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REVIEW

Enzymes for the biocatalytic production of rare sugars

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Abstract Carbohydrates are much more than just a source of energy as they also mediate a variety of recognition processes that are central to human health. As such, saccharides can be applied in the food and pharmaceutical industries to stimulate our immune system (e.g., prebiotics), to control diabetes (e.g., low-calorie sweeteners), or as building blocks for anticancer and antiviral drugs (e.g., L-nucleosides). Unfortunately, only a small number of all possible monosaccharides are found in nature in sufficient amounts to allow their commercial exploitation. Consequently, so-called rare sugars have to be produced by (bio)chemical processes starting from cheap and widely available substrates. Three enzyme classes that can be used for rare sugar production are keto–aldol isomerases, epimerases, and oxidoreductases. In this review, the recent developments in rare sugar production with these biocatalysts are discussed.

Keywords Rare sugars · Biocatalysis · Epimerase · Isomerase · Polyol dehydrogenase

Abbreviations

D-ADH	D-Arabitol 2-dehydrogenase
L-AI	L-Arabinose isomerase
CE	Cellobiose 2-epimerase
MDH	Mannitol 1-dehydrogenase
L-RI	L-Ribose isomerase
D-TE	D-Tagatose 3-epimerase
T6PI	Tagatose-6-phosphate isomerase
UDP	Uridine diphosphate
XDH	Xylitol 4-dehydrogenase

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Introduction

The International Society of Rare Sugars (ISRS) has classified monosaccharides and derivatives according to their abundance in nature [27]. In this classification, of all possible hexoses and pentoses, only seven (glucose, galactose, mannose, fructose, xylose, ribose, and L-arabinose) were considered to be present in significant amounts, whereas 20 hexoses and nine pentoses were described as rare sugars. Another large group of rare sugars consists of deoxygenated monosaccharides, which often play a crucial role as recognition elements in bioactive molecules [23, 65, 111]. Furthermore, secondary modifications, such as amination or methylation, can also occur. Rare sugars cannot be extracted from natural sources and thus have to be produced by (bio)chemical reactions. Nevertheless, several of these are now commercially available as bulk products, such as D-tagatose and D-sorbose. Others, in contrast, are specialty compounds that are used in high-value applications, which is the case for most L-sugars. It can be expected that more efficient production routes will increase the availability of rare sugars for research purposes, resulting in the discovery of new applications and/or as yet unidentified characteristics [27].

Despite their low natural abundance, rare sugars hold enormous potential for practical applications (Table 1). In the pharmaceutical industry, for example, L-ribose can be used as a building block for drugs against cancers and viral infections. Its most important application is in antiviral

Table 1 Overview of rare, unmodified monosaccharides and their applications (update from [1])

Sugar	Application(s)	Reference(s)
D-Allose	Treatment of cancer, in particular chronic myeloid leukemia	[3, 34, 72, 76, 116]
	Suppression of thrombus formation and reperfusion injury	[4, 35, 77]
	Cryoprotectant for mammalian cells and organs	[102]
	Immunosuppressant	[36]
D-Altrose	Synthesis of cyclic carbamates of derived glycosylamines (polymer chemistry)	[53, 55]
D-Arabinose	Synthesis of antitumor compounds, such as dehydroamino acid derivatives	[22, 74, 123]
	Production of D-erythroascorbic acid and oxalic acid	[66]
D-Gulose	Drug-formulation agent and food additive	[7]
D-Idose	Synthesis of cyclic carbamates of derived glycosylamines (polymer chemistry)	[53, 55]
D-Lyxose	Synthesis of antitumor and immunostimulatory agents	[75, 104]
D-Psicose	No-calorie sweetener, treatment of diabetes	[6, 32]
	Potential anthelmintic	[97]
	Precursor of xylosylpsicoses (used as prebiotics, cosmetics, and therapeutics)	[91]
D-Ribulose	Starting material for branched pentoses (useful in pharmaceutical chemistry)	[37]
D-Sorbose	Building block for industrial and bio-active products	[39, 40]
	Insect control agent	[40]
D-Tagatose	Low-calorie sweetener, treatment of diabetes	[19, 60, 67, 79]
	Improvement of human health (e.g., antiplaque, prebiotic)	[60, 67, 79]
	Additive in detergents, cosmetics, and pharmaceutical formulations	[60, 79]
D-Talose	Anti-tumor and anti-microbial activities, including marker of O-antigens	[78, 115]
D-Xylulose	Starting material for branched pentoses (useful in pharmaceutical chemistry)	[37]
L-Allose	Therapeutic agent for diseases involving vasculogenesis	[110]
L-Altrose	Component of biologically important oligo- and polysaccharides	[38]
L-Fructose	Potential inhibitor of various glucosidases	[56]
	Mixture of L- and D-fructose kills ants and house flies	[1]
L-Galactose	Potential in synthesis of L-nucleoside-based antiviral medications	[113]
	Component of saponins, with applications in food, cosmetics, and pharmaceuticals	[83, 113]
L-Glucose	Starting material for glycoconjugate vaccines against diseases caused by *Shigella sonnei*	[56]
	Cytostatic and cytotoxic properties with regards to neoplastic cells (cancer therapy)	[1]
L-Gulose	Building block of bleomycin A_2, a glycopeptide antibiotic (potential anticancer agent)	[18, 113]
	Synthesis of nucleosides that exhibit very potent activity against HBV and HIV	[18]
	Starting material for the production of L-nucleoside-based antiviral medications	[113]
L-Idose	Derivatives are required in the synthesis of sensitive substrates for α-L-iduronidase	[18]
	Derivatives are used as glycosyl donors in the synthesis of heparin oligosaccharides	[108]
L-Lyxose	Component of the antibiotic avilamycin A	[33]
	Potential L-fucosidase inhibitor	[11]
L-Mannose	Component of steroid glycosides	[38]
L-Psicose	Starting material for the production of L-fructose	[41]
L-Ribose	Building block for antiviral and anticancer L-nucleosides	[30, 70, 124]
	Building block for glycoconjugates, oligonucleotides and L-aptamers	[82]
	Starting material for the production of L-allose and L-altrose	[5]
	Potential against HBV and Epstein–Barr virus	[109]
L-Ribulose	Starting material for L-ribose production	[15, 16, 99]
L-Sorbose	Starting material for the production of L-tagatose	[41]
	Precursor for the synthesis of the potent glycosidase inhibitor 1-deoxygalactonojirimycin	[1]
	Starting material for the production of L-ascorbic acid, also known as vitamin C	[41]
	Starting material for the synthesis of L-talitol	[96]

Table 1 continued

Sugar	Application(s)	Reference(s)
L-Tagatose	Potential as a functional sweetener	[91]
	Potential in chemotherapy	[91]
	Precursor of complex materials, such as 1,2,3,4-diisopropylidene tagatofuranose	[91]
	Starting materials for the synthesis of L-deoxygalactonojirimycin	[40]
L-Talose	Precursor of L-talose nucleosides, inhibitors the in vitro growth of leukemia L1210 cells	[59]
L-Xylose	Starting material for the synthesis of the nucleosides against HBV	[69]
	Synthesis of L-ribofuranose derivatives	[8]
L-Xylulose	Potential inhibitor of various glucosidases	[56, 61]
	Synthesis of L-xylose and L-lyxose	[26]
	Indicator of hepatitis or liver cirrhosis	[105]

HBV Hepatitis B virus, *HIV* human immunodeficiency virus

therapy, where it is incorporated in L-nucleosides analogues [30]. The advantages of the L-enantiomer are increased antiviral activity, better metabolic stability, and more favorable toxicological profiles. Since the discovery of lamivudine (2′,3′-dideoxy-3′-thiacytidine, mostly referred to as 3TC), an increasing number of L-nucleoside analogues are undergoing clinical trials and/or preclinical studies, while several other L-sugars can be used to produce L-nucleosides, such as L-gulose, L-xylose, and L-galactose [30, 69, 113]. L-Sugars can also be used as an active compound on their own, for instance as glycosidase inhibitors [56] or as insecticides [1].

Other rare sugars, such as D-tagatose, can serve as low-calorie sweeteners, replacing classical table sugar in the food industry. A major advantage is that tagatose has a low glycemic index, making it suitable for diabetic patients [67]. In that respect, it is interesting to note that tagatose has entered phase III clinical trials to investigate whether it can be used as diabetic medication [19]. Similar effects have been attributed to D-psicose, which shows potential as a no-calorie sweetener as well as a diabetic and obesity control agent [6]. In contrast, D-allose, an isomer of D-psicose, displays rather different properties. In addition to its inhibitory effect on both carcinogenesis and cancer proliferation, it is also useful in surgery and transplantation as an anti-inflammatory agent, immunosuppressant, and cryoprotectant [64].

Enzymes for rare sugar production

Basically, three different types of enzymes can be used for the interconversion of monosaccharides (Fig. 1). Two of these are classified within the class of isomerases, i.e., keto-aldol isomerases (EC 5.3.1) and carbohydrate epimerases (EC 5.1.3). The former (often referred to as aldose isomerases or simply as isomerases) catalyze an

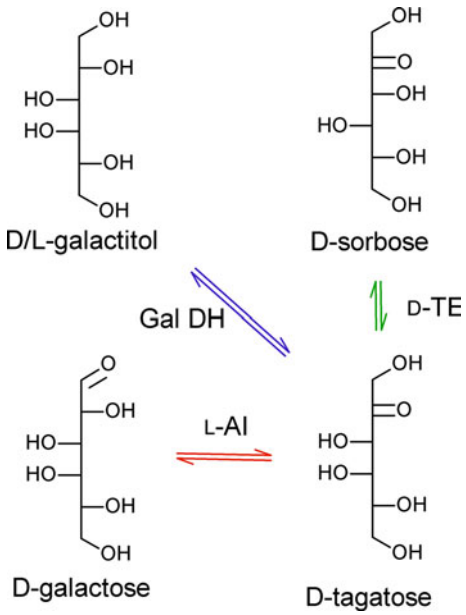

Fig. 1 The three different enzyme classes that can be used for rare sugar production. Oxidoreductases are exemplified by galactitol dehydrogenase (*Gal DH*), aldose isomerases by L-arabinose isomerase (*L-AI*) and epimerases by D-tagatose 3-epimerase (*D-TE*)

intramolecular redox reaction, exchanging the carbonyl functionality between the C1- and C2-positions [43]. The latter, in contrast, catalyze the re-orientation of a hydroxyl group, converting the substrate into one of its epimers [95]. The third group of enzymes consists of oxidoreductases (EC 1.1) that convert carbohydrates into their corresponding polyols, and vice versa [112]. Oxidoreductases acting on ketoses are typically designated as polyol dehydrogenases, whereas those that act on aldoses are known as aldose reductases [27].

All three of these enzyme classes have already been applied for the production of rare sugars (see overview in Fig. 2), but each has specific advantages and disadvantages

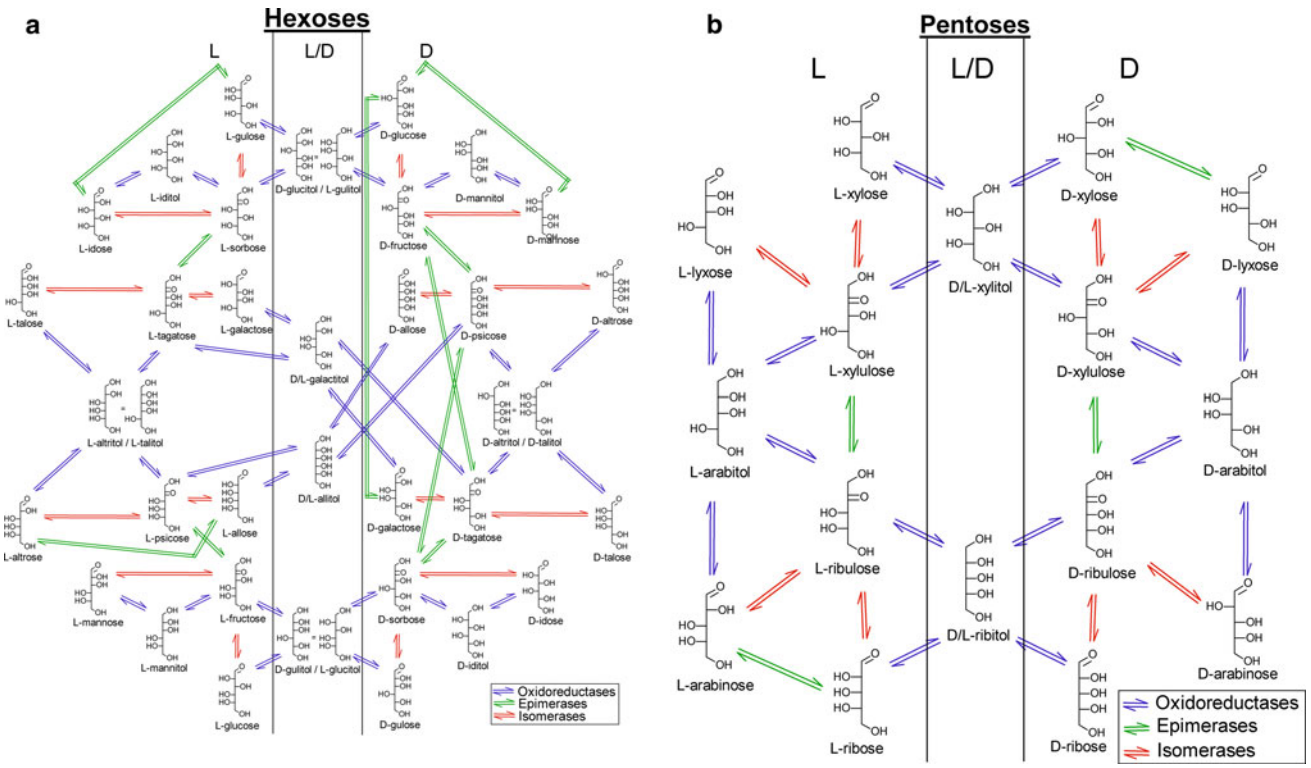

Fig. 2 Overview of the enzymatic interconversions of unmodified monosaccharides that have been reported to date: for hexoses (**a**) and for pentoses (**b**). Update of the Izumoring process described in [27]

Table 2 Comparison of three enzyme classes in rare sugar production

Enzyme	Advantages	Disadvantages
Isomerase	Substrates often unsubstituted	Product mixtures are sometimes formed
	Broad substrate specificity	
Epimerase	Shortcut in synthetic route	Substrates often substituted
	Potential bridge between D- and L-sugars	Substrate specificity is rather strict
Oxidoreductase	Bridge between D- and L-sugars	Need for cofactor regeneration
	Substrates often unsubstituted	

(Table 2). Isomerases, for example, are promiscuous biocatalysts that are active on a range of simple substrates, i.e., unsubstituted monosaccharides. However, promiscuity is not always an advantage because it can result in the formation of side products and complicate the downstream processing. This is nicely illustrated with the glucose-6-phosphate isomerase from *Pyrococcus furiosus*, which converts L-tagatose not only to L-talose but also to L-galactose (Fig. 3) [121]. Although both products are valuable, it would be more efficient to produce them separately with two different isomerases, each specific for one of the aldoses.

Epimerases are potentially the most useful biocatalysts for the production of rare sugars as they can give access to a wide range of structures, in contrast to the two other enzyme classes that are limited to modifications of the C1- and C2-positions. Unfortunately, most epimerases are only active on sugars that are substituted with a phosphate or nucleotide group, which drastically increases production costs. The recently discovered D-tagatose 3-epimerase is a noticeable exception that has allowed the production of D-psicose from the cheap substrate D-fructose [107]. The identification of new epimerases can create interesting shortcuts in current synthetic routes. A 2-epimerase, for example, can replace the double keto-aldol isomerization previously required for the conversion of D-xylose into D-lyxose (Fig. 2). Furthermore, 5-epimerases could form a new bridge between D- and L-hexoses, and the same is true for 4-epimerases acting on pentoses (Fig. 4). For instance, glucose could then serve as cheap substrate for L-idose production in a single reaction instead of the three-step process, which is currently the shortest route (Fig. 2).

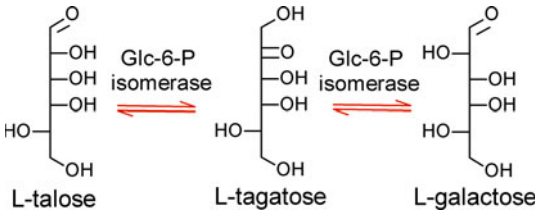

Fig. 3 Isomerization of L-tagatose with glucose-6-phosphate (*Glc-6-P*) isomerase. Because of the enzyme's low specificity, both L-talose and L-galactose are formed

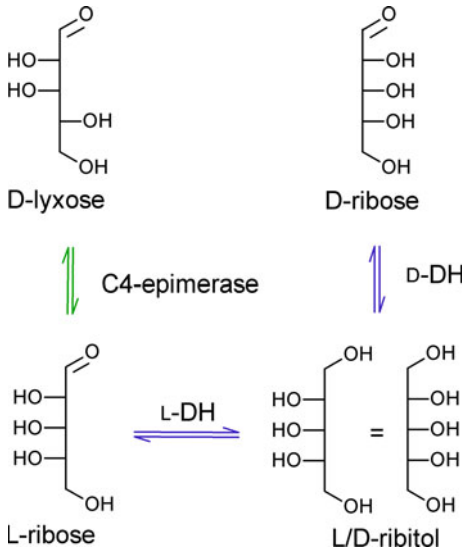

Fig. 4 Interconversion of D- and L-sugars. D-Ribose can only be converted to L-ribose through ribitol as intermediate, using oxidoreductases as biocatalysts. However, a putative C4-epimerase would be able to convert D-lyxose directly into L-ribose. *DH* Dehydrogenase

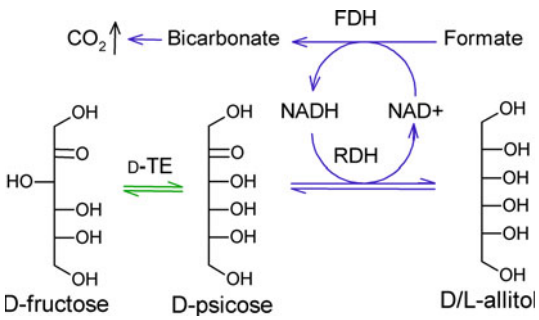

Fig. 5 The production of allitol from fructose. Allitol is produced from fructose by a coupling reaction using D-tagatose 3-epimerase (*D-TE*) and ribitol dehydrogenase (*RDH*). Cofactor regeneration is achieved with the help of the irreversible formate dehydrogenase (*FDH*) reaction

For the moment, D-sugars can only be converted into their L-isomers using the corresponding polyols as intermediate products. To that end, oxidoreductases are applied in a two-step process, i.e., sugar reduction followed by polyol oxidation (Fig. 4). However, polyols like xylitol and sorbitol are also valuable compounds in their own right and are used as sweeteners with a cooling sensation [24]. Despite their industrial relevance, oxidoreductases exhibit one great disadvantage, namely the need for the expensive cofactor NAD(P)H. As a result, reactions with oxidoreductases are often performed inside microbial cells so that the cellular metabolism can provide the reductive power. Alternatively, specific cofactor regeneration systems can be employed when isolated enzymes are to be used (Fig. 5) [17, 40].

Oxidoreductases

Since the development of the chemical hydrogenation method, xylitol has been used on a large scale as alternative sweetener [24]. However, research has also focused on developing microbial production methods for xylitol. To that end, oxidoreductases are often employed in metabolically engineered organisms, such as *Saccharomyces cerevisiae* and *Candida* strains [24, 25]. The main challenge with *S. cerevisiae* is to increase the uptake of xylose as substrate, as well as the regeneration of NADPH through the pentose phosphate pathway. Although *Candida* yeasts are relatively better at taking up xylose and maintaining the intracellular redox balance, their application in the food industry is hampered by the opportunistic pathogenic nature of some *Candida* species. In parallel, the use of xylitol 4-dehydrogenase (XDH) as the isolated biocatalyst for the production of xylitol has also been optimized. The immobilization of the enzyme from *Rhizobium etli*, for example, has resulted in a tenfold increased thermostability, a broader operational pH range, and excellent reusability [125]. In turn, xylitol can serve as substrate for the production of other rare sugars, such as L-xylulose and L-xylose. In a recent study, the XDH from *Bacillus pallidus* has been overexpressed in *Escherichia coli* for L-xylulose production. Although the conversion rates were lower than when the *B. pallidus* strain was used, the advantage of *E. coli* is that the formation of side products is drastically reduced [87, 105]. At higher temperatures, however, L-xylose started to accumulate instead of L-xylulose, perhaps due to the activity of endogenous D-arabinose isomerase in *E. coli*.

The combination of regio- and stereoselectivity for the C2 position allows the mannitol 1-dehydrogenase (MDH) from *Apium graveolens* to catalyze several interesting reactions, including the conversion of ribitol to L-ribose, D-sorbitol to L-gulose, and galactitol to L-galactose [100]. A recombinant *E. coli* harboring this MDH represents a significantly improved method for the large-scale production of L-ribose compared to previously used methods [114].

For example, a threefold higher productivity was obtained compared to the double isomerization of L-arabinose with xylose isomerase [45], and a much higher conversion rate was obtained compared to the chemical process, while the environmental hazards could be avoided [48].

Another dehydrogenase that shows high potential as a biocatalyst in rare sugar production is the recently discovered D-arabitol 2-dehydrogenase (D-ADH) from *Thermotoga maritime* [49]. This is the first hyperthermophilic D-ribulose-forming D-ADH and thus exhibits several industrial advantages. First, the enzyme can convert the inexpensive substrate D-arabitol with very high selectivity. Secondly, the risk of contamination in food production is dramatically lower as it can be used at increased temperatures. Lastly, it can be efficiently purified from recombinant *E. coli* by heat precipitation, providing a reliable and cost-effective supply of biocatalyst.

Oxidoreductases, and their producing strains, can also be used for the interconversion of deoxy-sugars. L-rhamnose is the only cheaply available deoxy-hexose, but it can be transformed into 1- and 6-deoxygenated D/L-psicose, D/L-fructose, and L-tagatose by *Enterobacter aerogenes* IK7 [28, 29]. Alternatively, *Enterobacter agglomerans* 221e can be applied in the synthesis of 1- and 6-deoxy-D-tagatose from both enantiomers of fucose [122]. In these processes, the deoxy-sugar is first chemically hydrogenated towards the corresponding polyol, which then serves as substrate for the enzymatic oxidation. Microbial oxidation can also be performed on methylated polyols, for instance for production of both enantiomers of 4-C-methyl-ribulose by *Gluconobacter thailandicus* NBRC 3254 [46, 92].

Isomerases

Due to the broad substrate specificity of isomerases, these enzymes can be applied in the synthesis of various aldoses and ketoses starting from their cheaper counterpart. A well-known example that illustrates their promiscuity is the fact that xylose isomerase is better known as glucose isomerase because it is mainly used for the production of high fructose corn syrup, rather than for its wild-type conversion (D-xylose to D-xylulose). The importance of substrate resemblance for side activities can be further illustrated with the following examples. The D-lyxose isomerase from *Providencia stuartii* was found to be active not only on D-lyxose/D-xylulose but also on nine other ketose–aldose couples. The highest specific activity was measured on aldose substrates with the C2 and C3 hydroxyl groups in the left-hand configuration, just as in the wild-type substrate. In contrast, very little activity could be detected on the mirror image substrates L-lyxose and L-mannose [54]. Many more isomerases have been shown to be active on sugars similar to their wild-type substrates [2, 12, 57, 71, 85, 86, 88, 94, 117].

The promiscuity of isomerases has not only been evaluated with resembling monosaccharides but also with respect to the presence of a phosphate group. Indeed, several isomerases that are naturally active on phosphorylated sugars have been shown to convert free carbohydrates as well. In particular, ribose-5-phosphate isomerase has been shown to be active on D/L-ribose, D/L-lyxose, D/L-talose, D/L-mannose, D/L-allose, L-lyxose, and L-tagatose [85, 118, 120]. Similarly, mannose-6-phosphate isomerase and galactose-6-phosphate isomerase have been found to convert free monosaccharide, such as L-ribose [119] and D-allose [86], respectively.

A second feature to take into account is whether the isomerase is specific towards one of the two aldoses. If the enzyme is not specific enough, mixtures will be obtained containing three sugars, namely the substrate, the desired product, and an undesired side product (Fig. 3). Consequently, the choice of the proper enzyme will result in both higher yields and easier purification. The L-rhamnose isomerase from *Bacillus pallidus* has been shown to convert D-psicose/D-allose, L-fructose/L-mannose, D-ribose/D-ribulose and L-talose/L-tagatose without the formation of by-products [88]. The opposite is true for the glucose-6-phosphate isomerase from *P. furiosus*, which converts L-talose to both L-tagatose and L-galactose, and D-ribulose to both D-ribose and D-arabinose [121].

To further outline the importance of isomerases in rare sugar synthesis, an overview is given from some isomerases that are currently in use or which show great potential in rare sugar conversion.

L-Arabinose isomerase for tagatose production

L-Arabinose isomerase (L-AI) has been thoroughly studied for the conversion of D-galactose into the low-calorie sweetener D-tagatose [9, 10, 14, 89, 94]. Tagatose is almost as sweet as sucrose but is metabolized in a different fashion, resulting in a caloric content that is about threefold lower [19, 60]. The enzyme from *Bacillus stearothermophilus* has just recently been successfully mutated into an improved industrial biocatalyst. Through rational design, three mutants were created based on previously reported data and sequence alignments. Acidotolerance and stability were improved by the mutation Q268K and a broader temperature range was achieved by the mutation N175H, with the combined double mutant displaying both characteristics [93]. In another study, ten positions identified through random mutagenesis of *Geobacillus thermodenitrificans* L-AI were examined in detail.

Five of the corresponding variants were shown to display a two- to threefold increase in specific activity. Double mutants were then created, with C450S/N475K generating a 20% higher tagatose conversion and a fourfold increase in specific activity compared to the wild-type enzyme [81]. Production processes have also been improved. The addition of boric acid was found to result in increased yields [63], whereas immobilization of the L-AI was performed to result in a stable and economic method for the industrial production of tagatose [80].

Isomerases for L-ribose production

Several isomerases have been shown to display a minor side activity on L-ribose, which has been the focus of enzyme engineering efforts. The activity of the mannose-6-phosphate isomerase from *Thermus thermophilus* for L-ribose production has, for example, been improved by the mutation R142N, resulting in a 1.4- and 1.6-fold increase in specific activity and catalytic efficiency (k_{cat}/K_M), respectively. This mutant was found through alanine scanning of several active-site residues, followed by partial randomization of the most important position. The catalytic efficiency of the resulting R142N mutant was 3.8-fold higher than that of *Geobacillus thermodenitrificans* mannose-6-phosphate isomerase, which had exhibited the highest catalytic efficiency reported to that date. The purified R142N mutant had a volumetric productivity of 107 g l^{-1} h^{-1} in a L-ribose production process [119]. However, a specific L-ribose isomerase (L-RI) has also been isolated from an *Acinetobacter* strain, with a K_M of 44 mM for L-ribose and a specific activity of 24.2 μmol mg^{-1} min^{-1} for L-ribulose formation [99]. Remarkedly, the enzyme's specific activity was about tenfold higher when it was recombinantly expressed in *E. coli* [73]. The L-RI could be a promising biocatalyst for the production of L-ribose, but has not yet been evaluated in such a process.

Isomerases for deoxygenated and other modified sugars

Two isomerases that prefer deoxy-sugars as their substrates are L-fucose isomerase [21, 47] and L-rhamnose isomerase [57, 58]. These enzymes also tolerate other modifications at centers higher than C3 and can use epimeric or functionalized sugars as substrates. Examples hereof are terminus-modified fuculose analogues for L-fucose isomerase [21]. Other related enzymes, including xylose (glucose) isomerase, have also been shown to accept deoxygenated and/or substituted sugars [20, 31, 90]. It can thus be expected that several isomerases will be applied for the production of modified sugars in the near future.

Carbohydrate epimerases

All biochemical reactions that can be used for the production of rare sugars and polyols from readily available raw materials, such as starch, wood, and lactose, have been summarized by Granstrom et al. in the Izumoring process [27]. At that time, only one epimerase was available for such conversions, namely the D-tagatose 3-epimerase. Since then, however, two other epimerases have been shown to be naturally active on unsubstituted sugars (i.e., cellobiose 2-epimerase and UDP-galactose 4-epimerase), whereas the L-ribulose-5-phosphate 4-epimerase has also been found to show promise for the production of rare sugars.

Ketohexose 3-epimerase

D-Tagatose 3-epimerase (D-TE) was found to catalyze the epimerization of various ketoses at the C3 position, making it a very useful enzyme for rare sugar production. D-TE has already been used for the synthesis of various carbohydrates, both in single and multiple enzyme reactions. At the laboratory scale, this enzyme has even been used to produce all possible ketohexoses [41, 42]. Furthermore, D-TE is a highly promiscuous enzyme that can accept a large range of unnatural substrates, such as C-4-methylated pentoses [92], C-5-methylated hexoses [46], 5-deoxy-ketohexoses [90], and several 1- and 6-deoxy-ketohexoses [29]. Alternatively, immobilized D-TE has been applied in the mass production of D-psicose from D-fructose [107]. The addition of borate to the reaction mixture results in the removal of the product as a psicose–borate complex and will thus result in improved yields [52]. Two examples of multiple enzyme reactions in which D-TE is applied are allitol and D-arabinose production, starting from fructose and xylose, respectively. To this end, the epimerase is combined with dehydrogenases or isomerases (Fig. 5) [103, 106].

A very similar enzyme has been described in *Agrobacterium tumefaciens*, namely D-psicose 3-epimerase (D-PE). Unfortunately, due to its short half-life (63 min at 50°C), this enzyme is inefficient for the industrial production of psicose; consequently, enzyme variants have been created by error-prone PCR and tested for improved thermostability [13]. Two single mutants (I33L and S213C) display an increased optimal temperature and kinetic stability. Combining both mutations in a single enzyme further improved these parameters, resulting in a 30-fold increase in half-life at 50°C. In a continuous production process with the immobilized double mutant, no decrease in activity could be observed after 30 days, suggesting that the I33L/S213C variant may be useful as an industrial producer of D-psicose.

UDP-Galactose 4-epimerase

The second enzyme that shows epimerase activity on free monosaccharides is the *E. coli* UDP-galactose 4-epimerase, which normally uses nucleotide-activated galactose and glucose (UDP-Gal/UDP-Glc) as substrates. However, the purified enzyme was found to also catalyze the 4-epimerization of free galactose, glucose, fructose, tagatose, psicose, and sorbose, albeit with very low specific activities (0.3–9.7 nmol mg^{-1} min^{-1}). Three residues were submitted to site-saturation mutagenesis, resulting in the identification of a N179S mutant with a twofold improved activity on fructose and tagatose. The enzyme was also tested for 4-epimerization activity on allose, gulose, altrose, idose, mannose, and talose but no activity was found on these aldohexoses [50].

Cellobiose 2-epimerase

A third enzyme that was found to catalyze (rare) carbohydrate epimerizations is the cellobiose 2-epimerase (CE) from *Caldicellulosiruptor saccharolyticus*. This thermophilic epimerase not only catalyzes the epimerization of cellobiose, but also shows low activity on aldoses harboring the hydroxyl group oriented in the left-hand configuration at the C3 position. This enzyme was found to exhibit the highest side activity for mannose (to glucose), although this was 20-fold lower than its activity on cellobiose. Low activities were also detected on D-xylose, L-altrose, L-idose, and L-arabinose [84]. Very recently, a new mannan catabolic pathway has been described in *Bacteroides fragilis*, including a CE that functions as mannobiose 2-epimerase in vivo. These findings are supported by a lower K_m and higher catalytic efficiency for mannobiose than for cellobiose, [98]. Further proof of its physiological role can also be found in its preference for mannose over glucose as a monosaccharide substrate, which was reported for the *C. saccharolyticus* CE [84]. Surprisingly, the latter enzyme also showed a slight isomerase activity on various monosaccharides when long reaction times and high amounts of enzyme were used [84]. It is well known that isomerase side activity will lower epimerization yield and complicate the purification process.

L-ribulose-5-phosphate 4-epimerase and related aldolases

Another epimerase that is promising for rare sugar conversions is L-ribulose-5-phosphate 4-epimerase. This enzyme is structurally and mechanistically related to the dihydroxyacetone phosphate-dependent L-fuculose-1-phosphate aldolase and L-rhamnulose-1-phosphate aldolase [44, 68]. Despite the fact that the wild-type substrates of all these enzymes are phosphorylated (deoxy-) sugars, this epimerase shows potential for the production of rare sugars that lack a phosphate group. Indeed, by applying error-prone PCR to L-rhamnulose-1-phosphate aldolase, variants could be created that display activity towards unsubstituted L-rhamnulose [101]. Since the two enzymes are very similar, it is not unreasonable to assume that the same or equivalent mutations would have an identical effect on the substrate specificity of the epimerase, thus making it active on free ketoses. Furthermore, aldolases can be used in the direct synthesis of rare sugars by aldol condensation of smaller carbohydrates. The previously mentioned L-fuculose-1-phosphate aldolase has been used in the production of D-psicose, D-sorbose, L-tagatose, and L-fructose, all starting from DL-glycerol 3-phosphate and D- or L-glyceraldehyde [62].

Conclusions and outlook

During the past decades, major progress has been made in research on rare carbohydrates. The discovery of new enzymes and the engineering of existing biocatalysts have generated new opportunities for their application in various industrial sectors. The discovery of a D-TE, for example, has not only allowed the synthesis of all possible ketohexoses, but also of methylated and deoxygenated sugars. For other biocatalysts, mutagenesis efforts have resulted in improved activity and stability, as illustrated by the R142N variant of mannose-6-phosphate isomerase and by the I33L/S213C variant of D-psicose 3-epimerase, respectively.

Epimerases, in particular, are exciting biocatalysts as they can facilitate rare sugar production through the introduction of major shortcuts in current production routes. One major challenge, however, is the need for epimerases that are active on free monosaccharides instead of nucleotide-activated or phosphorylated sugars. Obtaining such epimerases by redesigning the active site of known enzymes is not a trivial task. Indeed, the substituents are often essential for strong binding of the substrates and can even be crucial for the enzyme's activity through an induced fit mechanism. Alternatively, suitable epimerases could be identified by further screening in natural environments, which remains a powerful approach and regularly redefines our knowledge of microbial physiology.

A very important step in any enzyme engineering project is the choice of the most suitable template. In a recent study, L-AI and tagatose-6-phosphate isomerase (T6PI) were compared as starting points for increased isomerization activity on galactose [51]. Although this would require the loss of a phosphate group in the case of T6PI, the authors concluded that this enzyme was the best template for directed evolution because mutations are less likely to

diminish its activity. Indeed, L-AI makes use of a metal cofactor whose binding can be easily disrupted by random mutations. Nevertheless, L-AI will probably be the right choice for rational design studies because its crystal structure is available. Future work will reveal which enzyme and which strategy will generate powerful new biocatalysts for the production of rare sugars.

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