.2.2.3. Immobilization in beads

4.1.2.2.3.1. Immobilization by noncovalent techniques

Enzyme immobilization via physical adsorption is a widely used technique of enzyme immobilization [178]. Immobilizing enzymes via noncovalent techniques may have some advantages, such as the simplicity of immobilization protocols, the high stability of activated supports, and, most importantly, the possibility of reuse of the supports after the release of inactivated enzymes [121]. The main problem with these techniques is the risk of enzyme release during the use of biocatalysts [178]. Regarding enzyme stabilization upon immobilization [169], it is possible to stabilize enzymes mainly by the generation of enzyme-favorable nano-environments, such as the partitioning of some deleterious compounds from the environment [159,179,180], or by preventing enzyme subunit dissociation [130,181,182]. This is quite an important cause for the inactivation of the enzyme reviewed. However, except under affinity adsorption, the mechanisms of enzyme immobilization onto the supports are based on multipoint interactions [183], i.e., a single-point covalent immobilization in an inert support may be milder than physical adsorption [170]. In practical terms, this means that the support activation energy needs to be high if a strong enough adsorption process is to take place, and some negative enzyme-support interactions may be triggered during the process [184,185].4.1.2.2.3.1.1. Ion exchange

It can be seen in most literature reports that the physical processes for β-galactosidase immobilization are based on ion exchange. First, the enzyme was immobilized in Duolite A-568 to test the effect of microwave irradiation on the production of GOS [183]. Immobilizing of the enzyme onto this support increased the synthesis of GOS, with a further increment being achieved by reducing the water activity of the system. Microwave irradiation combined with the addition of cosolvents (e.g., hexanol) also resulted in an increase in GOS production [186].

Hierarchical porous particles and monoliths of silica were produced by polycondensation of sodium silicate in the presence of ethyl acetate and cetyltrimethylammonium bromide under hydrothermal conditions [187]. These supports were used to immobilize a Kl-β-gal by adsorption. Loading capacity and enzyme release were proved to be linked to hierarchical porosity and to the presence of ionized silanol groups on the support surface. Enzyme stability under stress conditions was improved [187].

The enzyme was also utilized as an example of how the employment of polymeric ion beds formed on supports can be a successful way to strongly adsorb enzymes, using aminated agarose coated with sulfate-dextran. In this work, 80% of the proteins present in crude extracts from *Acetobacter turbidans* or *Escherichia coli* were adsorbed onto these porous composites at pH 7 [188]. Finally, the enzyme was immobilized in amino acrylic resin and used to show the suitability of using 2′,4′-dinitrophenyl 2-deoxy-2-fluoro-β-D-galactopyranoside to titrate its active center, as previously discussed [56]. In this study, the adsorption of 2′,4′-dinitrophenolate on the support was not a problem.

4.1.2.2.3.2. Covalent immobilization4.1.2.2.3.2.1. Glutaraldehyde activated supports

The exploration of the glutaraldehyde chemistry to immobilize enzymes is perhaps one of the most used and versatile strategies to prepare immobilized biocatalysts from enzymes, despite this being a complex compound [189,190]. Activating a support with glutaraldehyde also renders it heterofunctional, enabling the first immobilization of the enzyme to take place via different modalities, which alters the enzyme orientation in relation to the support surface. It is possible to either use pre-activated glutaraldehyde supports or treat the immobilized enzymes with glutaraldehyde [191–195]. Each approach has advantages and drawbacks, as outlined in a recent review [189]. This method has been utilized to immobilize Kl-β-gal for many applications and onto various supports. For example, nylon-6 microbeads were activated with glutaraldehyde and utilized to Kl-β-gal immobilization [196]. The biocatalyst obtained was then utilized to hydrolyze lactose from skim milk in a spin-basket reactor. In less than 7 min at 34 °C, almost 89% of the lactose had been hydrolyzed. In another work, graphite was activated using glutaraldehyde and used to immobilize Kl-β-gal, promoting a 5-fold increase in the K_m value [101]. When utilized for hydrolysis at a concentration of 5% and at 37 °C, the conversion rate was 70% after 3 h, and the enzyme was stable under various storage and operation conditions. In a following work from the same research group, the effect of pH and temperature on the activity of the immobilized enzyme was analyzed [197]. After immobilization, the conditions for maximum activity were increased from 40 °C to 50 °C, while the optimum pH increased from 6.6 to 7.7.

β-Galactosidase was also immobilized using glutaraldehyde onto cotton fabric, and the biocatalyst was utilized to determine the kinetic parameters of the enzyme in the hydrolysis of lactose with special emphasis on the inhibitions and side-reactions occurring during GOS formation [92]. The model considering all these parameters explained the formation of glucose and galactose better than the previous models. The same biocatalyst was later utilized in a pilot-scale packed-bed reactor to hydrolyze lactose in whole milk in batch and continuous models [198]. The experimental data obtained were correlated with the simulation results using the kinetic model previously developed.

β-Galactosidase has also been immobilized using glutaraldehyde on silica gel [199]. When the enzyme was pretreated with lactose, the final activity recovered was 2.6-fold higher. The optimal temperature increased 5 °C after immobilization and the optimal pH increased from 7 to 7.5. The operational stability of the pretreated, immobilized enzyme increased, retaining almost 65% after 10 reuse cycles when compared to the untreated immobilized biocatalyst [199].

β-Galactosidase was immobilized on polysiloxane-polyvinyl alcohol activated with glutaraldehyde and used to produce GOS [21]. The immobilization yield and the expressed activity were next to 100% and 80%, respectively. The optimal reaction conditions were 40 °C, 270 $g{\cdot}L^{-1}$ initial lactose concentration, pH 7.1, and 6 U mL^{-1} enzyme concentration. Under these conditions, more than 25 $g{\cdot}L^{-1}$ of trisaccharides were produced [21].

The Kl-β-gal enzyme was also immobilized on collagen, treated with aluminum, glutaraldehyde, acetic acid, or a combination of aluminum and glutaraldehyde [200]. Immobilization yields were approximately 70% in all cases, and the four biocatalysts hydrolyzed 50% of lactose after 17 reuse cycles in milk and whey, using a batch reactor processing. Using a packed-bed reactor, the yields were improved after 48 h of operation. The biocatalyst exhibited good operational stability, especially when used in a packed-bed reactor in continuous mode.

Poltorak and coworkers [201] immobilized several β-galactosidases (from *Kluyveromyces fragilis*, *Escherichia coli*, *K. lactis*, and *Penicillium canescens*), showing that the immobilization promoted changes in the kinetic properties of the enzyme, and more interestingly, on its inactivation constant [201]. The results showed that the dissociative mechanism of thermal inactivation was operative in all cases [202].

Chitosan beads, a partially deacetylate derivate of chitin, are also

widely utilized to immobilize enzymes [77,80,203]. The amino groups of chitosan have a low pK and they are not adequate for enzyme immobilization via ion exchange. However, following activation with glutaraldehyde, their use has become very widespread to this end.

β-Galactosidase, produced by optimized process using the *Kluyveromyces lactis* strain NRRL Y1564, was immobilized onto chitosan activated with glutaraldehyde [100]. The immobilized enzyme was proven to be 8-fold more stable than its free form, enabling its reuse for 10 cycles and retaining 50% of its initial activity. At 4 °C and pH 7.0, the enzyme activity remains unchanged for 3 months. In another work, the immobilized enzyme was utilized in a packed-bed reactor for the continuous hydrolysis of lactose and the synthesis of GOS [60]. The immobilized enzyme increased the pH and temperature operation conditions, as a consequence of enzyme stabilization. Almost full lactose hydrolysis was observed for both milk whey and lactose solution at 37 °C, and a GOS concentration of 26 $g{\cdot}L^{-1}$ was achieved [60].

In another research, Kl-β-gal immobilized in glutaraldehyde-chitosan was used to produce lactulose using cheese whey supplemented with fructose as feedstock [77], reaching 17.3 g/L of lactulose. The same enzyme was also utilized to compare its immobilization potential as a support for a novel silica-chitosan structure compared to standard silica activated with 3-aminopropyltrimethoxysilane, both activated with glutaraldehyde [204]. The enzyme immobilized in the new support proved to be more efficient in the lactose hydrolysis, maintaining 90% of its initial activity after 200 h of use in continuous mode. The authors reported that the new support can bring together the enzyme stabilization possibilities of chitosan and the mechanical resistance of silica.

The use of magnetic materials to immobilize enzymes has some utilities, as it has been recently reviewed [205]. For example, this practice permits the biocatalyst recovery even from a substrate formed by a suspension, as is the case with milk. Some reports have studied the immobilization of this enzyme on magnetic macro-supports [206]. The β-galactosidase was covalently immobilized onto polysiloxane-polyvinyl alcohol magnetic particles via glutaraldehyde activation, without alteration of its kinetic properties. The immobilized enzyme exhibited a higher operational and thermal stability than the soluble enzyme, and can be utilized in suspension like milk, for example, with its recovery being made possible due to the magnetic properties of the system.4.1.2.2.3.2.2. Supports activated with genipin

Although glutaraldehyde is frequently used as support to immobilize enzymes, as previously discussed, it is a reactive molecule not approved by the Food and Drug Administration (FDA) to be used in products targeted at human consumption [207–212]. In this context, it has been replaced by the nontoxic alternative genipin. Genipin is a crosslinker agent of natural origin and, although its mechanism of action is not fully understood, its low toxicity makes it ideal as a support activation agent in the preparation of biocatalysts to be used in applications in biomedicine, food, among others [213].

Apparently, so far, there is only one study [214] in the literature using this reagent together with chitosan to immobilize the Kl-β-gal. Both compounds are natural and nontoxic and hence, this seems to be the ideal protocol to immobilize enzymes that are to be used in processes and products aiming at human consumption [208]. In this work [214], enzyme immobilization was optimized via an experimental design. Optimal conditions for enzyme immobilization were found to be 4.57 $mg{\cdot}mL^{-1}$ of support concentration and a substrate concentration of 10 mM. The thermal stability of the enzyme was improved and the biocatalyst could be utilized in four runs of lactose hydrolysis in diluted UHT milk [214].4.1.2.2.3.2.3. Glyoxyl activated supports

These supports are recognized as one of the most efficient in enabling multipoint covalent immobilization [215]. A bottleneck for using these materials is that usually enzymes must be immobilized at alkaline pH values [216]. However, multimeric enzymes are an exception, as they have several primary amino groups (those of the individual terminal amino groups) that, if present in the same plane, can interact with the support [217,218].

β-Galactosidase immobilization onto this support has also been studied. The enzyme was immobilized on glyoxyl sepharose with almost quantitative immobilization and showed an expressed activity of 82% [219]. This proved to be the most stable biocatalyst, 100-fold more stable than the enzyme immobilized in cyanogen bromide Sepharose.

In another research, several β-galactosidases (from *K. lactis*, *Bacillus circulans* and *A. oryzae*) were immobilized on glyoxyl macro-mesoporous silica [220], and yielded high enzyme immobilization levels and activity, with no enzyme lixiviation detected following adsorption [187]. The covalently immobilized biocatalysts were 120 times more stable compared to the free enzyme, including resistance to higher pH and temperatures [220].4.1.2.2.3.2.4. Epoxide activated supports

These supports are one of the most utilized for enzyme immobilization at industrial level. They are more stable, can react with many different groups of enzymes (thiol, imidazole, phenol, primary amino groups), and are already manufactured as activated matrices [221,222]. On the other hand, epoxy activated supports are not very reactive, requiring the first adsorption of the enzyme to the support to take place, before immobilization can happen [223–226].

One of the works on the Kl-β-gal immobilization described its immobilization on Eupergit C and optimization through a Plackett-Burman [227]. The ionic strength and immobilization pH were variables that showed a significant effect on the process. The optimal conditions were 25 °C, pH 6.6, ionic strength of 1.5 M, immobilization time of 8 h, 1 mM of divalent magnesium ion, and 0.4 mL of enzyme added. These conditions delivered an 85% immobilization yield. The latter was more stable than the former, and its use in a batch reactor for lactose hydrolysis from cheese whey provoked an increase in lactose conversion levels [227].

In another research, the enzyme was immobilized on Immobead 150 (an epoxide support) using different support modification strategies [228]. The support was submitted to different modifications via either acid hydrolysis of the epoxide groups or modification of the support with glutaraldehyde (the authors did not explain which groups react with glutaraldehyde). All preparations could be used in repeated batch hydrolysis of lactose and they were more stable than the free enzyme. The mechanism of immobilization on the different supports was not studied [228].4.1.2.2.3.2.5. Thiol exchange immobilization

The Kl-β-gal is an enzyme with a large amount of superficial Cys [27]. This has deemed this enzyme to be an ideal candidate to immobilization in supports via thiol-interchange. This process is a reversible immobilization, as the bond can be broken by the presence of either other thiolated compounds or of reducing agents [229]. Reports show that Kl-β-gal was immobilized onto thiolsulfinate/thiolsulfonate supports and retained 80% of the enzyme activity [230,231]. The blocking of the remaining reactive groups located in the support with glutathione multiplied the stability of the biocatalysts by 2-fold. The biocatalyst hydrolyzed 85–90% of a 50 g/L lactose solution in saline solution, skimmed milk, whey permeates, or whey either in packed beds or in batch. The enzyme was fully active for 10 months at 4 °C [230]. In another example, the enzyme was used as a model in the development of a bifunctional epoxy/thiol-reactive support [232]. After preparing the heterofunctional support by partially modifying commercial EP-Sepabeads with dithiothreitol, the enzyme was immobilized under neutral pH, via thiol-thiol interchange (reversible). After incubation under alkaline pH, the enzyme become immobilized via the epoxy groups, making the immobilization irreversible.

Posteriorly, the reduction of disulfide bonds by the enzyme was studied using solid phase reducing agents deriving from agarose, Toyopearl® and Eupergit®, all coated with mercaptohydroxypropyl-ether groups [232]. The use of this reducing agent enabled the increase of the content on free thiol groups of the enzyme by 3-fold, using a much lower thiol/enzyme ratio than that in solution. The reduced enzyme was reversibly attached to thiolsulfinate-agarose. Subsequently, the reduction of the enzyme and its following immobilization was performed in continuous mode in two connected reactors, the first

containing thiopropyl-agarose, where the enzyme was reduced, and the second, where the reduced enzyme was later immobilized on thiolsulfinate-agarose [233].

4.1.3. Comparison of different immobilization strategies to immobilize Kl-β-gal

To prepare an ideal biocatalyst from a specific enzyme, a comparison between different immobilization protocols seems convenient, as it is likely that the optimal immobilization protocol may be different for each enzyme, and even for each operation condition. Below, we discuss some examples from the literature where the Kl-β-gal has been immobilized following different strategies.

Enzyme covalent immobilization through assayed in CPC-silica and agarose has been reported [234]. Aminated silica was activated with glutaraldehyde, while agarose was activated with bromocianogen groups. Activated glutaraldehyde allowed for better immobilization yields, but the enzyme's K_m increased and V_{max} decreased. The enzyme immobilized in agarose was more stable than the enzyme immobilized in silica, but both were not more stable than the free enzyme [234].

In another article [90], the enzyme was immobilized in agarose beads following different protocols and the glucose inhibition constant was analyzed. K_m was maintained in the immobilized enzyme, while the noncompetitive constant increased significantly. Thus, when glutaraldehyde or glyoxyl agarose beads were used, the noncompetitive inhibition was greatly reduced. However, when the enzyme was immobilized via its glycosidic chains, the inhibition constant remained fairly similar. As such, using the soluble enzyme or the enzyme immobilized via the sugar chain for the hydrolysis of lactose in skinned milk (at around 5%), the reaction stopped when 90% of the substrate was hydrolyzed. On the other hand, when a glutaraldehyde or a glyoxyl biocatalyst were employed, more than 99% of the lactose in milk would be hydrolyzed.

Lastly, this enzyme was immobilized onto glyoxyl agarose, agarose coated with polyethylenimine, or glutaraldehyde-activated chitosan, previous to being utilized to produce lactulose [203]. The results show a potential effect of the immobilization protocol on the lactulose production capacity of the enzyme. The enzyme immobilized on glyoxyl supports did not produce lactulose under any conditions tested, while the enzyme immobilized on agarose coated with PEI, synthetized lactulose at 50 °C, but not at 25 or 37 °C. The enzyme immobilized in chitosan activated with glutaraldehyde, in turn, produced lactulose under all temperatures investigated, although a lower yield was obtained at 25 °C or 37 °C, when compared to that achieved at 50 °C. The operational stability of biocatalysts seems to be strongly dependent on the reaction medium, in addition to being dependent on the nature of the biocatalyst itself. In some instances, the lactulose production capacity would be canceled/ceased, while a high lactose hydrolysis activity was still maintained. This study is a clear example of the modulation of multimeric enzyme properties in kinetically controlled processes [203]. These treatments increased the enzyme stability considerably in the presence of organic cosolvents for all the studied enzymes by generating a "partition effect" that reduced the concentration of hydrophobic organic solvents in the enzyme environment [53].

5. Genetic modifications

In recent years, the interest in the use of biocatalysts comprising recombinant enzymes has increased. The main advantages of these biocatalysts are: i) greater enzyme stability and protection of active sites against deactivation; ii) possibility of large-scale production and ease of purification; iii) rigidity and permeability; iv) adaptation of enzymes for different purposes, among others [235].

Thus, enzymes such as recombinant β-galactosidases show desirable properties and a wide range of industrial applications, among which is an efficient conversion of lactose to obtain lactose-free dairy products [236]. Additionally, there are genetically modified thermostable β-galactosidases capable of better production of GOS in media with high initial concentrations of lactose and at higher temperatures [237]. This can be explained precisely because this technology protects the active sites responsible for catalyzing these reactions against enzyme inhibition. There are also β-galactosidases adapted to lower operating temperatures, which provide catalysts that require lower industrial energy expenditure and that also deliver food products with reduced nutritional and sensory changes [238]. Therefore, studies are being carried out using recombinant enzymes to enable its application on a large scale.

One of the major obstacles to increasing the yield of lactose hydrolysis is the strong inhibition caused by galactose, which may limit its use in some industrial processes. Andrade et al. [88] used computational techniques to evaluate the effect of point mutations on the active site of different GRAS microbial β-galactosidases, including Kl-β-gal, on lactose hydrolysis, and on galactose affinity. These authors studied direct mutations on the active site of β-galactosidases in order to reduce the product's binding energy (glucose or galactose) without impairing association with lactose. Twelve mutations were tested and all of them considerably reduced the binding energy to the final product. The point mutations produced on residues Tyr523, Phe620 and Trp582 delivered satisfactory results for this enzyme.

Protein engineering strategies were also used by Rodríguez et al. [239] when successfully trying to convert the intracellular Kl-β-gal protein to be secreted to the culture medium through the construction of a hybrid protein between *K. lactis* and *A. niger* β-galactosidases. The author also aimed to improve product secretion and facilitate its downstream processing by eliminating the cell extraction step. The final product presented biochemical characteristics of proteins of high biotechnological interest.

Many studies have also reported on the genetic sequencing of some strains of *K. lactis* [26,240,241]. However, the most promising from an industrial point of view are those that deal with the expression of genes from this yeast in other microorganisms. For example, genetic engineering procedures can reduce the formation of non-enzymatic browning products in lactose hydrolysis. The *Kl*-β-gal lac4 gene was expressed in *E. coli* as a soluble recombinant enzyme labeled with His- under optimized culture conditions [235]. The recombinant *Kl*-β-gal from this work was designed by carrying a His-tag fused at the C-terminal end in *E. coli*. The His-tagged-B-gal was important for the purification in order to obtain a highly efficient model for hydrolysis and transgalactosylation reactions with glucose and lactose as acceptors [84].

K. lactis has specific mechanisms to produce galactose from the hydrolysis of disaccharides containing galactose and that are found naturally in their corresponding environments [242]. Seeking to optimize the process of lactose hydrolysis, specific genes that coordinate the production of Kl-β-gal have been expressed in other microorganisms. Kim et al. [71] studied the expression of LAC4 Kl-β-gal in *Escherichia coli* and evaluated the optimized reaction conditions. These authors reported advantages of the method being the low-cost extraction and the possibility of a one-step purification in a commercial process. The optimal reaction temperature in oNPG was pinpointed between 37 and 40 °C, pH 7. Also, among the several divalent cations tested, Mn^{2+} was the most effective, producing increases of activity of 8.9 and 5.6-fold with oNPG and lactose as substrate. Lastly, with the aim of overcoming the inhibition of enzymatic activity by increasing the concentration of galactose during lactose hydrolysis, another study [17] presented the expression of a β-galactosidase called "M1" in recombinant *E. coli*. A lactose hydrolysis process was conducted to compare the performance of M1 with a commercial Kl-β-gal (GODO-YNL2), and an approximate conversion between them (above 99%) was observed.

6. Concluding remarks and future trends

Kl-β-gal has received much special attention due to its ability to catalyze the reaction of lactose hydrolysis, as well as to produce GOS. As this enzyme is produced by a microorganism generally recognized as

safe (GRAS), it presents great potential for use in the food industry. Thus, targeted efforts have been made by scientists to produce biocatalysts with high activity and stability for use in industrial conditions. Among the main applications, the use in the production of lactose-free products and in the obtainment of prebiotic products can be highlighted. In addition, some other processes have been studied in order to improve their stability and mitigate the effect of inhibitors, such as immobilization and genetic modification. A future trend regarding the use of Kl-β-gal involves its use in the catalysis of synthesis reactions of prebiotic oligosaccharides, which are high added-value compounds obtained through the reaction of lactose transgalactosylation. As this constitutes a kinetically controlled synthesis, modifications in this enzyme that favor such synthesis reactions are important to increase process yields.

CRediT authorship contribution statement

Tiago Lima de Albuquerque, Marylane de Sousa, Natan Câmara Gomes e Silva, Carlos Alberto Chaves Girão Neto, performed the initial literature search, all authors contributed to the writing and final editing of the paper. Luciana Rocha Barros Gonçalves¸ Roberto Fernandez-Lafuente, Maria Valderez Ponte Rocha designed the paper and supervised the writing.

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