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Conventional and non-conventional applications of β-galactosidases

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ABSTRACT

β-Galactosidase is one of the most important industrial enzymes, that has been used for many decades in the dairy industry. The main application of β-galactosidase is related to the production of low-lactose and lactose-free milk and dairy products, which are now common consumer goods in supermarket shelves. This is a well-established market that is expected to keep on growing as these products become more accessible to mid-income people worldwide. However, a fresh air has come into the β-galactosidase business as non-conventional applications arose in recent decades based on its transgalactosylation activity. This capacity is certainly a major asset for a commodity enzyme that can be used now as a catalyst for the upgrading of readily available and cheap lactose into high added-value glycosides in processes of organic synthesis in tune with green chemistry principles within the framework of sustainability. This is a reflection of a paradigm shift, where enzymes are now being considered as apt catalysts for the synthesis of valuable organic compounds. This article reviews the main applications of β-galactosidase, going from its conventional use related to its hydrolytic activity to the ongoing non-conventional applications in the synthesis of high added-value oligosaccharides based on its transgalactosylation activity.

1. Introduction

β-Galactosidase (β-D-galactoside-galactohydrolase, E.C. 3.2.1.23) catalyzes the hydrolysis of a glycosidic bond between a terminal non-reducing β-D-galactoside unit and an aglycone moiety [1]. Most applications of this enzyme are related to the hydrolysis of lactose, so it has been commonly referred to as lactase. However, its biological role is not limited to such reaction [2] and in fact not all the β-galactosidases are able to hydrolyze lactose [3]. Thus, this denomination should be avoided. β-Galactosidase (βG) is a quite ubiquitous enzyme, being present in a wide variety of microorganisms, plant, insect and animal cells; however, only microbial βGs are technologically relevant, since they can be produced at low cost in an intensive operation of fermentation at high yield and productivity [4].

The discovery of βG is a story full of stumbles. Martinus W. Beijerinck was the first in mentioning this enzyme in 1889, coining it as lactase [5]. Beijerinck developed a sophisticated bioassay to observe the fermentation of milk sugar based on the fact that *Photobacterium phosphoreum* is unable to metabolize disaccharides but luminesces when hexoses are provided as carbon source. Thus, he plated *Kluyveromyces marxianus* var. *marxianus* and *P. phosphoreum* together observing light emission around the yeast colonies. He concluded then that the fermentation of lactose was preceded by its hydrolysis produced by an enzyme secreted by *K. marxianus* to the environment. However, these observations were a matter of controversy for a few years, until Emyl Fischer, in 1894, proved the intracellular production of βG by *K. marxianus* using chemical methods [5], being the merit of discovering βG acknowledged to him [5].

Since then, βG has become one the most studied and marketed industrial enzymes [6]. A great impulse on the study of βG came from the work by Jacob Monod, who used *Escherichia coli* βG as a model for studying genetic regulation during the decade of 1950 [7]. On the other hand, its technological relevance arose primarily from the fact that over 60% of the human population has an impaired capacity to digest lactose, which produces symptoms such as bloating, nausea, abdominal cramping, and diarrhea [8]. This compelled the dairy industry during the decade of 1970s to develop products reduced in lactose or lactose-free by subjecting them to enzymatic hydrolysis with βG [9]. Nowadays, the industrial use of this enzyme has experienced a new impulse since βGs are able to catalyze the synthesis of prebiotic oligosaccharide compounds, such as galacto-oligosaccharides (GOS), by the kinetically controlled reaction of lactose transgalactosylation [10]. Transgalactosylation activity of βG is promising for the synthesis of high added value compounds from lactose, which is of great interest for the dairy

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industry and the cheese manufacturers [11]. Hence, this route of synthesis is being actively investigated for the production of molecules of interest other than GOS, namely: lactulose, fructosyl-galacto-oligosaccharides (fGOS) and alkyl-galactosides [12,13]. These novel applications are based on βGs substrate promiscuity, which allows the synthesis of a wide range of potentially attractive compounds.

Within this scenario, this article offers a critical review on the fundamentals of the technological applications of βG. A historic perspective is used in order to weigh the biotechnological relevance of this versatile enzyme and its evolution from a biocatalyst for hydrolysis to one for organic synthesis. Consequently, βGs applications in this review are classified as conventional and non-conventional. The former refers to those based on its hydrolytic activity, which have been utilized for a long time in large-scale industrial processes, while the latter refers to those newer applications based on their transgalactosylation activity. To unveil how this enzyme catalyzes both types of reaction, its catalytic mechanism is revised briefly in the next section.

2. Catalytic mechanism of β-galactosidases

Classification of glycosidases according to the International Union of Biochemistry and Molecular Biology Enzyme Commission (EC) provides little insight on the relationship between enzyme structure and reaction mechanism. Therefore, in the early 1990s, a hierarchical classification for glycosidases in families and clans based on the homology of their primary structure was proposed [14–16]. According to the Carbohydrate Active Enzymes database (CAZy), βGs are presently classified in the glycoside hydrolases (GH) families 1, 2, 35, 42, 59 and 147 [17]. All βGs have a catalytic mechanism with retention of the β-anomeric configuration of the substrate [17]. This feature is quite relevant in the synthesis of glycosides and oligosaccharides by transgalactosylation. The β-glycosidic bonds in prebiotic compound synthetized by βGs (GOS, fGOS and lactulose) prevent them from being hydrolyzed by the animal digestive enzymes in the upper part of the gastrointestinal tract [18]. Thus, they reach intact the large intestine where they are selectively fermented, which favorably modulates the gastrointestinal microbiota, providing health benefits to the host [19]. As shown Fig. 1, βGs catalytic mechanism can be simplified to a double-displacement scheme, where the breakdown of the glycosidic bond and the transferring of the galactose moiety is assisted by the carboxylic chains of two glutamic acid residues 5.5 Å apart [15,16]. The first step of the catalytic mechanism is the liberation of the aglycone and the formation of a galactosyl-enzyme complex acting as a reaction intermediate; the first catalytic residue acts as nucleophile attacking the anomeric center of the β-galactoside producing the galactosyl-enzyme complex, while the second residue acts as an acid delivering a proton to the oxygen atom in the glycosidic bond for activating the aglycone as leaving group [15,16]. In the second step, a nucleophile containing a hydroxyl group acts as acceptor of the galactose residue producing the liberation of the product from the active site of the enzyme. The nucleophile attacks the anomeric center of the galactose moiety by the same side in which the aglycone is released resulting in the retention of the anomeric configuration. The addition of the nucleophile is assisted by the second catalytic residue acting now as a base by removing a proton from the hydroxyl group in the galactose acceptor so recovering its acid form. The galactose acceptor nucleophile, now activated, attacks the galactosyl-enzyme complex setting free the active site of the enzyme [15,16]. According to CAZy, all the βGs classified until now have a $(\beta/\alpha)_8$ barrel structural fold [17]. The $(\beta/\alpha)_8$ barrel folding is quite common in nature being present in catabolic enzymes. It consists in eight parallel β-strands alternated with eight α helices, where the β-strands form the inner side of the barrel while the α-helices cover the outer side. In the enzymes of clan GH-A, to which most βGs belong, the catalytic acid/base residue is located in strand 4, while the catalytic nucleophile is in strand 7 [16], glutamic acid residues performing both functions [17].

3. Conventional applications of β-galactosidases

βGs catalyze both transgalactosylation and hydrolysis reactions at proportions depending upon the reaction conditions [10]. In this regard, lactose concentration is the most influential variable, having a strong effect on such proportion. Usually, at lactose concentrations lower than 300 mM (~10% w/w), water activity (a_w) is high enough to make hydrolysis prevail over transgalactosylation in most of the βGs. On the contrary, at concentrations higher than 980 mM (~30% w/w), a_w is low enough for transgalactosylation to prevail so that a significant fraction of lactose acts as nucleophile acceptor producing galactosyl-lactose [10]. Hence, the hydrolysis of lactose is the predominant reaction when βGs are added to milk or cheese whey, whose lactose content is around 130 mM. This is clearly shown in Figure 2, where the behavior of *Aspergillus oryzae and Bacillus circulans* βGs is represented at different concentrations of lactose in the reaction medium.

As mentioned before, lactose hydrolysis can be considered as a conventional application of βG, representing a mature technology already consolidated at commercial level [20]. Lactose can be hydrolyzed

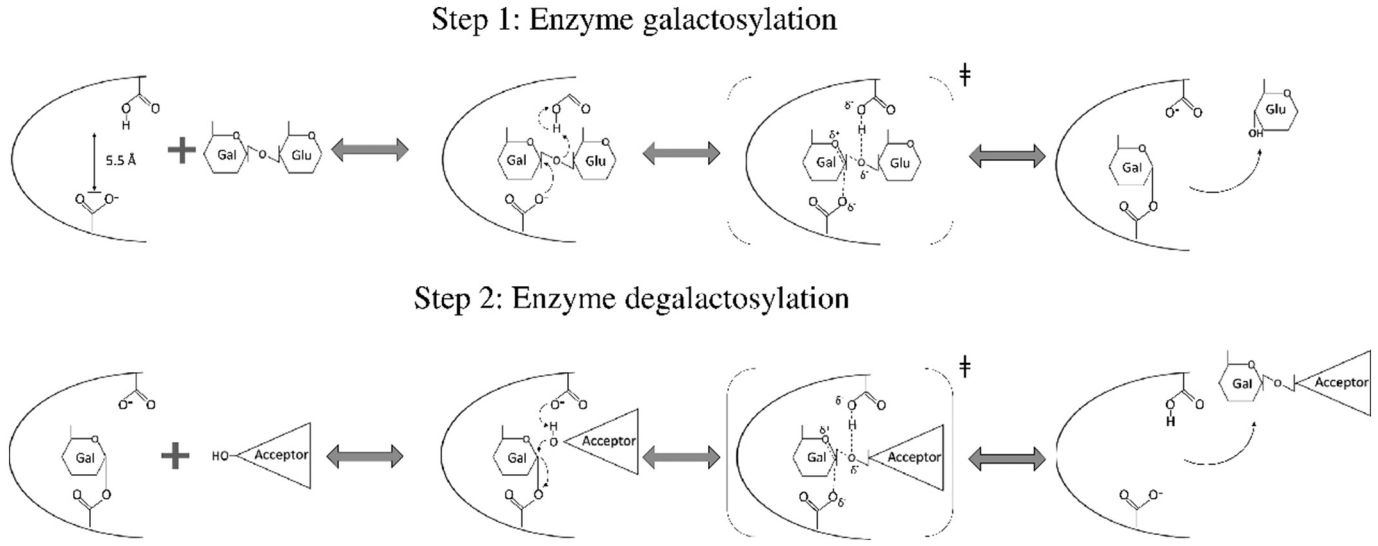

Fig. 1. Catalytic mechanism for lactose hydrolysis and transgalactosylation by β-galactosidase. Gal and Glu correspond to the galactose and glucose moieties, respectively. Acceptor: corresponds to a hydroxyl-containing nucleophile, which can be water (hydrolysis) or an organic compound (transgalactosylation).

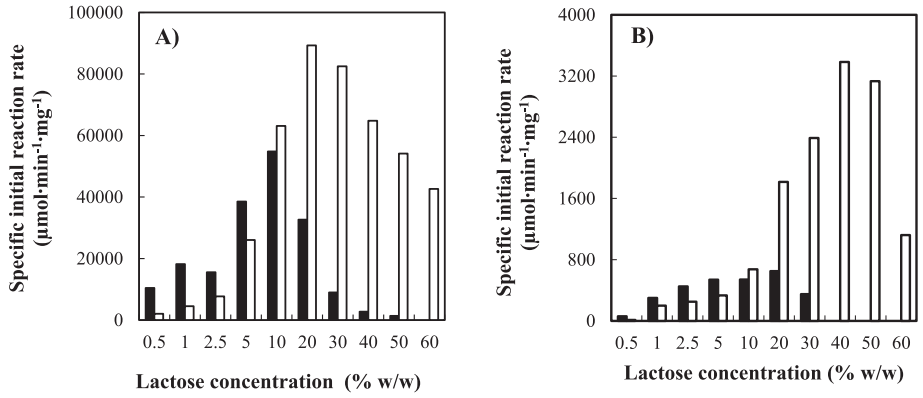

Fig. 2. Effect of lactose concentration on the specific rates of lactose hydrolysis (■) and transgalactosylation (□). A) *A. oryzae* β-galactosidase; B) *B. circulans* β-galactosidase. Adapted from Guerrero et al. [10].

by acid catalysis or enzymatically with βGs. The latter is the preferred option since acid hydrolysis requires the absence of protein and the product must be neutralized and extensively purified for removing unwanted side-products and colored compounds [21]. Main applications of βG for lactose hydrolysis are the production of low-lactose and lactose-free milk and dairy products, and lactase tablets for lactose-intolerant people. It is also used in the production of whey syrups, in upgrading the sensory properties and bioavailability of whey-derived products, and in dairy waste management [4].

3.1. Lactose-hydrolyzed milks

Products free of lactose or containing reducing amounts of it have gained importance both from commercial and health perspectives, driven by the increasing consciousness about lactose intolerance [21,22]. Lactose-hydrolyzed/defatted UHT milk was launched firstly in Italy during the decade of 1970s. It was produced by Centrale del Latte di Milano [20] using SNAM Progetti technology that employed *Kluyveromyces lactis* βG entrapped into cellulose triacetate fibers as catalyst. Lactose hydrolysis was carried out batchwise at low temperature until reaching the desired conversion, then milk was separated from the biocatalyst, sterilized and packaged [23]. Nowadays, the processes using immobilized βGs are among the largest using immobilized enzyme technology, with a production of about 10^5 ton per year, only surpassed by the processes using glucose isomerase for the production of high fructose syrup, nitrile hydratase for acrylamide manufacturing and lipases used in a broad range of industries: detergent, food, pharmaceutical, leather and paper-processing [6]. Despite the advantages of using an immobilized enzyme, the most common technology consists in dosing the (soluble) enzyme just before packaging and letting it act during storage [24,25]. The source of the βG employed in the production of lactose-hydrolyzed products is mainly constrained by the operational pH, the inhibitory effect of galactose and glucose and the cofactor requirement of the enzyme, as well as by sanitary regulations. Since the pH of bovine milk ranges from 6.0 to 7.0, commercial preparations of *K. lactis*, *Kluyveromyces fragilis* and *B. circulans* are preferred for lactose hydrolysis [20]. Global market for lactose-hydrolyzed products was estimated in US$ 6.7 billion in 2015, with an 8% increase in the last year. Market is expected to keep on growing at a compound annual growth rate of 6% with a projected figure of US$ 9.0 billion for 2020, USA representing the biggest market with a 29% share of global sales [26]. These products represent 62 % of all food products consumed by intolerant people in the USA and belong to two main categories: lactose-free milk and dairy products and lactose-free baby foods, with a market share of 47.7 and 52.3% respectively [27].

On the other hand, the use of low-lactose milk (LLM) in the formulation of different dairy products has several advantages over whole milk; an extensive review on the subject was published by Mahoney [28]. A major advantage is the reduction of sucrose addition by 20 to 40% in flavored milks, so reducing the calorie count without sacrificing sweetness [29]. In dairy products of low a_w (condensed milk, “dulce de leche”, ice-cream and frozen milk) the use of LLM prevents lactose crystallization avoiding the appearance of a sandy texture [30]. In fermented products, like yogurt and cheese, the use of LLM increases the production rate, since lactose hydrolysis is usually the limiting step [4,28,31].

3.2. β-Galactosidase supplements for lactose intolerant people

In the middle 1970's, SugarLo Company started marketing *Kluyveromyces marxianus* var. *lactis* lactase (βG) in the USA supplied by Gist-Brocades in a single-dose packet able to hydrolyze 70% of the lactose contained in a quart of refrigerated milk in 24 h [32]. This alternative was rapidly superseded by industrially produced LMM. Unfortunately, lactose intolerant people not always have the option of consuming such products. Therefore, in 1985 Lactaid Inc (formerly SugarLo Company) started marketing *A. oryzae* βG in capsules, claiming that two tablets before a meal could handle the lactose content equivalent to one glass of milk. In 1990, Sterling Drug Co launched a chewing tablet under the trade name Dairy-ease which is produced until now in formats containing *A. oryzae* βG together with digestive-aid enzymes, such as lipases and proteases [32]. Nowadays there are a number of enzyme supplements for alleviating the symptoms of lactose intolerance. Most of them contain *A. oryzae* βG [33], which withstands the low pH of the stomach [32]. The efficacy of exogenous βG supplementation for the removal of lactose from foods and the relief of symptoms associated to lactose malabsorption (flatulence, gas, abdominal pain, vomit and diarrhea) has been conclusively proven, being considered a good alternative for the treatment of lactose malabsorption and intolerance [34]. This is a relevant application for βG, since over 90% of the people from some ethnic groups (African Bantu, Asian Americans, Chinese, Southeast Asians, Thais and Native Americans) present lactose malabsorption [22].

3.3. Treatment and upgrading of effluents from the cheese industry

Global world production of whey was estimated in 180 million tons in 2013 [35], being no less than 40 % of it not industrially processed and simply used as feed or fertilizer, or disposed as a waste [36]. The appearance of progressively more stringent regulations on waste disposal has been a driving force for considering whey as a valuable by-product of cheese production, which can be used as raw material for the production of a gamut of high added-value products [12]. Lactose represents around 75% of whey solids [35], so lactose upgrading is a major issue that has attracted considerable technological attention [12]. As said before, direct utilization of lactose is hampered by its

physicochemical and nutritional properties, so its upgrade by chemical or biological transformations is the way to go [11,24,36]. Aside from the synthesis of transgalactosylated compound with βGs, which is analyzed in the following section, the basic and low added-value approach for lactose upgrading involves the enzymatic production of lactose-hydrolyzed syrups. Such syrups can be incorporated into dairy products like ice-cream, in confectionery and in bakery products [28,37]; it can also be used in animal feeding and as carbon source of different fermentation media [38,39]. The market of those syrups is rather elusive since they are hardly competitive with corn syrups and high-fructose corn syrups. However, their production may turn economically attractive under a scheme of circular economy, where the syrup is incorporated into products elaborated by the same company that produces it [40]. In the last decade, the production of D-tagatose has emerged as an attractive alternative for whey upgrading because of its high added-value. D-Tagatose is a rare natural carbohydrate that has gained interest as a low-calorie sweetener, endowed with prebiotic functionality and having a low glycemic index, which makes it attractive to the diabetic population [41,42]. Commercially, D-tagatose is produced by isomerization of D-galactose with L-arabinose isomerase as biocatalyst, where D-galactose derives from the enzymatic hydrolysis of lactose [43].

4. Unconventional applications of β-galactosidases

Within the framework of this review, unconventional applications refer to those based on the capacity of βGs to catalyze transgalactosylation reactions. Therefore, all these applications involve a strategy that favors transgalactosylation while depressing hydrolysis, namely, the use of very high substrate concentrations [44], the use of organic (co) solvents [13], the use of ionic liquids [45] or any other low a_w media [46]. However, it should be considered that the use of non-aqueous media may impair the enzyme activity and stability, severely reduce lactose solubility, and the solvent must be separated from the product, and most likely be recovered.

Overall, most glycosidases require values of a_w higher than 0.6 to be catalytically active [47], so that both hydrolytic and transgalatosylation activities will be present during the reaction. Hence, the synthesis of transgalactosylated compounds by βG is a kinetically controlled reaction where, at the beginning, transgalactosylation prevails and the concentration of the transgalactosylated compounds increases up to a maximum. At this point the rate of hydrolysis and transgalactosylation are equal, and afterwards the hydrolytic activity prevails and the concentration of the transgalactosylated compounds decreases [44]. So, the reaction must be quickly arrested when the maximum concentration of the transgalactosylated compounds is reached, which will be much easier if the enzyme is in immobilized form.

In the following sections the enzymatic synthesis of the major transgalactosylated compounds is analyzed in detail, making emphasis on the strategies used to promote the transgalactosylation activity of βG.

4.1. Synthesis of galacto-oligosaccharides (GOS)

GOS are the main lactose derived transgalactosylation products of βG, being produced on a large scale and having a well-established market [48]. They are non-digestible oligosaccharides (NDOs) with a well-documented prebiotic effect, being then a substrate that is selectively utilized by host intestinal microorganisms conferring a health benefit [19]. GOS can replicate the bifidogenic effect of human milk oligosaccharides (HMOs) by stimulating a healthy intestinal microbiota allowing to improve the intestinal motility, stimulating the immune system, promoting vitamin synthesis, reducing the levels of blood cholesterol and triglycerides and reducing the risk of colon cancer development [49]. Such health-promoting properties have boosted the interest of including them as functional ingredients in nutraceuticals and foods. Among them, milk formulas for newborns and follow-up formulas, specialized foods for the elderly, dairy and bakery products outstand [48,50]. [48,50]. A breakthrough in newborn infant formulas occurred in 2017, when Nestlé Spain introduced the first formula with two HMOs: 2′-Fucosyllactose and lacto-N-neotetraose [51,52]. Both HMOs are produced by fermentation with *E. coli.* K12 SRC6 and *E. coli.* K12 MP572, respectively. The genetic modification of both strains and the HMOs manufacturing process are described in detail in the GRAS notice 650 and 659 [53,54]. Despite that GOS cannot substitute HMOs functions completely, they have a similar functional effect [55]. Therefore, GOS incorporation into infant formulas will continue as the manufacture of synthetic HMOs is still challenging and expensive [55].

Early reports on the synthesis of GOS go back to the 1950s, when it was detected that the action of βG on lactose yielded not only products of hydrolysis (glucose and galactose) but also products of transgalactosylation, which were at that time considered undesirable in the production of low lactose milk [56]. However, knowledge about the health-promoting effect of these formerly undesirable contaminants, derived some twenty years later in the proposal of using GOS as substitutes of HMOs in infant formulas [57]. It was only in the 1990s that GOS began to be produced industrially and marketed in Japan [58]. At present, GOS market has expanded considerably and beyond Asia into Europe, America and Oceania [59]. GOS market has been forecasted to reach 175,700 tons by 2020 representing USD 1 billion. Europe represents about 59% of the present GOS market and expansion is estimated to be strongly supported by the Chinese and Indian markets [60].

In terms of molecular structure, GOS are oligosaccharides composed by two to eight galactose units β-linked to a terminal glucose unit, links being mostly β 1-4 and β 1-6 (see Fig. 3); galactose dimers are also considered in this category [12]. GOS can be chemically synthetized from lactose in a reversion process at severe reaction conditions of pH and temperature [61], producing complex mixtures of oligosaccharides with α and β linkages and anhydro sugars. This makes the chemical route unattractive because only oligosaccharides with β glycosidic linkages resist hydrolysis and are not absorbed in the upper part of the intestinal tract [62]. A better option for GOS synthesis is biocatalysis using either glycosyltransferases or βGs. Glycosyltransferases are highly regioselective and efficient catalysts for oligosaccharide synthesis, but they are quite expensive enzymes requiring nucleotide sugars as substrates making them technologically unattractive [63]. On the other hand, βGs can synthetize GOS from lactose in a kinetically-controlled reaction representing the preferred option for its production, since βGs are robust, readily available and inexpensive enzymes that have been used safely for decades in the food industry [12].

Fig. 4 presents a simplified reaction mechanism for GOS synthesis, where the trisaccharide (GOS 3) is the first product of transgalactosylation and then further galactose units are sequentially added to produce the longer chain GOS 4, GOS 5 and so on. Aside from the complex route of synthesis, GOS production also involves other technological challenges that need to be acknowledged: i) βGs are inhibited by the products of hydrolysis, mostly by galactose [64,65]; ii) βGs are labile catalysts under operation conditions [66,67]; iii) there is a limited number of βGs available commercially at large scale for their use in the food and pharmaceutical industry [20], and iv) the reaction yield is rather low (20 to 40 g GOS per 100 g of initial lactose), entailing several and costly purification steps for the removal of monosaccharides and unreacted lactose [48,68]. The presence of these sugars is considered undesirable because they restrain the use of GOS as ingredient in products for diabetic and lactose intolerant people and also for calorie counters [48,68].

Regardless of the reaction conditions, GOS yields rarely exceed 40 g of GOS per 100 g of initial lactose, except in those cases where there is a significant production of transgalactosylated disaccharides and yields tend to be higher [43,50]. Significant efforts have been made to optimize the process in terms of the most important operational variables, namely, temperature, pH, enzyme load, substrate concentration,

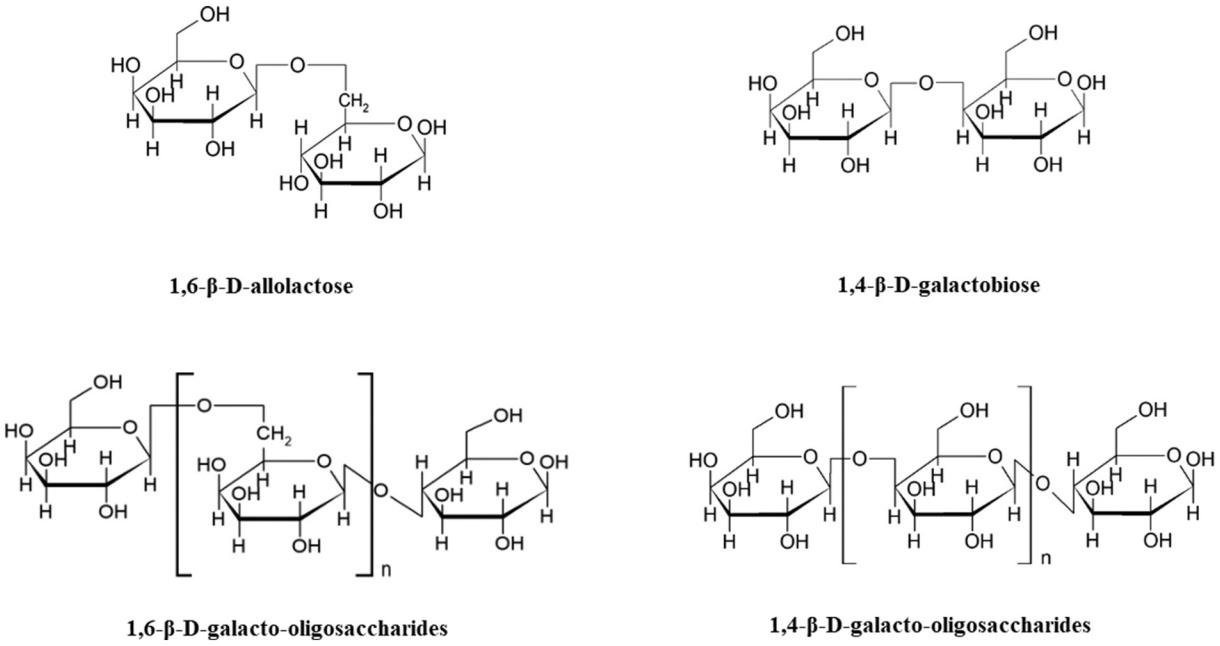

Fig. 3. Most frequent molecular structures of galacto-oligosaccharides.

enzyme source, a_w, immobilization strategy and reactor configuration [48,50,68]. Temperature exerts several effects in the reaction of GOS synthesis by modifying both kinetic and equilibrium constants [44,64,69]. These effects are very much dependent on the βG origin. For instance, in the case of *A. oryzae* and *E. coli* βGs, it has been shown that the activation energy (E_a) of both transgalactosylation and hydrolysis kinetic constants are quite similar, having values in the range from 12 to 14 kcal·mol^{-1} [64,69], so that temperature has a slight effect on yield [44]. On the contrary, a significant positive effect of temperature on GOS yield has been reported for the βG of the hyperthermophilic archaeon *Sulfolobus solfataricus* [70]. On the other hand, Michaelis constants for both hydrolysis and transgalactosylation reactions and the inhibition constant by galactose increased with temperature, having values of reaction enthalpy ranging from -20 to -5 kcal·mol^{-1} [64,65]. In addition, temperature affects the kinetics of βG inactivation and the concentration of lactose saturation, being their most important effects on GOS synthesis. Typically, the E_a value for the kinetic constant of inactivation varies between 70 and 180 kcal·mol^{-1} for the free βG, and between 7.7 and 55 kcal·mol^{-1} for the immobilized βGs [71]. Therefore, despite using immobilized βG, the inactivation of the enzyme during the reaction of synthesis is one of the most temperature-sensitive aspects. Lactose solubility in water is rather low in comparison with other carbohydrates, so reaching lactose concentration high enough to favor transgalactosylation reactions is a difficult task. Lactose solubility increases exponentially with temperature [72], thus GOS synthesis is usually carried out using supersaturated lactose dissolutions [44] at the highest temperature compatible with enzyme stability [48,50].

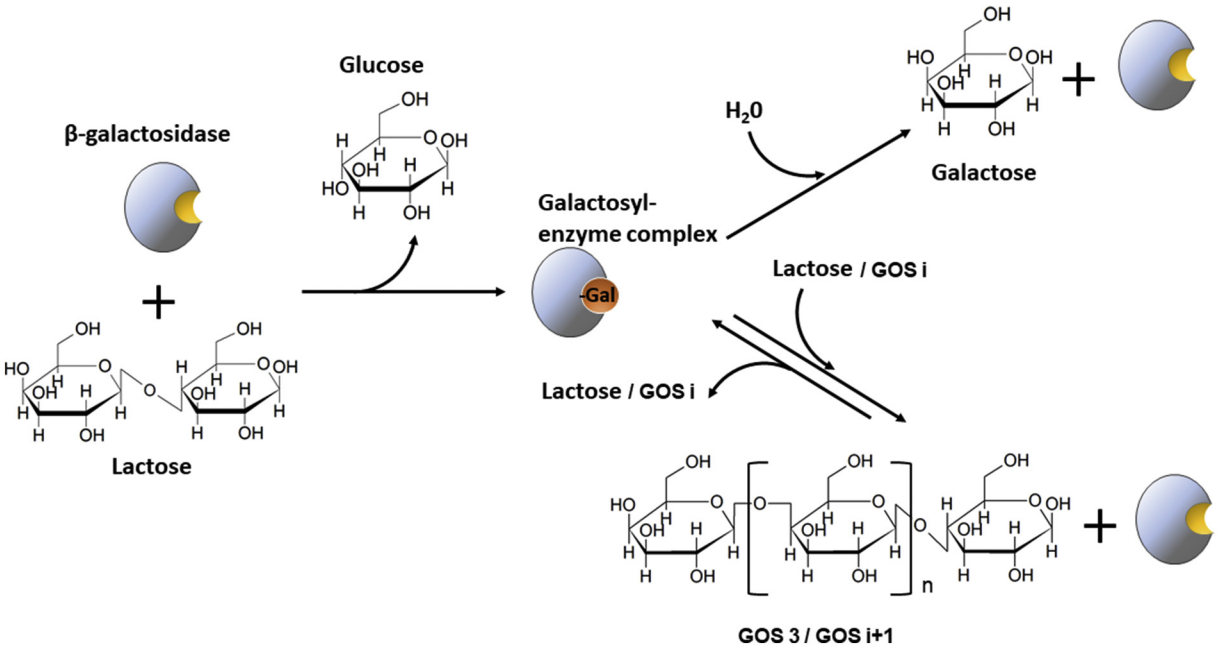

Fig. 4. Simplified mechanism for GOS synthesis. Typically, i goes from 4 to 7.

Numerous biocatalyst engineering techniques have been studied for increasing the biocatalyst stability during long-term synthesis of GOS, among which enzyme immobilization stands out. A number of authors have reviewed βG immobilization in depth, where the interested reader can found comprehensive information on the subject [48,50,73]. Even though this is not the focus here, it is worth saying that βG immobilization improves the biocatalyst stability, allowing its reuse for several reaction cycles [74] and long-term operation in continuous processes [75], and also simplifies the purification of the product [50]. βGs from different sources have been immobilized in different support materials by using strategies such as adsorption [76], entrapment [77], covalent binding [78] and crosslinking [79]. All the aforementioned immobilization techniques have advantages and disadvantages in terms of their preparation methodology, enzyme immobilization yield, thermal and mechanical stability of the biocatalyst and mass transfer limitations [50,73,80]. All those aspects should be weighed in order to select a proper immobilization strategy, having always in mind the final application of the catalyst. Major challenges that should be envisaged for large-scale immobilized enzyme usage are: protein adherence, channeling, microbial contamination in long term operation, hazardous, cumbersome and/or expensive immobilization process and difficult catalyst recovery [6,50]. Another strategy that indirectly involves enzyme immobilization is the synthesis of GOS in membrane bioreactors (MBR), which allows performing the reaction in continuous operation during a considerable period of time. Also, the use of MBR increases the enzyme availability to the substrate by raising their turnover, due to the continuous removal of the products (some of them are inhibitory) and their simultaneous replacement with fresh substrate under quasi-steady state operation [81]. MBR operations with commercial βGs, such as Maxilact 2000, Lactozym Pure 6500L, Biolactasa NTL-CONC and Lactase F, have shown to increase the reaction conversion, the enzyme productivity and, to some extent, the GOS yield with respect to reactions performed in conventional batch operation [82,83]. These positive effects have been attributed to the continuous recirculation of the enzyme, which is retained by an ultrafiltration membrane, and to the attenuated enzyme inhibition resulting from the continuous removal of galactose from the reaction medium [84]. Synthesis of GOS has been tested also by using MBR with the biocatalyst immobilized on the membranes: Palai and Bhattacharya [85] demonstrated that βGs of *B. circulans* immobilized in polyvinylidene fluoride membranes could be reused up to three times in the same membrane, performing the synthesis of GOS continuously during 30 hours each time. However, membrane tests showed a progressive reduction in permeability, thus reducing the permeate flux and the overall productivity of the system.

The interest in exploiting protein engineered βGs as biocatalysts is constantly increasing since they can be conveniently tailored within utility limits [86]. For example, Wu et al. [87] by cloning and site-directed mutagenesis of *S. solfataricus* P2 βG increased the GOS yields from 50.9% for the wild-type enzyme to 58.3% and 61.7% for the mutants F359Q and F441Y respectively. Since, *K. lactis* βG is less stable than other commercial sources of βG, it is not the preferred choice for the synthesis of GOS. However, Rico-Diaz et al. [88] introduced disulfide bonds in the interface between the enzyme subunits by using a rational mutagenesis strategy (R116C/T270C/G818C), yielding an enzyme 6.8 times more stable and twice more active than the wild type. Similarly, Zhang et al. [89] succeeded in decreasing the inhibition of *Aspergillus candidus* βG by galactose using a site-directed mutagenesis strategy. The Y364F mutant showed an inhibition constant by galactose 15.7 times higher than that of the wild-type and retained 90% of the activity. This is a quite relevant result because the strong inhibition by galactose of fungal βGs is their major disadvantages with respect to yeast and bacterial βGs.

Despite the technological potential of βGs from different sources, only few of them are approved by the corresponding sanitary authority. Dairy industry is rather conservative, so certified βG preparations from traditional and well established producers are preferred [25]. The βGs from *A. oryzae*, *B. circulans*, and *K. lactis* are the usual choice because of their commercial availability and customary use in the dairy industry [48,50]. Recently, Chr. Hansen has launched NOLA™ Fit, a *Bifidobacterium bifidum* βG expressed in *Bacillus licheniformis* [90], increasing the small number of βGs commercially available for large scale applications. Therefore, the application of biocatalyst engineering strategies to the currently available βGs for the food industry seems to be the most immediate option for the technological improvement of GOS production. Along with this, there are still many research opportunities referred to the enzymatic synthesis of GOS, enzyme immobilization, reaction medium engineering and the use of reactor configurations reducing the inhibitory effect of the reaction products. In spite of that, there are some remarkable cases of use of non-marketed βGs for GOS production. For instance, Clasado (United Kingdom/Malta) produces Bimuno® by means of permeabilized cells of *B. bifidum* NCIMB41171, which carries three βGs and one α-galactosidase, all of them contributing to GOS formation [91]. Nissin Sugar Co., Ltd. (Japan) manufactures Cup-oligo® using *Cryptococcus laurentii* βG and Yakult Pharmaceutical Industry Co., Ltd. (Japan) produces Oligomate® utilizing the βGs derived from *Sporobolomyces singularis* and *K. lactis* [68]. The decision of using a non-marketed enzyme will mainly depend on economic considerations, this is, whether the cost of producing the enzyme is compensated or not by an increase in the reaction yield and/or productivity. Also, it has to be considered if the industry using the biocatalyst has the interest, the capability, the resources or the know-how to produce it. A partnership with an enzyme manufacturer may be a suitable alternative in most cases.

4.2. Synthesis of lactulose and lactulose-derived oligosaccharides (fGOS)

Lactulose (4-O-β-D-galactopyranosyl-D-fructose), is a disaccharide formed by a molecule of galactose linked to a fructose residue by a β 1-4 glycosidic bond. Much attention has been given recently to lactulose because of its prebiotic effect, having several applications as a functional food ingredient, besides its well-established use as a medicine. This makes it a quite valuable lactose-derived product [92]. Presently, lactulose is produced commercially by alkaline isomerization of lactose [93,94]. Chemical synthesis of lactulose has the drawbacks of requiring high amounts of inorganic and environmentally objectionable catalysts, producing significant lactulose degradation and forming undesirable and hard to remove unwanted compounds, making product recovery cumbersome and costly [92,93]. In this scenario, production of lactulose by enzyme biocatalysis appears as an attractive option for overcoming the limitations of chemical synthesis [94]. Early this decade, the enzymatic synthesis of lactulose by direct isomerization of lactose was reported for the first time. The enzyme used was a promiscuous cellobiose 2-epimerase from *Caldicellulosiruptor saccharolyticus*, initially cloned in *E. coli*, obtaining a lactulose yield of 58% [95]. Since *E. coli* is an objectionable host for producing proteins for food or pharmaceutical use, efforts have been made to express this gene in a GRAS host, such as *Bacillus subtilis* [96]. Nowadays, this route seems to be the most promising for the industrial production of lactulose via biocatalysis. However, this enzyme is not commercially available so far, and does not count on the sanitary certifications for its intended use. Therefore, fructose transgalactosylation from lactose with βGs is the most common route for lactulose synthesis, that has been thoroughly evaluated [94]. First studies on lactulose synthesis with βGs were reported early this century. Lee et al. [97] were the first showing that lactulose could be synthetized from lactose and fructose using permeabilized cells of *K. lactis* containing βG as biocatalyst, obtaining 20 $g{\cdot}L^{-1}$ of lactulose with a volumetric productivity of 6.8 $g{\cdot}L^{-1}{\cdot}h^{-1}$. At about the same time, the production of lactulose with the βGs from *A. oryzae* and *S. solfataricus* and with the β-glycosidase from *Pyrococcus furiosus* were also reported [98]. Lactulose synthesis with βG is far more complex than the synthesis of GOS, not only because the reaction involves two substrates but

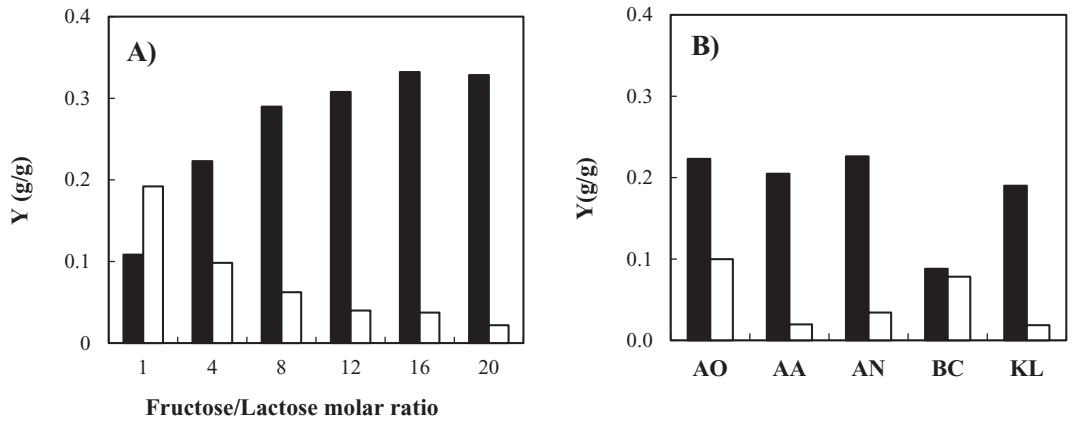

Fig. 5. Enzymatic synthesis of lactulose. A) Effect of fructose:lactose molar ratio on the yield of synthesis (Y) with *A. oryzae* β-galactosidase. B) Effect of the β-galactosidase source on Y at fructose:lactose molar ratio of 4. AO: *A. oryzae*, AA: *Aspergillus aculeatus*, AN: *Aspergillus niger*, BC: *B. circulans* and KL: *K. lactis* β-galactosidase. Black bars: lactulose yield; white bars: TOS yield. Adapted from Guerrero et al. [10,99].

also because, concomitantly with lactulose synthesis, transgalactosylated oligosaccharides (TOS), namely GOS and fructosyl-galacto-oligosaccharides (fGOS) are formed [94]. Most significant variables for the control of lactulose synthesis are the fructose:lactose molar ratio and the biocatalyst origin. The first one allows driving the reaction preferentially to the synthesis of TOS or to the synthesis of lactulose, by selectively increasing the rate of the desired reaction [99]. For instance, when *A. oryzae* βG is used, TOS synthesis is favored at fructose:lactose molar ratios lower than 1, while at values higher than 1 lactulose:TOS ratio continuously increases (see Fig. 5A). On the other hand, the origin of the enzyme determines its substrate preference for the different carbohydrates as galactose donor or acceptor [10] (see Fig. 5B). Within the βGs available for industrial use, higher selectivity for lactulose is obtained with the mold enzymes, while similar amounts of lactulose and TOS are produced with the *B. circulans* βG. Besides fructose:lactose ratio and enzyme origin, lactulose synthesis should be conducted at high carbohydrates concentrations to depress the reactions of hydrolysis in favor of transgalactosylation [10,99].

Considerations made in the previous section with regard to immobilized enzymes in GOS synthesis are equally applicable for the case of lactulose. However, it is worth mentioning, that both yield and selectivity of lactulose synthesis can be improved by using immobilized enzymes [80]. Different factors allow explaining the observed effects of βGs immobilization on lactulose synthesis: stiffening of the enzyme structure making it more robust, alteration of its three-dimensional structure, chemical modification, partition and mass transfer limitations [100]. The effect of immobilization on lactulose synthesis is illustrated in Fig. 6. Using βG immobilized by aggregation and cross-linking an increase in selectivity was obtained without reduction in yield (see Fig. 6A), being a consequence of the reduction in TOS formation, probably due to internal diffusional restrictions [80]. This effect is not always observed with enzymes immobilized to inert supports (see Fig. 6B) so that different immobilization techniques should be evaluated to select the most appropriate for a given reaction system.

4.3. Synthesis of lactosucrose

Lactosucrose (O-β-D-galactopyranosyl-(1,4)-O-α-glucopyranosil-(1,2)-β-D-fructofuranoside) is a synthetic trisaccharide mostly produced by transfructosylation of lactose in a reaction catalyzed by β-fructofuranosidase [101]. Lactosucrose exerts different health-associated physiological responses and its application as a functional food ingredient has rapidly expanded in Japan and Europe [15,102]. Lactosucrose is considered a potential prebiotic since it is not hydrolyzed in the upper gastrointestinal tract being metabolized by the colonic microbiota exerting a bifidogenic effect and inhibition of *Clostridia* [102]. At large-scale, lactosucrose is produced by transfructosylation of lactose using *Arthrobacter* sp. β-fructofuranosidase as biocatalyst. The reaction is conducted in the presence of an invertase-negative yeast for consuming the inhibitory compounds resulting from the glucose assimilation produced during lactosucrose synthesis, obtaining a product with 65% lactosucrose [103].

Synthesis of lactosucrose with βGs has been barely reported in the literature. In 2003, Farkas et al. [104] using *B. circulans* βG were the first demonstrating that βG can transgalactosylate sucrose, obtaining a lactosucrose yield of 17%. Later on, Li et al. [105] studied the effect of reaction conditions on the synthesis of lactosucrose with the same enzyme, obtaining under optimized conditions a maximum concentration of 146 $g{\cdot}L^{-1}$ of transgalactosylated products; 40% of which were lactosucrose. The production of lactosucrose has been evaluated also using *B. circulans* βG immobilized in chitosan, reaching a maximum concentration of 250 $g{\cdot}L^{-1}$ of transgalactosylated products, 32% of them being lactosucrose [106]. During the synthesis of lactosucruse with *B. circulans* βG iso-lactosucrose, 4′-galactosyl-lactosucrose and GOS were produced as well [107].

4.4. Synthesis of alkyl-β-galactosides

p-Nonylphenol and their ethoxyl108ated derivatives are nowadays considered “workhorse surfactants” given their cost-effectiveness and high performance in multiple industrial applications [108]. However, they have serious drawbacks: they are petroleum-based products, their production is environmentally threatening and toxic effects for the endocrine system have been claimed for them [109]. In view of this, they have been banned in the European Union and the Environmental Protection Agency (EPA) of the USA launched a campaign in 2010 for promoting the replacement of p-nonylphenol and their ethoxylated derivatives [108–110]. Alkyl-β-galactosides are biodegradable, non-toxic and hypoallergenic non-ionic surfactants that can be produced from renewable raw materials [47]. Therefore, they are considered as a sound alternative for the substitution of p-nonylphenol and their ethoxylated derivatives.

At industrial scale, alkyl-glycosides are produced by Fischer glycosylation of a fatty alcohol usually containing from 8 to 14 carbon atoms [47]. The process is conducted at severe conditions of pH and temperature, and may require hazardous catalysts [47,111] under eco-threatening conditions. Since many equally reactive hydroxyl groups are present in the glycoside, the product of reaction is a complex mixture of isomers and anomers of alkyl polyglycosides [112]. Being so, the enzymatic synthesis of alkyl-glycosides is a promising technology for overcoming the drawbacks of the chemical synthesis, allowing the production of anomerically pure alkyl-glycosides, which is quite

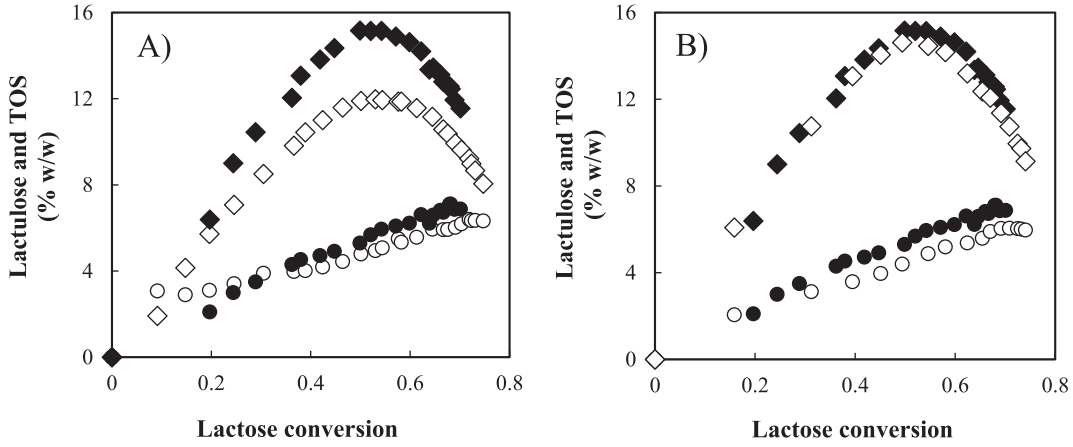

Fig. 6. Synthesis of lactulose with immobilized β-galactosidases. A) enzyme immobilized by aggregation and crosslinking; B) enzyme immobilized by multipoint covalent attachment to glyoxyl- agarose. Diamonds: transgalactosylated oligosaccharides (TOS); circles: lactulose. Open symbols: free β-galactosidase; closed symbols: immobilized β-galactosidase. Adapted from Guerrero et al. [80].

cumbersome by the chemical route [43,101]. βGs have been reported as appropriate catalysts for alcohol transgalactosylation reactions since the mid-1970s [113]. However, almost 30 years elapsed before its application in the synthesis of alkyl-β-galactosides was considered viable from a technological perspective [111,114,115].

Industrial applications of alkyl-β-galactosides require alkyl chains from 8 to 14 carbon atoms due to their detergent properties [116]. Regrettably, glycosidases generally produce lower yield and productivity of alkyl-glycosides synthesis as the chain length of the fatty acid used as acceptor increases, being quite poor for alkyl chains of more than 8 carbon atoms [47,117,118]. This is the main reason precluding the adoption of the enzymatic technology, which is also considered more complex than the chemical synthesis [112]. In this regard, Vera et al., [13] demonstrated for the synthesis of hexyl-β-galactosides with *A. oryzae* βG that the Michaelis constant (K_M) for the donor substrate plays a key role in determining the reaction yield. Donor substrates with low K_M values reduce the hydrolysis of synthetized alkyl-β-galactoside, increasing the reaction yield. These results suggest that secondary hydrolysis may be in part responsible for the low yield obtained in the synthesis of long-chain alkyl-glycosides. Table 1 listed some examples of this application and the reaction conditions required for synthesis.

The use of immobilized βGs is an attractive option for the synthesis of alkyl-β-galactosides, since such robust catalysts can withstand the presence of organic solvents in the reaction medium [120,126,127]. For instance, Hronska et al. [126] reported the use of *A. oryzae* βG immobilized into polyvinylalcohol hydrogel for the production of ethyl- and propyl-β-D-galactopyranosides. The biocatalyst preserved 50% of the initial transgalactosylation activity after 25 repeated batch runs in the production of ethyl-β-D-galactopyranoside. When 5% (v/v) propanol was used as an acceptor, the enzyme activity remained constant for 25 repeated batch runs. In the same way, Vera et al. [120,127] performed the synthesis of propyl- and butyl-β-D-galactopyranoside with *A. oryzae* βG immobilized in glyoxyl-agarose during ten successive batches. They demonstrated that the cumulative productivity obtained with the immobilized βG surpassed the one obtained with the free enzyme after the third and fourth successive batches of propyl- and butyl-β-D-galactopyranoside synthesis respectively. However, the reuse of the immobilized βG was rather limited, since its stability was similar than the one of the free enzymes. This occurs because stabilization of βG achieved by its immobilization is less significant when a high content of organic (co)solvent is present in the reaction medium [128].

Table 1
Synthesis of alkyl-β-galactosides with β-galactosidases from different sources.

β-Galactosidase source	Donor substrate	Donor substrate concentration (mM)	Acceptor	Temperature (°C)	pH	Yield (mol/mol)	Reference
Arthrobacter sp. *32cB*	Lactose	29 to 438	2-Propanol	30	8	NR	[119]
			1-Butanol			NR	
			1-Hexanol			NR	
Aspergillus oryzae	Lactose	13.3	1-Propanol	25	4.5	0.87	[120]
	Lactose	29	1-Butanol	45		0.79	[121]
	Lactose	16.6	1-Hexanol	35		0.06	[13]
	Butyl-β-galactoside	16.6	1-Hexanol			0.49	
	Propyl-β-galactoside	16.6	1-Hexanol			0.47	
	o-Nitrophenyl-β-D-galactopyranoside	16.6	1-Hexanol			0.59	
	Lactulose	16.6	1-Hexanol			0.12	
Bacillus pseudofirmus	Lactose	150	1-Hexanol	40	9.5	0.5	[122]
		50	1-Octanol			0.26	
Kluyveromyces lactis	Lactose	500	Methanol	40	6.8	0.71	[115]
		1000	Ethanol			0.36	
Penicillium canescencs	Lactose	174	1-Octanol	20	5.5	0.45	[123]
Pseudoalteromonas sp. *22b*	Lactose	487	1-Butanol	30	8	0.18	[124]
			1-Hexanol			0.11	
			1-Octanol			0.05	
Sulfolobus solfataricus	Lactose	93	1-hexanol	75	7	0.51	[125]
Aspergillus oryzae	Lactose	290	Ethanol	40	4.5	0.27	[126]
			Propanol			0.25	

4.5. Synthesis of galatosyl-polyhydroxyalcohols

Galactosyl-polyhydroxyalcohols (gal-polyols) are produced by enzymatic transgalactosylation of polyhydroxyalcohols, such as sorbitol, glycerol, xylitol, isomaltitol, lactilol, and mannitol [129–131]. These sugar alcohols, or polyols, are nutritive sweeteners having diverse applications in food and pharmaceutical products [132]. The synthesis of gal-sorbitol, gal-xylitol, gal-erythritol and, gal-lactitol was reported for the first time by Klewicki [129], who used *K. fragilis* β-gal as biocatalyst. The concentrations of galactosyl derivatives of polyols obtained were 0.31, 0.22, 0.18 and 0.14 M for gal-xylitol, gal-sorbitol, gal-lactitol and gal-erythritol, respectively. The enzyme origin, the lactose to polyhydroxyalcohols molar ratio, the total dry matter content and the ionic strength were the main variables determining the reaction yield [129,131,133]. The β-gal from *Kluyveromices* sp. [131], lactose to polyhydroxyalcohols molar ratios between 1:3 to 1:1 [131,133], high ionic strength (0.75 M NaCl) [133] and total dry matter content from 20 to 40% w/w [129] have proved to be suitable values for these variables. *A. oryzae* βG immobilized in glutaraldehyde-agarose was also reported as catalyst for the synthesis of galactosyl-ethyleneglycol, galactosyl-glycerol and galactosyl-erythritol [134]. This biocatalyst showed a preference for primary alcohols, consequently producing mixtures of stereoisomers in the case of using glycerol and erythritol as acceptors [134].

Despite of being scarcely reported, the synthesis of transgalactosylated compounds by reverse hydrolysis can be catalyzed also by βGs. Different from transgalactosylation, synthesis by reverse hydrolysis is thermodynamically controlled. Therefore, the yield of synthesis is determined by the composition at the reaction equilibrium [135]. To shift the reaction equilibrium toward the synthesis of β-galactosides, the water activity must be low. Regrettably, glycosidases typically need a_w values over 0.6 to be catalytically active [47], so, yield and productivity are usually lower than obtained by transgalactosylation [47,111]. An interesting example of galactoside synthesis by reverse hydrolysis was reported by Wei *et al* [136]. The authors evaluated the synthesis of galactosyl-glycerol from galactose and glycerol using *K. lactis* βG as catalyst. After 24 h of reaction, the authors obtained 116.47 $g{\cdot}L^{-1}$ of galactosyl-glycerol with a galactose conversion of 56%. Galactosyl-glycerol and acylated glycolipid-like derivatives may find interesting applications in food, cosmetic and health-care products, and even as antitumor drugs [136].

4.6. Synthesis of other transgalactosylated compounds

The capability of βGs to transfer a galactosyl moiety to compounds containing hydroxyl groups has been used to synthetize a wide range of new compounds with improved bioactive properties [137–139]. There are several related studies in pharmacology, where it has been observed that galactosyl derivatives of certain prodrugs usually have improved specificity, cellular permeability or pharmacokinetic profile as well as reduced toxicity compared to the unmodified substance [140]. For example, the unpleasant odor and taste of commercial vitamin B_1 hydrochloride (thiamin·HCl) have been removed by the synthesis of β-galactosylthiamin with *A. oryzae* βG [141]. Also, salicin-β-galactoside has been synthetized using *A. oryzae* βG; due to its structural resemblance with galectin inhibitors, analgesics and antipyretics, this molecule might have physiological activity [142]. Recently, 19-O-β-galactosyl andrographolide has been produced using a βG from bovine liver, obtaining molar yields ranging from 22 to 52%. The galactosylated andrographolide, unlike the non-glycosylated andrographolide, showed antibacterial activity and 702-fold higher solubility in water [143]. Also, Wojciechowska et al., have recently reported the trangalactosylation of glucoheptonic acid [138], gluconic acid [139] and ascorbic acid [137] using *K. lactis* βG.

Other interesting application of βGs is the galactosylation of phenolic compounds. These are secondary metabolites present in most plant tissues, which are characterized by having at least one phenol unit in their structures [144,145]. Interest relies on their appealing physiological and pharmacological functions such as: antioxidant, antibacterial, anti-inflammatory and protective effects on cardiovascular or cerebrovascular diseases [144,145]. Glycosylation of phenolic compounds increases their solubility and stability in water [146,147]. Also, it may prevent their oxidation, enhance their bioavailability and decrease their toxicity [148,149]. All these positive effects contribute to overcome the major limitations for their technological application [144,145,150]. The enzymatic galactosylation of phenolic compounds is an emerging field, where only a couple of cases have been reported. This is probably due to the fact that most glycosidases present a low reactivity on phenols, preferring primary and secondary hydroxyl groups [111]. Reported cases show that βGs are suitable biocatalyst for galactosylation of hydroquinone. The synthesis of hydroquinone galactoside (a potential skin whitening) was accomplished by using βGs from *A. oryzae, B. circulans. Thermus* sp. *and K. lactis*; the highest conversion of 60.3 % was obtained with the latter [151]. More recently, Lu et al. [152] used a mutated (W980F) βG from *Lactobacillus bulgaricus* L3 for the galactosylation of hydroquinone, pyrogallol, catechol and phenol, obtaining yields of 65, 30, 18 and 16 %, respectively.

5. Conclusions

βGs have been a matter of study both from a scientific and a technological perspective. The βG from *E. coli* was a preferred model of study for the cell regulation of enzyme synthesis; key metabolic control phenomena, like induction and catabolite repression, were mostly studied on the lac operon that controls βG synthesis. On the other hand, GRAS yeast βGs have been traditionally used in the food industry for the production of milk and dairy products with reduced lactose content. This application has been a major success and now such products are common consumer goods in supermarket shelves all over the world. This is a robust and still expanding market for βGs and βG genes from different origins have been cloned in GRAS hosts for producing enzymes with special properties (i.e. enzymes able to delactose milk at high or low temperatures).

Market for βG has received a strong input as novel non-conventional applications arose in recent decades that exploit its transgalactosylation activity, which can be efficiently expressed under particular reaction conditions, usually involving a low water activity. In such media, βGs hydrolytic activity is arrested so that the enzyme catalyzes the formation of β-glycosidic linkages instead of breaking them. This capacity is a major asset for a commodity enzyme, like βG, that opens up the possibility of using it in high-added value processes leading to the synthesis of different oligosaccharides and glycosides. Synthesizing such products will require rather harsh reaction conditions, like very high substrate concentrations and non-aqueous reaction media, that may significantly impair the activity and operational stability of βGs. Robustness under such conditions is then a must for their use as catalysts for transgalactosylation reactions. This challenge is being successfully tackled by a combination of protein engineering (molecular redesign by site-directed mutagenesis and directed evolution), biocatalyst engineering (immobilization and chemical modification) and medium engineering (performance in neoteric solvents and at very high solute concentrations) strategies. The production of lactose-derived prebiotics and health promoting food and feed ingredients and the production of biodegradable surfactants illustrate quite well the perspectives of βGs, which is certainly an enzyme of the past, with a well-consolidated present and a more promising future.

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