Review

Advances and Prospects of D-Tagatose Production Based on a Biocatalytic Isomerization Pathway

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Abstract: D-tagatose is a low-calorie alternative to sucrose natural monosaccharide that is nearly as sweet. As a ketohexose, D-tagatose has disease-relieving and health-promoting properties. Due to its scarcity in nature, D-tagatose is mainly produced through chemical and biological methods. Compared to traditional chemical methods, biological methods use whole cells and isolated enzymes as catalysts under mild reaction conditions with few by-products and no pollution. Nowadays, biological methods have become a very important topic in related fields due to their high efficiency and environmental friendliness. This paper introduces the functions and applications of D-tagatose and systematically reviews its production, especially by L-arabinose isomerase (L-AI), using biological methods. The molecular structures and catalytic mechanisms of L-AIs are also analyzed. In addition, the properties of L-AIs from different microbial sources are summarized. Finally, we overview strategies to improve the efficiency of D-tagatose production by engineering L-AIs and provide prospects for the future bioproduction of D-tagatose.

Keywords: D-tagatose; production; enzyme; L-arabinose isomerase; biocatalysts

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1. Introduction

In recent years, traditional high-absorption and high-calorie sugars have gradually been replaced by low-calorie and low-absorption rare sugars. As a representative of such rare sugars, D-tagatose is a naturally occurring monosaccharide with 92% of the sweetness of sucrose but only one-third of the calories.

D-tagatose, with the molecular formula $C_6H_{12}O_6$, is the ketose form of D-galactose and the epimer of D-fructose at the C-4 position (Figure 1). D-tagatose is very rare in nature and small amounts of D-tagatose are found to be naturally present in cheese, yogurt, hot cocoa, sterilized powder milk, and other dairy-derived products [1].

The functions of D-tagatose have been extensively evaluated for disease amelioration (obesity control, antidiabetes, and regulation of blood metabolites) and health promotion (anti-aging, antioxidant, and prebiotic properties) (Figure 2) [2]. Diabetic patients have been shown to experience gradual weight loss through long-term treatment with D-tagatose [3]. This is because D-tagatose does not lead to fat deposition. Research by Buemann et al. [4] showed that osmotic effects caused by unabsorbed D-tagatose lead to intestinal distension. This would potentially mediate an acute appetite suppressant effect and help to reduce energy intake; D-tagatose holds potential in blood sugar control with a glycemic index (GI) value of 3 [5]. When individuals consume D-tagatose before meals, approximately 20% of it is absorbed by the body, converting blood glucose into glycogen and slowing down the rate of decomposition of glycogen into glucose [6]. The reason for this may be due to

its liver metabolism process, which is similar to that of D-fructose. D-tagatose is phosphorylated to tagatose-1-phosphate by fructokinase. Its slower rate of breakdown results in the accumulation of tagatose-1-phosphate. Tagatose-1-phosphate stimulates glucokinase and promotes the conversion of glucose to glucose-6-phosphate. Glucose-6-phosphate stimulates liver glycogen synthase to accelerate glycogen formation. The remaining 80% is not absorbed and may compete with or partially inhibit glucose transporters in the small intestine, thereby inhibiting and preventing glucose absorption [7,8]. D-tagatose helps improve blood health [9] by strengthening key blood factors, such as the red blood cell count, prothrombin time, and activated partial thromboplastin time, as well as increasing coagulation factors. Therefore, it is a candidate for the treatment of anemia and hemophilia. D-tagatose also has the potential to prevent oral diseases [10]. Mayumi et al. [11] found that it selectively inhibits the growth of oral pathogens by affecting their glycolysis and downstream metabolism. Hasibul et al. [12] discovered that D-tagatose prevents the cariogenic species *Streptococcus mutans* from growing and forming biofilms. Dietary restriction through D-tagatose maintains lower blood sugar and insulin levels in the body, thereby delaying age-related disease development and further extending the lifespan of those who practice it [13,14]; Additionally, D-tagatose is an excellent prebiotic. Research has shown [15] that unabsorbed D-tagatose will enter the large intestine, where it is selectively fermented by microbial flora. This fermentation process promotes the proliferation of beneficial flora while inhibiting the growth of harmful flora, thereby improving the gut microbiota. At the same time, the fermentation of D-tagatose yields large amounts of beneficial short-chain fatty acids such as butyric acid [16], which is a good source of energy for colon epithelial cells.

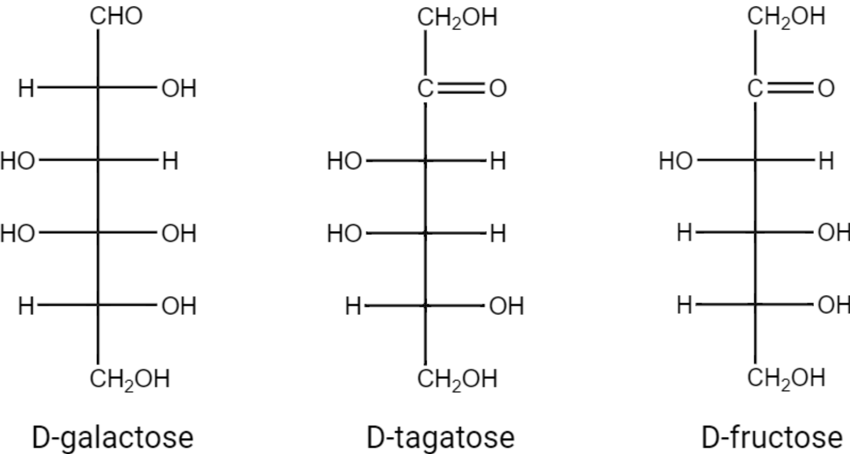

Figure 1. The structural formula of D-galactose, D-tagatose, D-fructose.

D-tagatose is an emerging low-calorie sweetener with potential to replace sucrose [17]. It can provide sweetness in a wide range of concentrations without adding an undesirable taste. The addition of D-tagatose to low-sugar products can provide them with desirable organoleptic properties, which means it can be beneficial to health without changing the flavor of the product [18,19]. Since D-tagatose has been approved as "generally recognized as safe" (GRAS), it can be used in confectionery, beverages, nutraceuticals, and dietary products. D-tagatose can also be used as an additive in prescription medications and as a sweetener in toothpaste, mouthwash, and cosmetics.

D-tagatose was discovered in the gum of *Sterculia setigera* as early as 1949 [20]. However, its scarcity makes it difficult to extract D-tagatose directly from nature on a large scale. In order to make D-tagatose production more economical and efficient, chemical and biological methods are usually used (Figure 3).

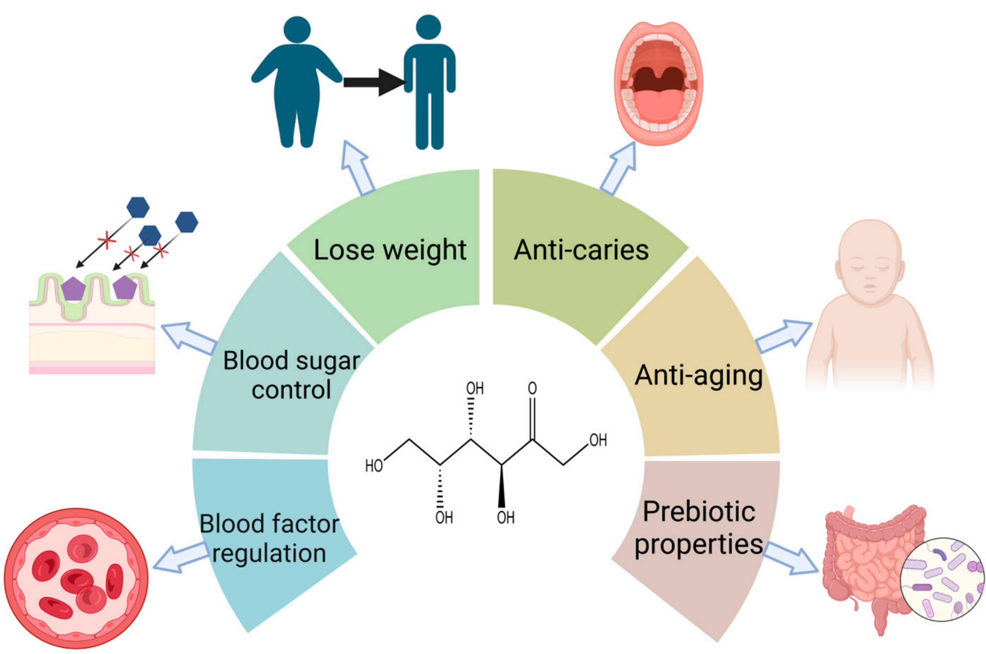

Figure 2. Various physiological functions of D-tagatose on the human body.

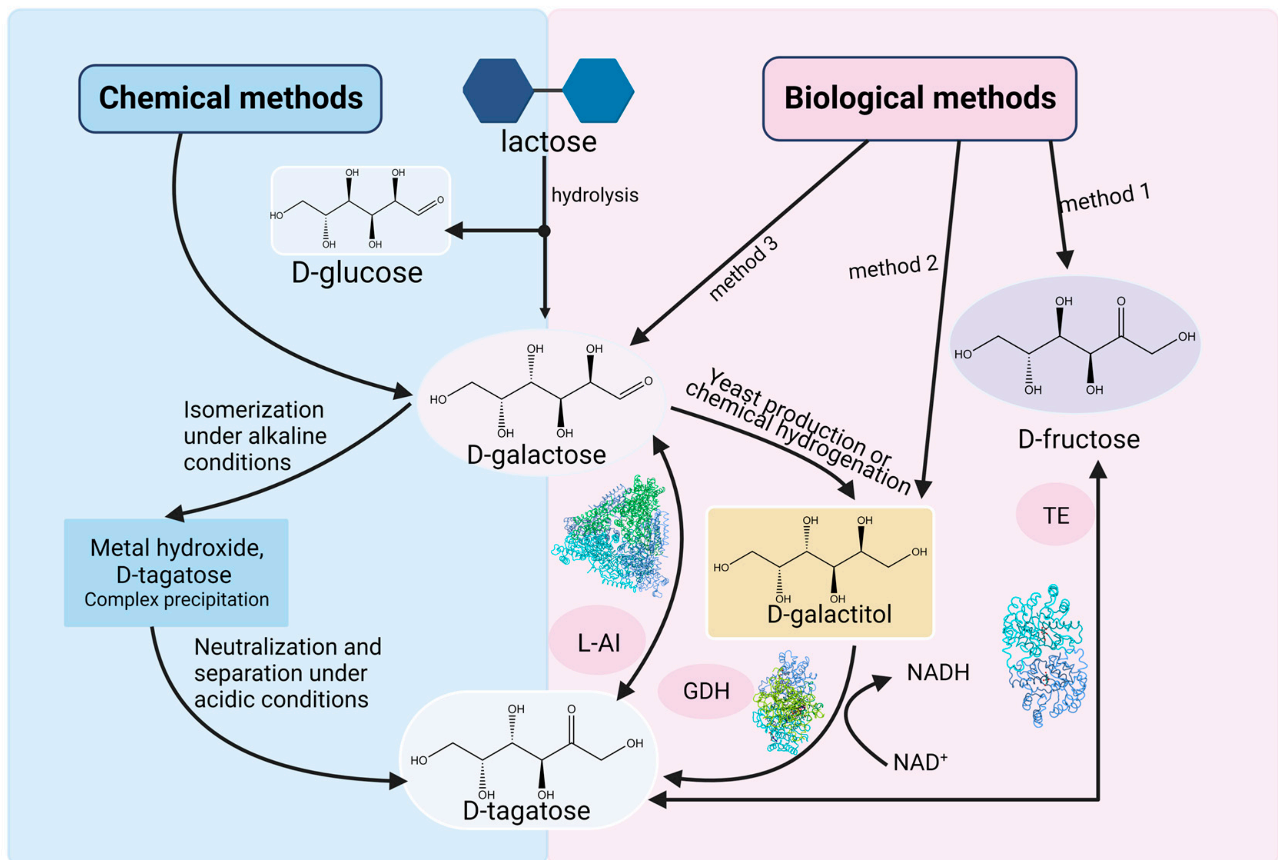

Figure 3. Current methods for producing D-tagatose via feasible chemical or biological routes. L-AI: L-arabinose isomerase, GDH: Galactitol dehydrogenase, TE: Tagatose 4-epimerase.

The typically used chemical method, also known as non-enzymatic isomerization [21], uses metal hydroxides as catalysts. This method consists of three steps: firstly, an insoluble D-tagatose complex is generated when D-galactose interacts with metal hydroxides, which

are stable under alkaline conditions; in the second stage, the insoluble D-tagatose complex is neutralized with an acid to produce an insoluble salt; finally, filtering is used to separate D-tagatose from the insoluble salt. During the isomerization, the metal hydroxides perform two functions: isomerizing D-galactose to D-tagatose and degrading D-galactose into dicarbonyl compounds and acidic substances. This method has a high yield (>70%) but includes the following disadvantages: severe D-galactose degradation leads to a reduction in the yield of D-tagatose, while the decrease in the quality of the syrup makes the production of crystalline D-tagatose difficult; the removal of the degradation products requires complex extraction steps; and a large number of metal hydroxides and acids are consumed in the reaction, which ultimately has many negative impacts on the cost and the environment. There are also other methods such as supercritical fluid (<24%) [22], triethylamine (<34%), arginine (<16.8%) [23], hydrotalcite (<27%) [24], Sn-β zeolite (<26%), etc. However, all of these non-enzymatic pathways have low D-tagatose yields [25]. Moreover, these methods require reactions under extreme conditions, which can lead to increased energy consumption and damage to substrates and products.

Biological methods use whole cells and isolated enzymes as catalysts. The advantages of such methods include mild reaction conditions, environmental friendliness, few byproducts, and easy purification. Thus, the production of D-tagatose by biological methods has advantages over chemical methods in many aspects. In biological methods, the lzumoring strategy is often used to produce various rare sugars. This strategy uses different types of enzymes including epimerase, oxidoreductase, and aldose–ketose isomerases to achieve interconversion between monosaccharides and sugar alcohols [26]. D-tagatose can also be produced by these three types of enzymes, including tagatose 4-epimerase, galactitol dehydrogenase, and L-arabinose isomerase.

Tagatose 4-epimerase can epimerize D-fructose into D-tagatose. However, there are few reports of enzymes with D-fructose epimeric activity at the C-4 position in nature. Therefore, obtaining such enzymes by screening new ones or modifying old ones is the main work in this production pathway. Shin et al. [27] developed a new tagatose 4-epimerase through rational design and directed the evolution of the tagaturonate 3-epimerase from *Thermotoga petrophila*. The modified enzyme exhibited 184-fold-higher epimeric activity towards D-fructose compared to the original enzyme. Under optimal conditions of 80 °C, pH 8.5, and 1.5 mM Ni^{2+}, the enzyme was able to produce 213 g/L of D-tagatose from 700 g/L of D-fructose within 2 h, with a conversion rate of 30%. Jeon et al. [28] expressed tagatose 4-epimerase from *Thermotoga neapolitana* in *Corynebacterium glutamicum* and improved the enzyme expression level by optimizing plasmid copy numbers. Under conditions of 60 °C and a specific amount of metal ions, the conversion rate reached 21.7%.

In contrast to isomerization reactions, redox reactions require cofactors. D-tagatose is produced by oxidizing the C-2 position of D-galactitol using galactitol dehydrogenase (GDH). By coupling the cofactors NADH/NAD^+ or NADPH/$NADP^+$, this method avoids unnecessary thermodynamic reaction equilibrium issues caused by aldose–ketose isomerases and epimerase. The advantages of this pathway are low reaction temperatures and high conversion rates as well as independence from metal ions [29]. For instance, Jagtap et al. [30] characterized galactitol dehydrogenase from *Rhizobium leguminosarum* with an optimal pH and temperatures of 9.5 and 35 °C, respectively. Generally, D-galactitol is produced by chemical hydrogenation or one of several yeast organisms from D-galactose [31], such as the oleaginous yeast *Rhodosporidium toruloides* IFO0880 [32]. However, due to the high cost of D-galactitol, this method is not currently suitable for large-scale production. In addition, D-galactose can be converted into D-galactitol using xylose reductase. On this basis, many researchers have developed the two-enzyme redox pathway to convert D-galactose into D-tagatose. Zhang et al. [33] constructed a pathway in *Bacillus subtilis* by introducing a heterologous xylose reductase and the identified RoGDH, and then increased the yield of D-tagatose through promoter engineering and cofactor regeneration. After 120 h, the final D-tagatose concentration reached 39.57 g/L and the conversion rate was 55%. Liu et al. [34] used an engineered yeast strain with oxidation-reduction enzyme

reactions. They deleted the gal1 gene encoding galactokinase and introduced heterologous xylose reductase and galactitol dehydrogenase (GDH) to produce D-tagatose from lactose. The engineered yeast can use lactose to produce D-glucose and D-galactose within its cells. D-glucose was used for cell growth and maintenance, while D-galactose was converted into D-tagatose. The final D-tagatose concentration reached 90%. However, this method produced D-tagatose with a relatively low concentration (37.7 g/L), and the fermentation cycle was long (300 h). Therefore, the two-enzyme redox pathway needs further research and improvement for economic feasibility.

In addition to the Izumoring strategy, other non-Izumoring enzymatic technologies can be used for the production of D-tagatose, such as the phosphorylation–dephosphorylation cascade. This method can efficiently convert D-fructose into D-tagatose through a series of enzymatic reactions. Lee et al. [35] successfully converted D-fructose into D-tagatose through a three-step enzyme cascade reaction. This process required hexokinase, ATP, D-fructose 1,6-bisphosphate aldolase, and phytase. Ultimately, it produced 0.8 M D-tagatose from 1 M D-fructose in 16 h with a conversion rate as high as 80%. To further reduce costs, Han et al. [36] successfully realized the production of D-tagatose using cheap starch as a substrate. This pathway of multienzyme-catalyzed reaction contains α-glucan phosphorylase, phosphoglucomutase, phosphoglucose isomerase, tagatose 6-phosphate 4-epimerase, and tagatose 6-phosphate phosphatase. In addition, the authors constructed an artificial shell of the organosilicon network using an immobilization method and, finally, developed a highly efficient and stable semi-artificial cell factory, with the final conversion rate of D-tagatose exceeding 40.7%.

2. The Production of D-Tagatose by L-AIs

L-arabinose isomerase is a key enzyme in the microbial pentose phosphate pathway and is considered an important biocatalyst in the production of rare sugars. This enzyme isomerizes L-arabinose into L-ribulose. Due to the structural similarity between L-arabinose and D-galactose, L-AIs can also isomerize D-galactose into D-tagatose [37]. A comparison of different methods is shown in Table 1. Among these methods, the large-scale production of D-tagatose by L-AIs utilizing D-galactose as a substrate is considered to be the most economical and feasible solution. Advantages of this method include a low cost, mild reaction conditions, high yields, and many enzyme sources.

Table 1. Comparison of different chemical and biological methods.

Methods		Advantages	Disadvantages	Significance
Chemical methods		Low cost	High temperature and pressure; environmental pollution; Low yield	D-galactose to D-tagatose
Biological methods	Tagatose 4-epimerase	Low cost; Mild reaction conditions	Low yield; Rare sources	D-fructose to D-tagatose
	Galactitol dehydrogenase	High yield; Mild reaction conditions	High cost; Need for cofactor Rare sources	D-galactitol to D-tagatose
	L-arabinose isomerase	Low cost; Mild reaction conditions; High yield; Many sources	Need for metal ions	D-galactose to D-tagatose
	phosphorylation-dephosphorylation cascade	High yield; Mild reaction conditions	Need for multiple enzymes and steps; Need for ATP	D-fructose or starch to D-tagatose

2.1. Molecular Structure and Catalytic Mechanism of L-AIs

The crystal structures of L-AIs from *E. coli* (PDB code: 2AJT) [38], *Lactobacillus fermentum* CGMCC2921 (PDB code: 4LQL) [39], *Geobacillus kaustophilus* (PDB code: 4R1O), and *Thermotoga maritima* MSB8 (PDB code: 7CWV) have been determined. Taking the L-AI from *E. coli* (ECAI) as an example [40], it is a hexamer with a total molecular weight of 336 kDa. Three asymmetric units of L-AI subunits form a trimer, as shown in Figure 4a. Two such trimers stack together to form the complete hexamer. Each subunit contains three structural domains: the N-terminal domain, the central domain, and the C-terminal domain. Within the complete hexamer of ECAI, there are six active sites located at the monomer–monomer interfaces (Figure 4a) and situated in the conjugate regions of adjacent subunits.

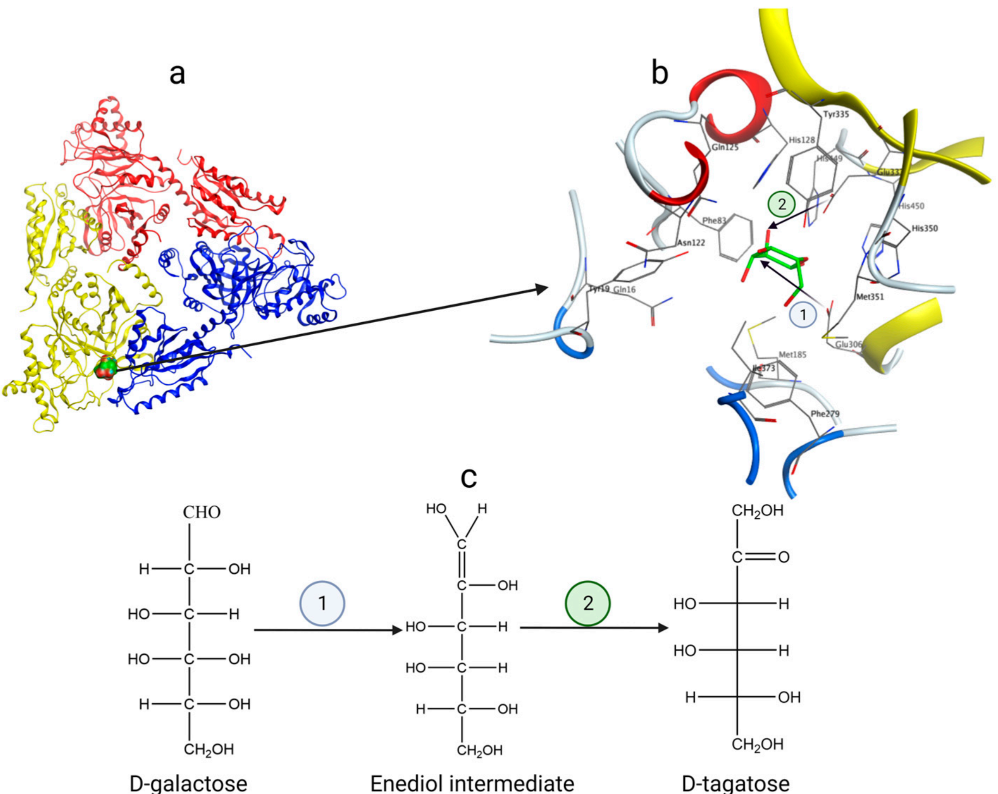

Figure 4. (**a**) The substrate and active site are located near the interface of two identical protein subunits; (**b**) ECAI catalytic residues Glu306 and Glu333 and amino acid residues in the region surrounding the active site; (**c**) Catalytic mechanism of L-AI—enediol intermediate.

The catalysis of D-galactose to D-tagatose by L-AIs follows the mechanism of enediol intermediate formation [41]. Taking ECAI as an example (Figure 4b), the residues E306 and E333 act as essential catalytic residues, while H350 and H450 stabilize the active site together with manganese ions. During isomerization (Figure 4), the $O^{\varepsilon 2}$ of Glu306 nucleophilically attacks the hydrogen atom of C-2 of D-galactose to deprotonate it and form a carbon–carbon double bond. The carbon–oxygen double bond is broken to form an oxygen anion, which combines with a proton to form an enediol intermediate; the $O^{\varepsilon 2}$ of Glu333 nucleophilically attacks the hydroxyl hydrogen of C-2 of D-galactose to generate a carbon–oxygen double bond at the C-2 position. This results in the cleavage of the carbon–carbon double bond between C-1 and C-2, producing a carbon anion that combines with a proton, ultimately yielding D-tagatose. This mechanism involves two proton transfers in D-galactose, one from O-2 to O-1 and the other from C-2 to C-1.

2.2. Properties of L-AIs

In order to efficiently produce D-tagatose, it is crucial to delve into the properties of L-AIs from various sources. The enzyme comes from a wide range of microbial sources, including *Lactobacillus plantarum* NC8 [42], *Anoxybacillus flavithermus* [43], *Bacillus coagulans* NL01 [44], *Pediococcus pentosaceus* PC-5 [45], *Clostridium hylemonae* [46], *Lactobacillus sakei* 23K [47], *Lactobacillus fermentum* CGMCC2921 [48], *Bifidobacterium adolescentis* [49], *Thermo toga maritima* [50], *Thermotoga neapolitana* [51], *Lactococcus lactis* [52], *Bacillus thermoglucosidasius* [53], *Arthrobacter* species 22c [54], *Shewanella* species ANA-3 [55], *Bacillus licheniformis* [56], *Bacillus subtilis* 168 [57], etc. The properties of these enzymes are shown in Table 2. Currently, amino acid sequences for approximately a hundred microbial sources of L-AIs are known, and these sequences share from about 40% to 60% or even higher homologies [58].

Table 2. Properties of L-AIs from various microbial sources.

Microbial Source	Temparature Optima (°C)	pH Optima	Metal Ion Requirement	D-Tagatose Yield (%)	k_{cat}/K_M (mM^{-1} min^{-1}) (D-galactose)	Reference
Lactobacillus plantarum NC8	60	7.5	Mn^{2+}, Co^{2+}	30	1.6	[42]
Anoxybacillus flavithermus	95	10.5	none	60	5.16	[43]
Bacillus coagulans NL01	60	7.5	Mn^{2+}, Co^{2+}	32	1.0	[44]
Pediococcus pentosaceus PC-5	50	6.0	Mn^{2+}, Co^{2+}	50	2.9	[45]
Clostridium hylemonae	50	7.5	Mg^{2+}	46	3.69	[46]
Lactobacillus sakei 23K	35	5	Mn^{2+}, Mg^{2+}	36	10.3	[47]
Lactobacillus fermentum CGMCC2921	65	6.5	Mn^{2+}, Co^{2+}	55	9.02	[48]
Bifidobacterium adolescentis	55	6.5	Mn^{2+}, Fe^{2+}, Zn^{2+} Ca^{2+}	56.7	9.3	[49]
Thermotoga maritima	90	7.5	Mn^{2+}, Co^{2+}	56	8.5	[50]
Thermotoga neapolitana	85	7.0	Mn^{2+}, Co^{2+}	68	3.24	[51]
Lactococcus lactis	50	8.0	Mg^{2+}, Mn^{2+},Co^{2+}	42.4	NA	[52]
Bacillus thermoglucosidasius	40	7.0	Mn^{2+}	45.6	2.8	[53]
Arthrobacter species 22c	52	8.0	Mg^{2+}, Mn^{2+}, Ca^{2+}	30	0.14	[54]
Shewanella species ANA-3	15–35	5.5–6.5	Mn^{2+}	34	NA	[55]
Bacillus licheniformis	50	7.5	Mn^{2+}, Co^{2+}	NA	slight activity	[56]
Bacillus subtilis 168	32	7.5	Mn^{2+}	NA	NA	[57]

NA—Not Available.

L-AIs from different microbial sources have different optimal temperature ranges. L-AIs sourced from mesophilic microorganisms exhibit optimal temperatures between 30 and 50 °C; those from thermophilic microorganisms have optimal temperatures ranging from 50 to 80 °C; and L-AIs from hyperthermophilic microorganisms have optimal temperatures exceeding 80 °C. Although L-AIs from hyperthermophiles have higher optimal temperatures and excellent thermal stability, their need for Co^{2+} to stabilize their structure at ultrahigh temperatures limits their application in the food industry [59,60]. Therefore, in actual industrial applications, L-AIs from thermophilic microorganisms are usually preferred.

L-AIs from different microbial sources exhibit variations in their optimal pH. Most reported L-AIs display their maximum activity under neutral or alkaline conditions. However, L-AIs with an optimal pH in the weakly acidic range offer advantages in industrial applications, including faster reaction rates and reduced by-product formation.

Metal ions play a crucial role in the activity and stability of L-AIs. Although not all L-AIs necessarily rely on the divalent metal ions to maintain their activity [61], for most L-AIs, divalent metal ions, especially Mn^{2+} and Co^{2+}, are essential for exerting their activity and maintaining thermal stability. Since Co^{2+} is not permitted in the food industry, the addition of Mn^{2+} is more appropriate. Choi et al. [62] explored the structure of the L-AI

from *Geobacillus kaustophilus* and found that the addition of Mn^{2+} transforms the L-AI structure from low oligomers to complete hexamers, indicating the significant role of metal ions in the oligomerization process. The authors also determined the melting temperatures of the hexamers by differential scanning calorimetry (DSC) and showed that the complete hexameric form is thermodynamically more stable.

Although most of the reported L-AIs were specific for L-arabinose and D-galactose, the specificity for L-arabinose was significantly higher than that for D-galactose. However, there are some exceptions. For example, the L-AIs from *Bacillus subtilis* 168 and *Pseudoalteromonas haloplanktis* [63] showed unique substrate specificity exclusively for L-arabinose. In addition, there are some enzymes classified as D-galactose isomerase because their optimal substrate is D-galactose instead of L-arabinose, such as the L-AI from *Bifidobacterium adolescentis*.

In addition to L-AIs, various sugar phosphate isomerases are also general aldose-ketose isomerases that can catalyze the biotransformation of non-phosphorylated monosaccharides. Patel et al. [64] characterized the phosphoglucose isomerase from *Pseudomonas aeruginosa* PAO1. This enzyme can also isomerize D-galactose to D-tagatose. The D-tagatose yield was 56% and the optimal activity was observed at 60 °C and pH 7.

A high temperature and slightly acidic conditions are considered ideal conditions for the efficient isomerization of D-galactose into D-tagatose. This is because the D-galactose isomerization process requires a high Gibbs free energy (4.96 kJ/mol) [65]. Therefore, the proper thermal conditions are necessary to overcome this energy barrier, which usually requires high temperature conditions [37]. High temperature conditions provide several advantages in this process. Firstly, within a specific range, higher temperatures lead to faster reaction rates, favoring the production of D-tagatose and improving the conversion; increasing the substrate solubility; reducing the viscosity of the reaction mixture, which improves the mass transfer efficiency and makes the reaction more efficient; and causing the risk of microbial contamination to be relatively low. However, it should be noted that extremely high temperature conditions above 80 °C may cause browning of the product and the generation of unnecessary by-products [66]. Therefore, the temperature range between 60 °C and 80 °C is usually chosen for industrial production. D-tagatose exhibits stability under acidic conditions and can remain relatively stable within the pH range of 3–7 [67], while side reactions will increase under alkaline conditions [68]. In addition, a high temperature and high D-galactose concentration may result in a decrease in the pH due to the Maillard reaction [69].

In conclusion, the L-AI from *Lactobacillus sakei* 23K showed optimal conditions at a low temperature and under acidic conditions, making it particularly suitable for the conversion of D-galactose to D-tagatose during the storage of milk and yogurt [70]. Xu et al. screened the L-AI from *Lactobacillus fermentum* CGMCC2921 and found its optimum temperature of 65 °C and the optimal pH of 6.5, which was within the range of suitability for industrial applications.

2.3. Production of D-Tagatose Using Lactose as Raw Material

D-galactose can usually be easily obtained from raw materials containing lactose. Therefore, it is necessary to combine lactose hydrolysis with the enzymatic isomerization of D-galactose to D-tagatose. This process can be accomplished under optimal conditions for hydrolysis and isomerization or through a one-pot method where hydrolysis and isomerization are carried out simultaneously [71]. The stepwise method allows hydrolysis and isomerization to be optimized separately under their respective optimal conditions, but high concentrations of D-glucose and D-galactose can lead to feedback inhibition of lactose hydrolysis, ultimately resulting in low conversion rates and yields. In such cases, the one-pot method is more advantageous as it simplifies the operations and minimizes the accumulation of D-galactose, thus improving the final yield of D-tagatose.

Furthermore, fully utilizing the residual D-glucose and D-galactose in the process is also an important consideration. These residues are typically wasted, and the similar physicochemical properties of D-galactose and D-tagatose introduce complex purification

steps in downstream processes. These residual substances can serve multiple purposes. They can be used as energy sources to sustain cell viability or further converted into other products such as D-fructose and ethanol. Rhimi et al. [72] successfully co-expressed the L-AI from *Bacillus stearothermophilus* US100 and D-glucose isomerase from *Streptomyces* SK in *E. coli* to isomerize the remaining D-glucose into D-fructose. Zheng et al. [73] expressed the L-AI from *Bacillus coagulans* NL01 in *E. coli* and combined it with self-expressed β-galactosidase for the crude enzyme conversion of lactose at 50 °C, and then the residual D-glucose and D-galactose were further fermented to bioethanol using *Saccharomyces cerevisiae* NL22. This approach enhanced the ethanol production competitiveness and simplified the purification process of D-tagatose. Zhang et al. [74] utilized cheese whey for D-tagatose-production through whole-cell biotransformation in *E. coli*. Then, they fermented the residual D-glucose and D-galactose into D-arabitol and D-galactitol using *Metschnikowia pulcherrima* E1 yeast to maximize the conversion of lactose in the cheese whey into three high-value rare sugars (Figure 5).

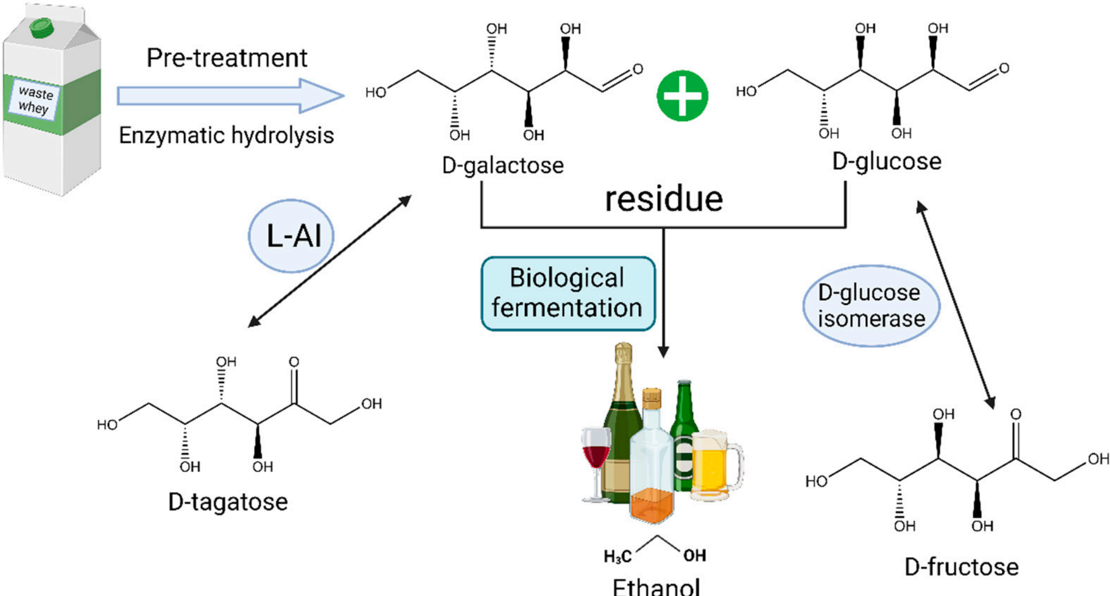

Figure 5. Utilization of lactose raw materials to produce D-tagatose, D-fructose, and bioethanol.

3. Strategies for Improving the Production of D-Tagatose by L-AIs

There are several strategies to improve the production of D-tagatose, including protein engineering [75], immobilization, and the application of chemicals. (Figure 6).

3.1. Protein Engineering

To improve the catalytic activity and substrate specificity towards D-galactose, modifications are typically performed on L-AIs. Usually, the natural substrate of L-AIs is L-arabinose, whose catalytic efficiency is much higher than that of D-galactose, and some even have no isomerizing activity towards D-galactose. This discrepancy may be attributed to the larger size of D-galactose compared to L-arabinose, as D-galactose has six carbon atoms instead of five. Therefore, it is necessary to modify the binding pocket of L-AIs to improve their substrate specificity for D-galactose. Jayaraman et al. [76] increased the isomerase activity of D-galactose by replacing the bulky Phe residue with a smaller hydrophilic residue in the L-AI from the *Shewanella* species ANA-3. Laksmi et al. [77] modified the binding pocket of the L-AI from *Geobacillus stearothermophilus* by selecting residues that would not significantly affect its affinity for mutation and, finally, obtained the high-affinity mutant H18T. Its catalytic efficiency was 1.45 times that of the wild type, significantly increasing its ability to bind to D-galactose. Kim et al. [78] conducted molecular docking of the L-AI from *Geobacillus thermodenitrificans* and D-galactose. They identified three key

residues near D-galactose O-6: Met186, Phe280, and Ile371. Through site-directed mutagenesis of these residues, they obtained the F280N variant enzyme, which exhibited approximately 2.3-times-higher D-galactose isomerization activity. In addition to rational modifications, Kim et al. [79] used error-prone PCR technology to irrationally transform the L-AI from *Geobacillus stearothermophilus* and successfully screened out the mutant enzyme M322V/S393T/V408A. It exhibited 1.9 times the D-galactose isomerization activity compared to that of the wild type enzyme under optimal conditions.

Isomerization reactions are reversible reactions affected by thermodynamics, meaning that the enzyme catalyzes both the substrate and the product. The proportion of the products no longer changes after reaching chemical equilibrium. This chemical equilibrium is related to the temperature. The isomerization reaction from D-galactose to D-tagatose is a heat-absorbing reaction, which means that increasing the temperature favors the conversion to D-tagatose. Most L-AIs are multimers, including dimers, tetramers, and hexamers. Previous research has indicated that hyperthermophilic L-AIs tend to form tetramers, while thermophilic L-AIs form hexamers [37]. Zhao et al. [80] obtained an engineered thermostable sucrose synthase (mutant M4) from *Nitrosospira multiformi* by using computer-assisted engineering. It exhibited significant improvements in thermal stability during UDP-glucose synthesis. This enhancement was attributed to the newly formed assembly interface of hydrophobic interactions in the tetramer mutant, which can result in a more compact subunit arrangement. Han et al. [81] categorized the L-AI family into two subfamilies based on the presence or absence of an α-helix at the C-terminus. They observed that the three α-helices of L-AIs in a trimer interact through a hydrogen bond network, which may make the entire enzyme more stable. Then, they introduced nine different α-helices at the C-terminus of the L-AI from *Lactobacillus fermentum* CGMCC2921. Among them, mutant no. 4 significantly improved its thermal stability during the catalytic process.

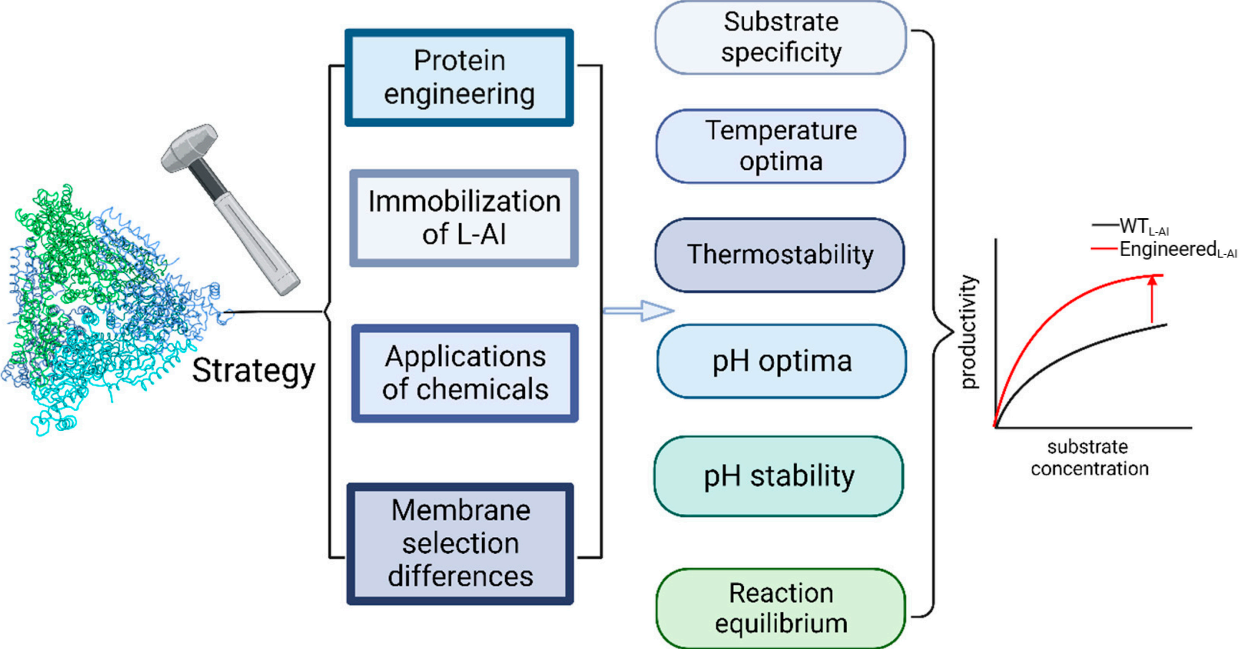

Figure 6. Strategies for improving D-tagatose production efficiency.

The industrial production of D-tagatose requires pH values between 5.0 and 6.0. However, most of the reported L-AIs exhibited their maximum activity under neutral or alkaline conditions. Therefore, lowering the optimum pH and increasing their stability in acidic conditions become particularly important. Xu et al. [82] replaced the aspartic acid residue with alkaline residues in the L-AI from *Lactobacillus fermentum* CGMCC2921, which significantly altered the optimum pH. Among these mutants, the D268K/D269K/D299K

variant exhibited optimal activity at pH 5.0 and demonstrated a broader optimum pH range from 4.5 to 6.0. Rhimi et al. [83] constructed the *Bacillus stearothermophilus* US100 mutant Q268K through sequence alignment with the L-AI from *A. acidocaldarius*. Its optimal pH was reduced from 7.5–8.0 to 6.0–6.5. This mutant enzyme exhibited improved stability under acidic conditions compared to the wild type enzyme.

3.2. Immobilization

Immobilized L-AIs provide a stable and economical method for the industrial production of D-tagatose because the reaction requires high temperature conditions and the long-term recovery and reuse of the enzyme. Immobilization can enhance the biochemical properties of L-As, including their optimum temperature and stability, thereby improving their performance in industrial applications. Liang et al. [84] overexpressed the L-AI from *Thermoanaerobacter mathranii* and immobilized it in calcium alginate beads. The optimal conditions for the immobilized enzyme were changed from pH 8.0 and 60 °C for the free enzyme to pH 7.5 and 75 °C. Bortone et al. [85] immobilized the L-AI from *Thermotoga maritima* on beads of copper-chelate epoxy supports (Eu-Cu). This method stabilized the multi-subunit structure and improved its stability by reacting $(His)_6$-tagged enzyme with epoxy groups to form covalent linkages and copper ions capable of physically adsorbing protein. The immobilized derivatives were post-treated with mercaptoethanol to remove residual copper ions that could affect their activity, thus further enhancing their biocatalytic activity.

Surface display systems immobilize enzymes on the surface of microbial cells to improve their catalytic activity and stability. *Bacillus subtilis* is an ideal strain for food-grade surface display systems because of its well-established genetic information, structural features, and advanced genetic modification techniques. Liu et al. [86] displayed the L-AI from *Lactobacillus* CGMCC2921 on the spore surface of *Bacillus subtilis* 168 with the anchor protein CotX to produce the fusion protein CotX-AI. This fusion protein was used for the production of D-tagatose, and the optimal temperature for the anchored L-AI reached 70 °C. Guo et al. [87] displayed the L-AI from *Lactobacillus brevis* on the spore surface of *Bacillus subtilis* DB403 using the anchor protein CotG and a peptide linker (Gly-Gly-Gly-Gly-Ser). The anchored L-AI showed excellent thermal stability with an optimal temperature of 67 °C, maintaining over 90% relative activity at temperatures between 60 °C and 70 °C, and over 60% relative activity at 80 °C.

3.3. Application of Chemicals

Jebors et al. [88] studied the interactions between supramolecular systems and various proteins. They discovered that adding 1 mM Noria and 1 mM NoriaPG (novel derivatives of Noria) did not significantly alter the enzymatic activity of the L-AI from *Lactobacillus sakei*, but did greatly increase its stability in low-pH and high-temperature conditions.

Traditional protein engineering methods have difficulty in directly altering the conversion equilibrium between substrates and products, as they are primarily controlled by the reaction temperature. Therefore, to achieve higher conversion rates, other strategies need to be adopted [89]. One approach to shift the reaction equilibrium is adding boric acid to the reaction mixture. Boric acid has a much higher affinity for ketose than aldose. Due to the ketose-rich nature of D-tagatose, it can form complexes with boric acid and then dissociate from the reaction system. This disrupts the reaction equilibrium, driving the reaction towards production and significantly increasing the final product yield. Lim et al. [90] used a mutated enzyme of the L-AI from *Geobacillus thermodenitrificans* and added boric acid under optimal conditions to react with 300 g/L D-galactose for 20 h, resulting in 230 g/L D-tagatose. The yield and conversion rate were 1.5 times higher and 24% higher than without boric acid.

3.4. Membrane Selection Difference

Another strategy to alter the reaction equilibrium is to utilize the selectivity of the cell membrane. The cell membrane separates substrates and products from enzymes coupled with membrane selectivity, which results in different concentrations of substrates and products inside and outside the cell, thus changing the reaction equilibrium. Kim et al. [91] showed that the cellular uptake of D-galactose was twice as fast as the uptake rate of D-tagatose, whereas the release of D-tagatose was 4.4 times faster than the rate of D-galactose release. This difference shifted the reaction equilibrium towards the production of D-tagatose, thereby increasing the yield of D-tagatose. Finally, the strategy resulted in a 68% D-tagatose yield at 37 °C compared to the 36% D-tagatose yield of purified L-AI.

3.5. Multiple Strategy Combinations

Usually, modifications to L-AIs are directed at only one aspect, including enhancing enzyme stability, substrate specificity for D-galactose, catalytic activity, and altering the equilibrium. Simultaneously improving the stability, thermodynamics, and kinetics is the direction required to enhance the industrial potential of L-AIs. For example, the use of permeabilized cells in whole-cell catalysis can offer multiple benefits. Firstly, it can provide a stable environment so that intracellular enzymes can perform catalytic functions under more favorable conditions, thereby improving their catalytic efficiency and stability. Secondly, permeabilized cells allow small and large molecular substrates to freely enter and exit the cells, enhancing the mass-transfer efficiency. Shin et al. [92] used *Corynebacterium glutamicum* to express the L-AI from *Geobacillus thermodenitrificans*. The activity of permeabilized *Corynebacterium glutamicum* cells treated with Triton X-100 was 2.1 times higher than that of untreated cells. Bober et al. [93] encapsulated the L-AI from *Lactobacillus sakei* into chemically permeabilized *Lactobacillus plantarum*, which not only improved the stability of the enzyme but also changed the properties of the cell membrane. This approach allowed D-galactose to preferentially enter the cell, thereby changing the reaction equilibrium. Finally, treatment with the chemical permeant SDS was also used to maintain cell membrane selectivity and improve the mass transfer efficiency. The reaction ultimately proceeded at high rates, high conversion rates, and high temperatures.

4. Prospects

In the future, the production of D-tagatose must develop in a sustainable direction. Waste utilization will play a crucial role in reducing high costs and achieving zero waste. D-galactose is an abundant carbohydrate monomer in nature and is widely found in large algae, plants, and dairy waste. Among these, D-galactose from dairy waste is particularly suitable as a raw material for the enzymatic production of purified value-added food ingredients [94]. Dairy industry by-products such as whey have been developed as a low-cost and attractive raw material, containing up to 75% lactose [95]. In addition to lactose, Kim et al. [96] utilized onion juice residue (OJR) as a substrate to produce D-tagatose. Sha et al. [97] produced D-galactitol by chemically hydrogenating waste xylose mother liquor and further produced D-tagatose by biologically oxidizing D-galactitol. These strategies made full use of carbon sources, reduced costs, and minimized waste generation, driving the production of D-tagatose in a more sustainable and efficient direction.

Despite the widespread application of L-AIs in the production of D-tagatose, there are still some problems and challenges involved. These include a low conversion efficiency, the requirement for metal ions, insufficient thermal stability, and a relatively low affinity for D-galactose. Furthermore, the tetramer structures of thermophilic and hyperthermophilic L-AIs remain unresolved. Addressing these structures is necessary to better understand the interactions between D-galactose and L-AIs as well as the relationship between L-Ais' structures and functions. In addition, protein engineering and genomic tools should be used more extensively to alter the functional properties of L-AIs, thereby improving D-tagatose production efficiency.

To ensure safety, researchers have started to explore ways to express L-AIs in food-grade hosts. Typically, L-AI expression is conducted in non-GRAS microorganisms such as *E. coli*. However, recombinant *E. coli* cells may cause safety issues due to the presence of endotoxins. Therefore, researchers are attempting to express L-AIs in GRAS microorganisms. Examples of these include *Lactobacillus plantarum* [98], *Bacillus subtilis* [99], and *Corynebacterium glutamicum*. In addition, antibiotics are not allowed in the food industry. Zhang et al. [100] successfully produced D-tagatose in recombinant *Bacillus subtilis* whole cells by constructing a heterologous gene expression system that did not require additional antibiotics. These methods are expected to be more widely used in the food industry in the future to meet safety and quality requirements.

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