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Review

Advances in Biological Production of D-Tagatose: A Comprehensive Overview

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Abstract: D-tagatose is a rare, naturally occurring low-calorie hexose, with a sweetness of 92% sucrose but only 1/3 of the calories. It has beneficial functions in lowering blood sugar, controlling obesity, preventing dental caries, and improving intestinal flora. In recent years, biotechnological routes to D-tagatose production from renewable raw materials have been regarded as very promising approaches. In this review, we provide an overview of the properties and applications of D-tagatose, with a focus on the current developments in the production of D-tagatose using enzymatic transformation and whole-cell catalytic synthesis. The biosynthetic pathways and the types and characteristics of the catalytic enzymes involved have been summarized, providing a reference for the design of D-tagatose synthesis pathways. We also expect that rapid developments in the fields of systems biology and synthetic biology will accelerate protein and metabolic engineering for microbial D-tagatose production in the future.

Keywords: D-tagatose; functional sugar; in vitro enzymatic transformation; whole-cell catalysis; biosynthetic pathways

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1. Introduction

In recent years, sugar intake has become a major public health issue and is becoming increasingly popular among people of all ages. However, excessive sugar intake increases the risk of metabolic diseases, including diabetes, dental caries, obesity, and other health problems [1,2]. Due to people's demand for nutrition and health, it has been discovered that sugar substitutes, which can be used as substitutes to maintain sugar intake, can benefit consumers by reducing calorie and sugar intake [3]. The U.S. Food and Drug Administration (FDA) has stated that the use of artificial sweeteners within the acceptable daily intake (ADI) range is safe [4]. Nowadays, sugar substitutes have been widely used in the food, medical, and cosmetics industries [5,6], and 25% of children and 41% of adults use sugar substitutes in the United States [7]. According to statistics, the global market size of sugar substitutes reached USD 18.96 billion in 2022, and the popularity of sugar substitutes has been continuously increasing [8].

Sugar substitutes are divided into natural and artificial substitutes according to their sources and processing methods. Artificial sugar substitutes are made through chemical synthesis and have the advantages of high sweetness, low calories, and good stability, and include saccharin, aspartame, sucralose, etc. Compared with artificial substitutes, natural

sugar substitutes have higher safety and are unanimously considered a more promising type of sweetener [9,10].

In natural sugar substitutes, those with the broadest market prospects include stevia [11], D-allulose [12], D-tagatose [13], and Brazilian sweet protein [14], among others. Stevia has a sweetness 200–300 times that of sucrose, with only about 1/250 of the calories. However, compared to sucrose, stevia exhibits a noticeable aftertaste of bitterness, which affects its further application [15]. D-allulose is a type of monosaccharide that exists naturally but in very small amounts. It has a sweetness level of 70% that of sucrose, yet contains only 0.3% of the calories of sucrose, boasting advantages such as a low calorie content, high stability, and non-hygroscopicity. With the further improvement of production efficiency driven by synthetic biology, its market size is gradually expanding [16]. Brazilian sweet protein is a sweet-tasting protein extracted from the fruit of a wild plant native to West Africa. With a sweetness intensity ranging from 500 to 2000 times that of sucrose, it is one of the sweetest natural substances known to date, devoid of metallic or bitter aftertaste, and offering advantages such as long-lasting sweetness and flavor enhancement [17]. Due to the high cost of traditional plant extraction methods, the development of a biological fermentation process for the production of Brazilian sweet protein is urgently needed.

D-tagatose is a naturally occurring rare monosaccharide with a structure similar to D-allulose. Its sweetness is 92% that of sucrose, and it contains only one-third of the calories of sucrose. As a natural sugar substitute, D-tagatose is a substance derived from the gum of a tropical evergreen tree called *Sterculia setigera*, and it has also been found in limited amounts in lower plants, including mosses and lichens, and in other dairy products exposed to heat [18–20]. Studies have shown that D-tagatose can inhibit oral bacterial growth by affecting glycolysis and its downstream metabolism [21], so it has great potential for preventing oral diseases [22].

In 2022, the global D-tagatose market reached hundreds of millions of dollars and is projected to continue growing at a compound annual growth rate of 6%. The market agencies predict that the global tagatose market size will expand from USD 155 million in 2023 to USD 243 million by 2033. However, with the increase in market demand, effective methods for D-tagatose production have become an urgent issue. Currently, the main producers of D-tagatose include Sukrin (Suriname), Living Fuel (U.S.), Damhert Nutrition NV (Belgium), CJ Cheiljedang Corporation (Korea), Bonumose (U.S.), Linlu Biotechnology (China), and other companies. The methods of production of D-tagatose mainly include chemical synthesis and biological methods [23,24]. Chemical synthesis involves using soluble alkali metal or alkaline earth metal salts as catalysts to promote the formation of D-tagatose from D-galactose under alkaline conditions, resulting in a metal hydroxide–tagatose complex, which is then neutralized with acid to obtain D-tagatose [25]. CJ Cheiljedang Corporation employs this method, with an annual production capacity exceeding 3000 tons of D-tagatose. Although chemical synthesis has achieved industrial production, they have many unfavorable production factors—for example, the production of many chemical pollutants and by-products under alkaline conditions, complex production processes, high energy consumption of intermediates, environmental pollution, etc. [26]. The biological method for producing D-tagatose utilizes biological enzymes or enzyme-containing cells as catalysts to catalyze the conversion of substrates into D-tagatose at a certain concentration, followed by separation and purification to obtain the D-tagatose product. Biosynthesis offers mild reaction conditions, safety, high efficiency, and production of the least amount of by-products, and is currently the mainstream research approach [27]. Currently, Linlu Biotechnology (Huang shi, China) Co., Ltd., has reached a production capacity of 2000 tons of D-tagatose using the bioconversion method. In this review, we primarily focus on the research progress of biological production of D-tagatose and divide

it into two types: in vitro enzymatic conversion and whole-cell catalytic synthesis. For each class, we summarize the synthetic pathway, the enzymes and its sources, substrate specificity, and catalytic efficiency. Finally, we discuss further improvements needed for the production of D-tagatose using protein engineering, systems biology, and synthetic biology strategies.

2. Overview of D-Tagatose

2.1. Structure and Physicochemical Properties of D-Tagatose

As a food sweetener, D-tagatose has 92% of the sweetness of sucrose, but the calorific value is only 1.5 kcal/g, which is 30% of the calorific value of sucrose [13]. The molecular formula of D-tagatose is $C_6H_{12}O_6$ and it has a relative molecular mass of 180.16 g/mol [28], which is the isomer of D-galactose, the C-3 diastereoisomer of D-sorbose and the C-4 diastereoisomer of D-fructose (Figure 1) [6]. Pure D-tagatose is a white crystalline substance with no particular smell and a melting point of 134 °C. It is readily soluble in water and slightly soluble in ethanol [19], and the solubility of D-tagatose is 58% at room temperature [26]. This reducing sugar has a low hygroscopicity but a high moisturizing capacity and stability. Similar to other monosaccharides, D-tagatose remains stable within the pH range of 3–7. Minimal loss of D-tagatose and browning were observed in citrate and phosphate buffers at pH 3.0. Furthermore, at 40 °C and in 0.1 M buffer (pH 3.0), a significant loss of approximately 5% of D-tagatose was observed over a period of more than six months, with a slight browning color [29].

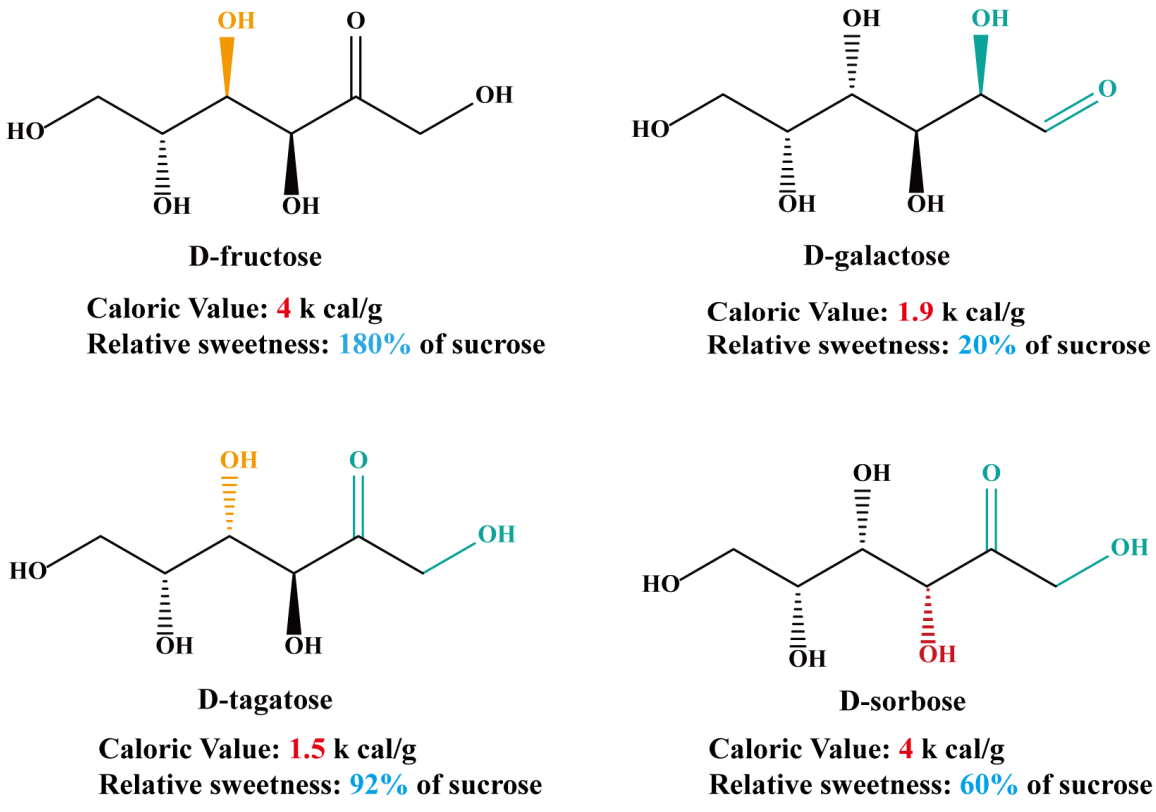

Figure 1. Chemical structures of D-tagatose, D-fructose, D-galactose, and D-sorbose. The caloric value refers to the amount of heat (in kcal) produced by consuming one gram of sugar, and the relative sweetness is compared with sucrose as the standard.

D-tagatose has also some alkalinity and heat resistance but is susceptible to the Maillard reaction at high temperature [30]. D-tagatose reacts with amino acids in a Maillard reaction to produce volatile substances such as 2-acetylfuran, 2-ethylpyrazine, and

2-acetylthiazole, which are more volatile than reducing sugars such as D-glucose and D-galactose [28]. These unique features enable D-tagatose to interact with other sweetening substances, such as stevia and sucralose, resulting in a more desirable sweetness perception through synergistic effects [31]. Saunders et al. showed in a study with mice that 68% of D-tagatose was finally broken down to CO_2 [32]. Kruger et al. conducted a tartar feeding experiment on mice and found that D-tagatose did not cause chronic or genotoxic lesions in mice at the experimental dose and was therefore safe and reliable for use as food. Other extensive safety toxicology studies on D-tagatose have also shown that it is safe and harmless to consume [33].

2.2. Physiological Function and Applications of D-Tagatose

Based on the physicochemical properties of D-tagatose mentioned above, it has the following four major functions: hypoglycemia [29], low energy consumption [34], improvement of intestinal flora [25], and caries prevention (Table 1) [35]. Studies have shown that only a small fraction (20–25%) of the D-tagatose that enters the body is digested and absorbed in the small intestine, and that it also inhibits the absorption of D-glucose in the small intestine, thus effectively suppressing an increase in blood glucose in patients with diabetes and reducing the chance of related complications [23]. D-tagatose can be catabolized by the 6-phosphate tagatose pathway, but this pathway is only present in specific microorganisms [36]. Furthermore, during the metabolic process, a part of the energy is lost due to the increased excretion of biomass from the flora [37]. Therefore, the energy produced by the decomposition and metabolism of D-tagatose is much lower than that of sucrose, which is beneficial for reducing food calories and obesity [38]. In addition to reduced absorption in the small intestine, D-tagatose is hardly broken down and utilized by microorganisms in the oral cavity, and trace amounts of D-tagatose that adhere to the tooth surface can form a protective film, thus effectively protecting dental health and preventing dental caries [21,34,35]. Moreover, D-tagatose undergoes selective fermentation in the colon by certain microflora, which stimulates the growth of *lactic acid bacteria* and *Lactobacilli* during the fermentation process and inhibits the growth of various pathogenic bacteria in the intestine, resulting in a favorable probiotic effect [25]. Some studies have shown that a daily intake of 4.5 g or more of D-tagatose can significantly improve intestinal flora [39].

Table 1. The functions and applications of D-tagatose.

Characteristic	Health Functions	Applications
1/3 of the caloric value of sucrose	Low energy	Low-sugar foods, diet foods, and grain foods
Low calorie, difficult to metabolize	Reduces blood sugar	Exclusive food for type II diabetic patients
Can be fermented by intestinal flora in the colon	Improves intestinal flora	Dairy products, juice drinks, and effervescent tablets
Difficult to be utilized by microorganisms in the oral cavity	Anti-caries	Gum, toothpaste, and mouthwash
Easy to caramelize, heat and moisture absorption resistant	Adds flavor	Bread, beverages, and sweets
	Medical field	Cough syrup and denture adhesive
Wettability and stability	Improves and prevents skin roughness	Cosmetic moisturizers
Inhibits the growth of some phytopathogens	Protects plants	Plant protection products

In addition to these common functions, studies have shown that D-tagatose has other physiological functions, including the enhancement of crucial blood factors that facilitate the promotion of blood metabolic circulation in humans [40]. It effectively inhibits the harmful effects of chemicals, such as cocaine and furantoin, on the liver, while enhancing the sensitivity of liver cells to toxic substances and acting as a hepatoprotective agent [41]. It improves the chances of pregnancy in female rats and has tremendous potential for the treatment of infertility [33]. It is able to inhibit the growth of numerous microorganisms, and it has been proposed as a promising plant protection product [42].

Based on the characteristics and physiological functions of D-tagatose, it has been granted Generally Recognized as Safe (GRAS) status by the U.S. Food and Drug Administration (FDA) since 2001 [43]. The European Union also approved the marketing of D-tagatose in Europe in 2003, and PepsiCo officially used D-tagatose as a flavor enhancer in Sprite beverages in August 2006, marking the first time D-tagatose entered the commercial arena. In 2007, Miada Sports Nutrition Foods of New Zealand applied D-tagatose to the development of chocolate products, which were released to Australian and New Zealand supermarkets in May. D-tagatose is currently used in large quantities in the U.S. as a substitute for white sugar in health drinks as well as in yogurt and juice products. D-tagatose products are now approved for use by food hygiene authorities in the U.S., Australia, Japan, and Korea.

3. Production of D-Tagatose by Biological Methods

The amount of D-tagatose in nature is extremely low, and therefore it is difficult for naturally extracted D-tagatose to meet the actual production demand. D-lactose, as a relatively inexpensive and abundant raw material, has been used for the industrial production of D-tagatose for the first time [44]. The current method of producing D-tagatose is primarily chemical synthesis, which involves using alkaline metals as catalysts, with D-lactose as the substrate, such as conversion of D-galactose to D-tagatose using a calcium catalyst. Under the catalysis of metal hydroxides and metal salts, the isomerization of D-lactose produces a D-tagatose–metal ion complex intermediate precipitate. Then, the acid neutralization method can be used to separate and purify the D-tagatose [41]. D-lactose can be hydrolyzed to produce D-glucose and D-galactose, and then chemically processed to produce D-tagatose. Although the method has a high yield, it has many disadvantages. Traditional manufacturing methods can only convert about 30% of D-galactose [45], and the cost of separating D-tagatose is high, leading to a persistently high retail price for tagatose. According to data from Dutch industry consulting firm FutureBridge, in 2020, the retail price of D-tagatose was USD 26/kg, while the retail price of sucrose was only USD 0.05/kg. Furthermore, alkaline conditions hinder the isomerization reaction of aldoketose. Additionally, the reaction process is prone to browning and the production of a large amount of by-product. This in turn leads to a complicated process of product separation and purification, increasing production costs and causing environmental pollution and resource waste, and the same molar amounts of D-galactose and Ca^{2+} must be used to obtain high yields of D-tagatose [46]. Severe degradation of D-galactose led to a decrease in D-tagatose yield, while a decrease in syrup quality allowed for the production of D-tagatose. Therefore, it is not a safe or long-term method for the preparation of D-tagatose [47]. Although there are other chemical methods to produce D-tagatose, such as supercritical fluid (<24%) [48], arginine (<16.8%) [49], hydrotalcite (<27%) [50], etc., these methods produced low yields of D-tagatose. Due to these disadvantages of chemical synthesis, the production of D-tagatose by biotechnology has become a hot topic of research [51].

In contrast, the production of D-tagatose by biological methods is superior to chemical methods in many ways, including enzymatic conversion and whole-cell catalysis. Enzy-

matic conversion methods, which use enzymes as catalysts, have advantages over chemical methods because they are substrate specific and produce products without any by-products, thus simplifying the product purification process. The whole-cell method allows a wide range of bacteria to be used as whole-cell factories. In addition, D-galactitol dehydrogenase can be used to convert D-galactitol to D-tagatose. In summary, the bioprocess for D-tagatose production offers advantages such as simple steps, mild reaction conditions, safety and efficiency, high conversion efficiency, specificity, and fewer by-products [52,53]. The production of D-tagatose using biotechnological methods includes enzymatic transformation and whole-cell catalysis.

3.1. Synthesis of D-Tagatose by Enzymatic Transformation In Vitro

Enzymatic conversion is the process of using specific enzymes extracted from microorganisms, animals, and plants to catalyze the conversion of substrates into desired products under suitable conditions. Depending on the substrate and enzyme adopted, these strategies can be categorized into single-, dual-, and multi-enzyme methods.

3.1.1. Synthesis of D-Tagatose by a Single Enzyme

Currently, the single-enzyme method is the main approach for the enzymatic synthesis of D-tagatose. The types of enzymes include L-arabinose isomerase (L-AI) [47,54–59], D-tagatose 3-epimerase (DTEase) [60], D-arabinose 3-epimerase (DPEase) [61], and D-galactose dehydrogenase (GDH) [62,63] catalyzing the one-step conversion of substrates to D-tagatose. The most extensively studied method is the one-step synthesis of D-tagatose from D-galactose catalyzed by L-AI, an intracellular enzyme that is widely present in microorganisms. Patrick et al. [64] isolated L-AI from *E. coli*, and it was found that L-AI consists of six identical subunits with a relative molecular mass of 36,200 Da. The enzyme is composed of an N-terminal domain, a middle domain, and a C-terminal domain, and its structure includes 16 β-sheets and 17 α-helices. Within its complete hexamer, six active sites are located at the subunit–subunit interface and in the bridging region of adjacent subunits. Manjasetty [65] observed the morphology of L-AI with an electron microscope. He found that six subunits were composed of three subunits, which formed symmetrical triangles, and then the triangles were stacked into triangular prisms. There was an equivalent cleft on the surface of the triangular rhombohedron. The cleft in the subunit was located between domains, forming a strong electron density abyss, which was presumed to be the enzyme's active center (Figure 2). L-AI catalyze not only the isomerization of L-arabinose to L-ribulose but also D-galactose to D-tagatose (Figure 3A) [66–68]. In 1993, Cheetham et al. [69] not only reported for the first time that L-AI from microbial sources could catalyze the isomerization of L-arabinose to L-fructose but also successfully used L-AI from *Mycobacterium phlei* and *Lactobacillus gayonii* to convert D-galactose into D-tagatose. Since then, L-AI from microbial sources has become one of the most widely studied enzymes in the research on the synthesis of D-tagatose.

Depending on the catalytic temperature, the microbial sources of L-AI can be classified as mesophilic, thermophilic, or hyperthermophilic microorganisms. The optimum reaction temperature of mesophilic microbial sources, including *Lactobacillus brevis* [30], *Bacillus coagulans* [43], *Lactobacillus fermentum* [70], *Pediococcus pentosaceus* [71], *Lactobacillus plantarum* [72], and *E. coli* [73], is 30–50 °C [25]. For thermophilic microbial sources, including *Geobacillus stearothermophilus* [74,75], *Geobacillus thermodenitrificans* [40,76], *Bacillus stearothermop hilus* [77,78], and *Arthrobacter psychrolactophilus* [79], the optimum reaction temperature is 60–80 °C. The optimum reaction temperature of L-AI from hyperthermophilic microbial sources, including *Thermotoga neapolitana* [41,80] and *Thermotoga maritima* [81,82], is 85–90 °C. The properties of L-AI from various microbial sources shown in Table 2.

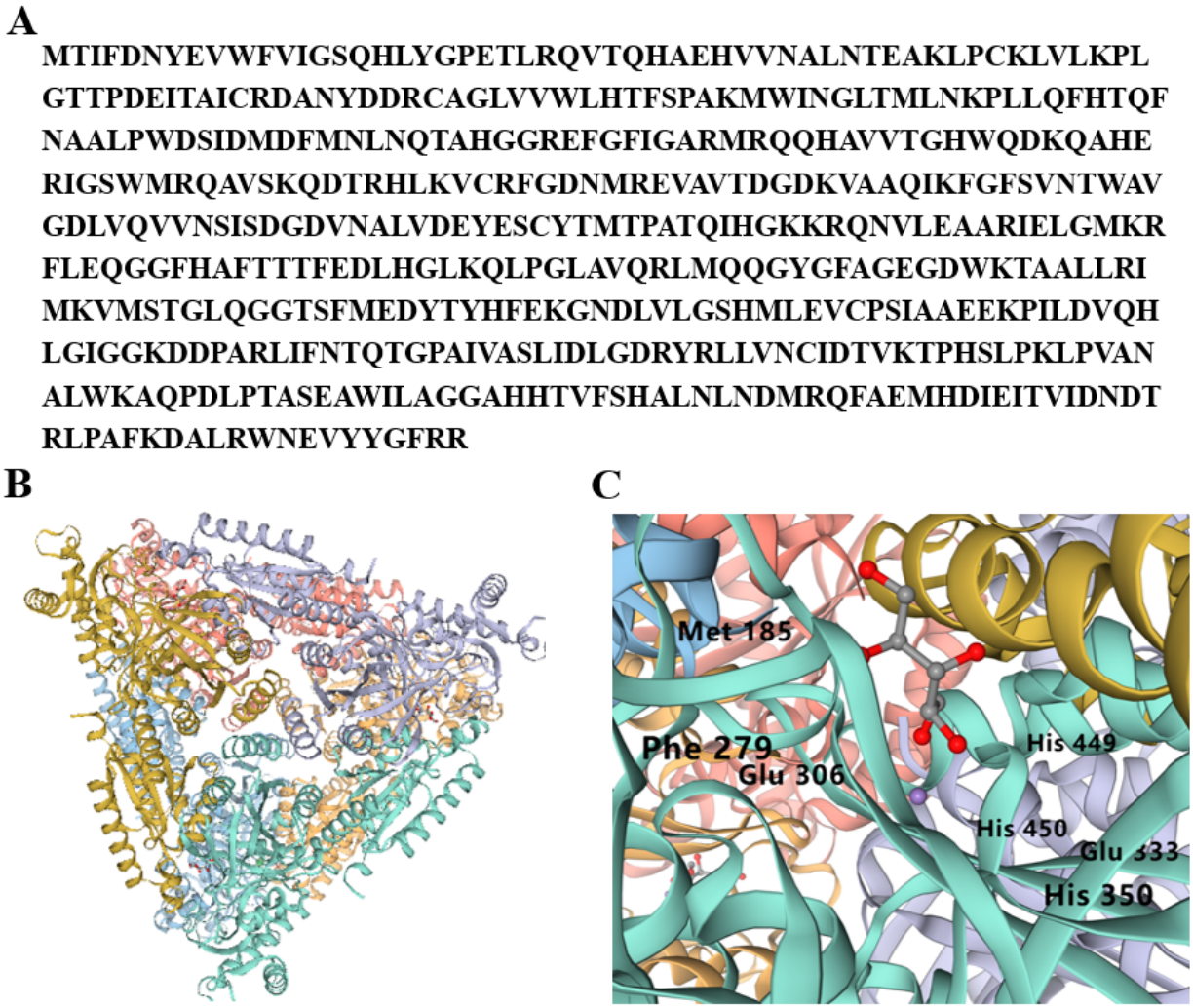

Figure 2. The protein sequence, crystal structure, and active center of *E. coli* L-AI. (**A**) Protein sequence, (**B**) crystal structure of *E. coli* L-AI monomer prediction of active centers, and (**C**) related amino acids.

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

Microbial Source	Substrate	Optimum Temperature (°C)	Optimum pH	Metal Ion Requirement	References
Thermoanaerobacterium brockii	D-galactose	65	6.9	Co^{2+}	[83]
Thermotoga neapolitana 5068	D-galactose	80	7.0	Mn^{2+}, Co^{2+}	[56]
Thermotoga maritima	D-galactose	90	7.5	Mn^{2+}, Co^{2+}	[81]
Lactobacillus plantarum NC8	D-galactose	60	7.5	Mn^{2+}, Co^{2+}	[72]
Thermophilic bacterium IM6501	D-galactose	60	8.0	Zn^{2+}, Ni^{2+}	[84]
Lactobacillus plantarum CY6	D-galactose	50	6.5	Mn^{2+}	[13]
Bifidobacterium adolescentis	D-galactose	55	6.5	Mn^{2+}, Fe^{2+}, Zn^{2+}, Ca^{2+}	[65]
Arthrobacter species 22c	D-galactose	52	8.0	Mg^{2+}, Mn^{2+}, Ca^{2+}	[85]
Clostridium hylemonae	D-galactose	50	7.5	Mg^{2+}	[86]
G. thermodenitrificans	D-galactose	60	9.0	None	[55]
Bacillus coagulans NL01	D-galactose	60	7.5	Mn^{2+}, Co^{2+}	[43]
Pediococcus pentosaceus PC-5	D-galactose	50	6.0	Mn^{2+}, Co^{2+}	[71]
Bifidobacterium longum NRRL B-41409	D-galactose	55	6.0–6.5	Ca^{2+}, Mg^{2+}	[10]
Anoxybacillus flavithermus	D-galactose	95	9.5–10.5	Ni^{2+}	[9]

The conversion of D-galactose varies depending on the monosaccharide isomerase and the conditions used. The reaction temperature is an important parameter for catalyzing the aldoketose isomerization reaction in vitro, and the isomerization equilibrium between D-galactose and D-tagatose gradually favors D-tagatose as the reaction temperature increases. Additionally, intracellular enzymes that are not involved in D-tagatose synthesis are less active under high-temperature conditions, which reduces the occurrence of side reactions and is more conducive to purification for downstream engineering [13]. The anaerobic thermophilic bacterium *Thermoanaerobacterium brockii*, produced and characterized in *E. coli* [83], produced D-tagatose from 300 g/L of D-galactose at 65 °C, with a yield of 126 g/L and a conversion rate of 42%. Kim BC et al. [56] cloned the *araA* gene encoding L-AI from the thermophilic bacterium *Thermotoga neapolitana* 5068 and expressed it in *E. coli*. The recombinant enzyme was purified by heat treatment, ion exchange chromatography, and gel filtration at an isomerization temperature of 80 °C, and the recombinant enzyme produced 1.22 g/L of D-tagatose from 1.8 g/L of D-galactose, with a conversion rate of 68%. The temperature exceeding 80 °C may result in undesirable Maillard reactions, which can decrease sugar production. Therefore, the temperature for D-tagatose production is usually set at 60–70 °C for industrial production, and heat-resistant enzymes are preferred.

L-AI is also affected by pH. The optimal pH of L-AI from different microbial sources varies. Most L-AI exhibits maximum activity under neutral or alkaline conditions. For instance, the *araA* gene encoding L-AI was cloned from the hyperthermophilic bacterium *Thermotoga maritima* and had maximum activity at 90 °C and pH 7.5 [81]. Chouayekh H et al. [72] cloned and expressed the gene *araA* encoding L-AI from *Lactobacillus plantarum* NC8. It exhibited 68% of its maximum activity at pH 5.5 and retained 89% of its activity after incubation at pH 5 for 24 h. The purified L-AI NC8 achieved a biotransformation rate of 30% for the conversion of D-galactose into D-tagatose after 6 h of reaction at 60 °C.

In addition, metal ions are essential cofactors of L-AI, especially Mn^{2+} and/or Co^{2+}, which play an important role in the activity and stability of L-AI [43]. These metal ions bind to the active center of the enzyme and help stabilize its protein structure, enabling effective catalysis. For example, Kim JW et al. [84] cloned L-AI from the *thermophilic bacterium* IM6501 using the PCR method. The purified L-AI has heat resistance and exhibits the highest activity when reacted for 30 min at 60 °C and pH 8. The Zn^{2+} and Ni^{2+} deactivated the catalytic activity of L-AI, while 5 mM Mn^{2+} increased the biotransformation rate by 90%. Lee DW et al. [81] cloned the *araA* gene encoding L-AI from the hyperthermophilic bacterium *Thermotoga maritima*, it is more thermostable in the presence of Mn^{2+} and/or Co^{2+} than in their absence, the enzyme achieved a conversion rate of 56% for the isomerization of D-galactose into D-tagatose at 80 °C, with a reaction time of 6 h.

To improve the efficiency of producing D-tagatose with L-AI, some researchers have used immobilized enzyme methods to enhance production efficiency. Kim P et al. [87] immobilized L-AI from *E. coli* on agarose to produce D-tagatose. The immobilized L-AI produced an average of 7.5 g/(L·d) D-tagatose for 7 days, with a yield exceeding that of free enzymes. Using an expanded immobilized enzyme system, 99.9 g/L D-tagatose was produced within 48 h from D-galactose, with a balance of 20%. Kim H J et al. [88] immobilized L-AI Gali152 on alginate carriers and optimized the conditions for the D-galactose isomerization reaction. Under stable conditions of pH 8.0 and 60 °C, the immobilized enzyme produced 58 g/L of D-tagatose from 100 g/L of D-galactose in an intermittent reaction over 90 h. In contrast, free enzyme, due to its lower stability, produced only 37 g/L of D-tagatose. In a continuous circulation mode, Gali152 immobilized in alginate beads was used in a packed-bed bioreactor with galactose as a raw material, producing 230 g/L of D-tagatose from 500 g/L of D-galactose, with a productivity of 9.6 g/(L·h) and a conversion rate of 46%.

Despite the efficiency and simplicity of the catalytic reaction between D-galactose and L-AI, the high cost of D-galactose currently poses a challenge to its widespread industrial production. To mitigate this issue, D-lactose or whey is commonly used as a raw material in the industrial production of D-tagatose. The initial step involves the acid hydrolysis of D-lactose or whey, which converts it into a mixture of D-glucose and D-galactose. Subsequently, L-AI is utilized to convert the D-galactose into D-tagatose, resulting in a significant reduction in the cost of D-tagatose production. It was reported that Zhang et al. [13] used recombinant *E. coli* to express L-AI from *Lactobacillus plantarum* CY6. The net yield and volumetric productivity of D-tagatose after two-stage SSB were 0.7 mol/mol D-lactose and 0.54 g/(L·h), respectively. Shin KC et al. [89] identified a D-tagaturate 3-epimerase from *Thermotoga petrophila*, which has potential 4-epimerase activity towards D-fructose. Through genetic engineering, the enzyme was modified to enhance its 4-epimerase activity towards D-fructose. At 80 °C, the enzyme produced 213 g/L of D-tagatose from 700 g/L of D-fructose within 2 h, with a conversion rate of 30%.

3.1.2. Synthesis of D-Tagatose by Dual Enzymes

Although the single-enzyme method is relatively simple to operate and does not require complex steps and multiple enzymes, the method may be limited by various factors, such as the characteristics of the enzyme itself, the properties of the substrate, and the reaction conditions, which can affect the efficiency and accuracy of the reaction. The dual-enzyme method can optimize the disadvantages of the single-enzyme method to a certain extent, thereby improving the efficiency of producing D-tagatose. The synthesis of D-tagatose by dual-enzyme catalysis relies on D-lactose as the substrate. First, D-lactose is hydrolyzed into D-galactose by β-galactosidase. Subsequently, D-galactose is converted into D-tagatose using L-AI as the catalyst (Figure 3B). Zheng et al. [73] used a recombinant *E. coli* expressing L-AI to establish a one-pot biosynthesis process for D-lactose hydrolysis and D-galactose isomerization at 50 °C. Using whey permeate containing 100 g/L of D-lactose as the raw material, they produced 23.5 g/L of D-tagatose and 26.9 g/L of bioethanol, with a conversion rate of 23.5%. This process does not require the addition of extra β-galactosidase, simplifies the biotransformation process, and reduces production costs. The *gatz* gene from *Caldilinea aerophile* (a filamentous thermophilic bacterium found in Japanese hot springs) was heterologously expressed in *E. coli* BL21, and the purified recombinant *gatz* was coupled with phosphatase (PGP) to construct a two-enzyme catalytic system that transformed F6P to produce D-tagatose [90]. The *gatz* has the properties of 6-phosphofructose 4-isomerase with an optimum pH of 8.0, is a metal-dependent enzyme with an optimum metal ion of Mg^{2+}, and has a high thermal stability with an optimum temperature of 70 °C. After holding at 60 °C for 30 h, it can still maintain more than 80% of the initial enzyme activity. When PGP was added together with *gatz*, the final yield was approximately twice that when PGP was added after the *gatz* reaction, yielding 1.23 g/L and 0.60 g/L of D-tagatose after 6 h, respectively. In addition, the reaction reached equilibrium at 50 and 150 min, with final yields of 4.1% and 2.0%, respectively [91]. Therefore, the consumption of 6-phosphate D-tagatose by PGP would drive the equilibrium of the *gatz*-catalyzed reaction towards D-tagatose production and increase the final concentration of D-tagatose.

3.1.3. Synthesis of D-Tagatose by Multi-Enzyme

In recent years, with the discovery of more enzyme preparations with novel functions, methods for synthesizing rare sugars based on multi-enzyme catalytic systems have gradually emerged. These new methods have the advantages of high efficiency, simplicity, and low cost. For the synthesis of D-tagatose, various enzyme conversion methods have been studied. Among the isomers of D-tagatose, D-fructose is the cheapest. However, there are no naturally occurring enzymes that can directly convert D-fructose into D-tagatose. While the conversion of D-fructose to D-tagatose can barely be achieved by the single-enzyme method, D-tagatose can be produced by a multi-enzyme catalytic system based on the Izumoring strategy and a phosphorylation–dephosphorylation multi-enzyme catalytic system. The Izumoring strategy was used for the biological production of all hexoses—16 aldohexoses, 8 ketohexoses, and 10 hexanols using enzymatic and microbial reactions that can cycle and prepare all monosaccharides using three enzymes (D-tagatose 3-epimerase, polyol dehydrogenases, and aldose isomerases) [92].

The phosphorylation–dephosphorylation multi-enzyme catalytic system involves a series of enzymes, starting with kinases or phosphatases, that react to produce phosphorylated sugars. Subsequently, the sugars are gradually converted using isomerase, differential isomerase, or synthase, and the phosphate groups are removed using phosphatase to obtain the target product (Figure 3C). Dai et al. [93] used this system to obtain D-tagatose by a three-step enzymatic cascade reaction involving hexokinase, D-fructose-1,6-bisphosphate aldolase (FbaA), and phytase (a phosphatase), with D-fructose as the substrate, and its conversion rate could reach 80%. Although this approach reduces the production costs of D-tagatose, the starting enzyme, kinase, requires the participation of ATP during the reaction, which is unstable at high temperatures and results in difficulty in purifying the product, making it unsuitable for practical production. In contrast, Wichelecki et al. [94] employed different functional phosphorylases as starting enzymes to synthesize high-purity D-tagatose in one step, where maltodextrin, cellulose, or sucrose were used as substrates after a multistep enzyme-catalyzed conversion. Han et al. [95] synthesized D-tagatose using starch as a substrate. This reaction pathway involves α-glucan phosphorylase (αGP), phosphoglucomutase (PGM), phosphoglucose isomerase (PGI), D-tagatose 6-phosphate 4-epimerase (TPE), and D-tagatose 6-phosphate phosphatase (T6PP), and this method further reduces the production cost of D-tagatose. Zhang et al. [96] synthesized D-tagatose from maltodextrin using a four-step enzymatic reaction. They immobilized α-glucan phosphorylase (αGP) and phosphoglucose mutase (PGM) on Duolite A568 exchange resin, constructing a catalytic cascade system to generate the D-tagatose precursor glucose 6-phosphate (G6P). The co-immobilized αGP&PGM can serve as a catalyst for one-pot production of G6P, which can serve as an initial pathway for further production of D-tagatose. Currently, over 30 phosphorylases have been explored and identified [97], which are anticipated to facilitate the one-step production of D-tagatose while reducing costs.

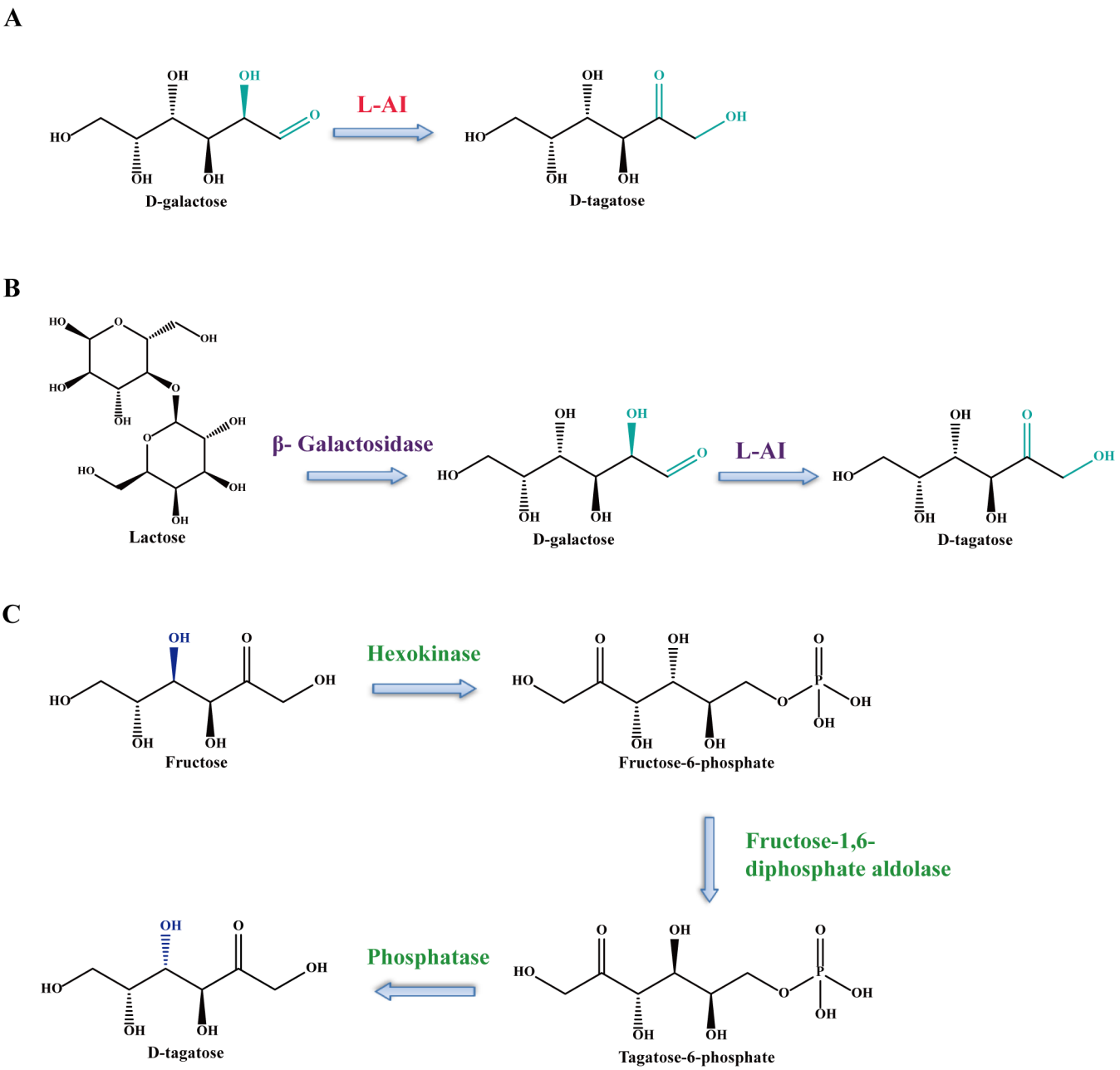

Figure 3. Enzymatic synthesis of D-tagatose. L-AI catalyzes D-galactose isomerization to D-tagatose (**A**). Lactose undergoes hydrolysis with β-galactosidase, resulting in the formation of D-galactose. The D-galactose is transformed to D-tagatose using L-AI as the catalyst (**B**). Fructose is converted by hexokinase into fructose-6-phosphate, which is then converted by fructose-1, 6-diphosphate into taglose-6-phosphate, which is finally catalyzed by phosphatase into D-tagatose (**C**).

3.2. Whole-Cell Catalytic Synthesis of D-Tagatose

Compared with in vitro enzymatic methods, whole-cell catalysis does not require cell fragmentation or enzyme isolation and purification, and the cell membrane prevents enzyme damage by shear forces, which is conducive to improving enzyme stability and reducing production costs. In addition to the use of L-AI for in vitro catalysis, whole-cell catalysis has been extensively studied, with *E. coli* being one of the most commonly used hosts [98–100]. Figure 4 shows the construction of the metabolic pathway for the synthesis of D-tagatose in *E. coli* in vivo. The whole-cell catalysis of multi-enzyme co-expressing recombinant strains has the advantages of high efficiency, low cost, and good reaction stabil-

ity, and has been widely used in the biosynthesis of many important chemicals [97,101–103]. Yuan et al. [104] demonstrated the overexpression of L-AI, which was derived from *Lactobacillus parabuchneri* in *E. coli* BL21, and the recombinant bacterium was employed to catalyze 140 g/L D-galactose to produce 54 g/L D-tagatose with a conversion efficiency of 39% and a yield of 0.54 g/(L·h). Du et al. [105] adopted a single-factor experiment to optimize the process conditions for the cell-catalyzed production of D-tagatose by *Lactobacillus brevis sp*, and the yield of D-tagatose was 3.916 g/L at pH 7.0 and 55 °C for 48 h, catalyzing 9 g/L D-galactose with a conversion rate of 43.5%. It was shown that the catalytic time was 48 h when cells were used as catalysts, and the cells could be reused three times to the maximum extent. Therefore, the use of cells as catalysts in the industrial production of D-tagatose could save a lot of cost and facilitate the stable production of D-tagatose. The reaction time of L-AI as a catalyst was relatively short, at 23 h, but the conversion efficiency per unit time was relatively high. However, the process of purification of L-AI required a long time for cell wall breaking, the purification process required a lot of energy, and the yield of L-AI was lower compared with that of cell catalysis after two instances of reuse. Together, these results suggest that the cell conversion method is more suitable for the industrial production of D-tagatose. Many researchers have found that a variety of microorganisms can catalyze the production of D-galactitol to D-tagatose, including *Mycobacterium smegmatis* [62,68], *Enterobater agglomerans* [106], *Klebsiella pneumoniae* [107], *Acetobacter* [108], and *Arthrobacter globiformis* [109]. However, owing to the relatively high costs of D-galactose and D-galactitol, their fermentation processes as substrates are difficult to apply for industrial production.

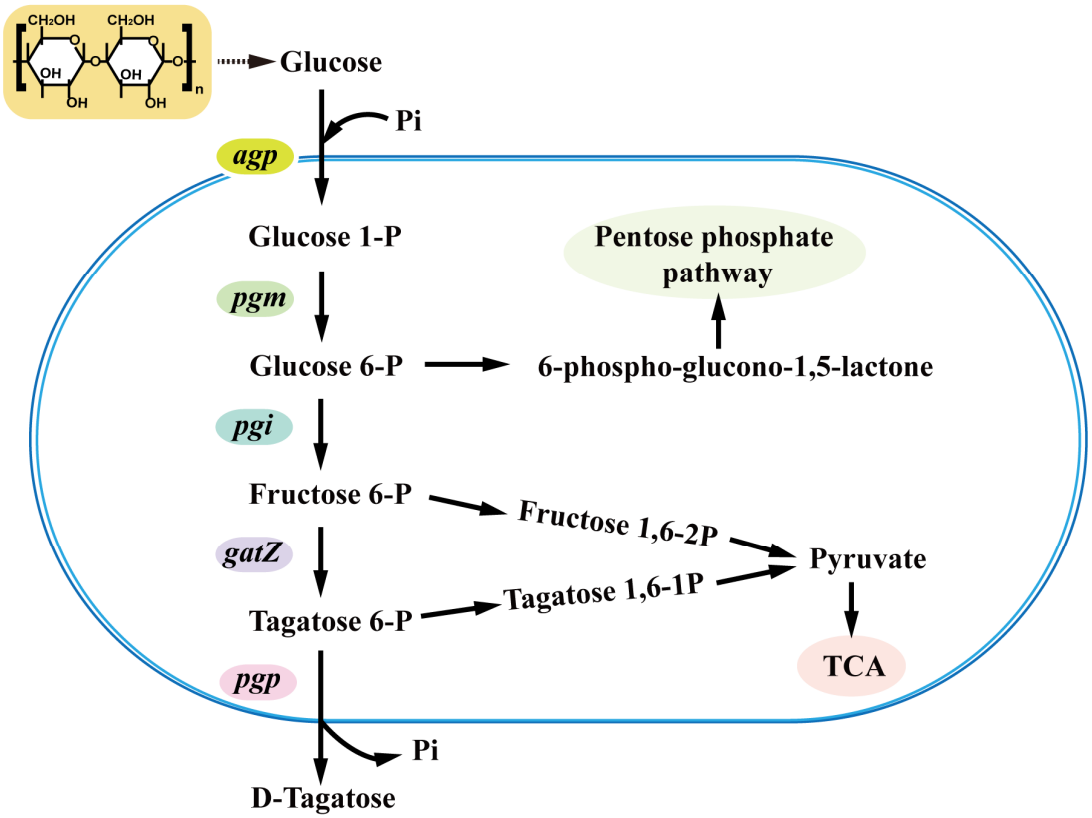

Figure 4. Biosynthesis of D-tagatose using starch-based ingredients as substrate through an in vivo multi-enzyme system. *agp*, a-Glucan phosphorylase; *pgm*, phosphoglucomutase; *pgi*, glucose 6-phosphate isomerase; *gatZ*, tagatose 1,6-bisposphate aldolase subunit *GatZ*; *pgp*, phosphoglycolate phosphatase.

Some research has shown that by expressing a multi-enzyme catalytic system consisting of five heat-resistant enzymes, *E. coli* was able to synthesize D-tagatose using maltodextrin as a substrate, with a yield of 1.88 g/L and a conversion rate of 18.8%, which was 5.9% higher than the conversion rate of the in vitro multi-enzyme catalytic system under the same experimental conditions [87]. Under optimal reaction conditions, recombinant cells were co-expressed for 24 h with 10 g/L and 20 g/L maltodextrin as substrates, yielding 2.08 g/L and 3.2 g/L D-tagatose [110]. There is a growing interest in the use of whole-cell catalysts in chemical synthesis [102,111]. The application of whole-cell catalysts can avoid the need to supplement expensive cofactors and preparation steps such as enzyme purification and isolation [112].

D-tagatose can be synthesized through two pathways: epimerization or oxidoreduction [113]. Many D-tagatose production routes involve epimerization. In the epimerization synthesis pathway, L-AI can convert D-galactose to D-tagatose. Co-expression of β-D-tagatose galactosidase and L-AI in *Saccharomyces cerevisiae* produces D-tagatose from D-lactose and achieves a conversion rate of 30%, which is higher than that of most in vitro enzymatic reactions [85]. D-tagatose can be produced from D-fructose through epimerization. However, although a large number of UDP-hexose 4-epimerases have been identified in various organisms, enzymes with excellent catalytic activity for the epimerization of D-fructose are rare [114,115]. Thus, D-tagatose can be produced by modifying some enzymes. In order to reduce production costs, a whole-cell biocatalytic system involving α-glucan phosphorylase, phosphoglucomutase, D-glucose 6-phosphate isomerase, D-tagatose 1,6-bisphosphate aldolase, and phosphoglycolate phosphatase was constructed to produce D-tagatose from maltodextrin [110]. However, the biotransformation efficiency of this whole-cell catalyst was low because of the unbalanced ratio of each enzyme and metabolic flux of the intermediates. By optimizing vectors and improving the expression of rate-limiting enzymes through the construction of multi-copy genes to regulate expression levels, the conversion rate of D-tagatose increased from 20.8% after 24 h to 25.2% after 3 h using 10 g/L maltodextrin as the substrate. Furthermore, the genes in the bypass pathways in *E. coli*, *pfka* and *zwf*, were deleted to increase the accumulation of intermediates. The strain ER-2*GatZ* (ΔpΔz) produced 3.383 g/L D-tagatose using 10 g/L substrate after 3 h, which was 1.34 times that of the wild strain [116]. However, due to the chemical equilibrium limitations of the epimerization reaction, epimerization was replaced by the oxidoreduction process. In the oxidoreduction pathway, D-galactose is first converted into D-galactitol, which is then converted into D-tagatose under the catalysis of galactitol-2-dehydrogenase from *Rhizobium leguminosarum* (GDH) and D-xylose-1-reductase isolated from *S. stipites* (XYL1). D-tagatose was ultimately extracted from D-lactose at 37.69 g/L [117]. With this route, although D-galactose is efficiently converted into D-tagatose without being limited by the equilibrium, further optimization is required.

Rhimi et al. [118] successfully catalyzed the production of D-tagatose and D-fructose from D-lactose hydrolysates using sodium alginate-embedded engineered bacteria co-expressing thermophilic L-AI and D-glucose isomerase. It was shown that the optimal temperature for D-galactose and D-glucose isomerization was 80 °C and 85 °C, respectively, and the activity of both isomerases was optimal at pH 7.5. The cell encapsulation significantly enhanced the acid resistance of both isomerases and their stability at a high temperature, but the isolation and purification of the sugar fractions in the mixture remained at the exploratory stage. Therefore, although whole-cell catalysis eliminates the enzyme extraction steps, the production process is simpler; however, it poses difficulties for downstream extraction due to the complexity of its fermentation products (Table 3).

Table 3. Research progress of D-tagatose production by chemical and biological methods.

Production Method	Reagent/Enzyme	Optimum Temperature (°C)	Substrate	D-Tagatose (g/L)	Conversion Rate (%)	References
Chemical method	Supercritical ethanol	180	D-galactose	80	24	[48]
	$CaCl_2$, triethylamine	60	1 mmol/L of methanol, D-glucose	38	32	[119]
	Sn/β zeolite	110	D-galactose	29	24	[46]
	Magnesium aluminates	110	D-galactose	16	18–27	[46]
	Sn/deAl-β zeolite	110	D-galactose	89.5	28.3	[120]
Enzyme catalysis	L-AI from *E. coli*	30	100 g/L D-galactose	28.8	28.8	[85]
	L-AI from *Lactobacillus plantarum*	35	100 g/L D-galactose	39	39.0	[55]
	L-AI from *G. stearothermophilus*	60	100 g/L D-galactose	30.6	30.6	[89]
	L-AI from *G. thermodenitrificans*	60	300 g/L D-galactose	158	52.7	[55]
	L-AI from *Thermus* sp.	60	1.0 g/L D-galactose	0.54	54.0	[84]
	L-AI from *Thermoanaerobacter mathranii*	65	300 g/L D-galactose	126	42.0	[82]
	L-AI from *B. stearothermophilus*	70	0.9 g/L D-galactose	0.43	48.0	[57]
	L-AI from *Thermotaga maritima*	80	1.8 g/L D-galactose	1.0	56.0	[81]
	L-AI from *Thermotoga neapolitana*	80	1.8 g/L D-galactose	1.22	68.0	[56]
	Tagaturonate 3-epimerase from *Thermotoga petrophila*	80	700 g/L D-fructose	213	30.0	[88]
	Hexokinase from *Saccharomyces cerevisiae* FbaA from *E. coli* Phytase from NR	50	180 g/L D-fructose	144	80.0	[93]
	L-AI and β-galactosidase from *E. coli* BL21	50	100 g/L lactose	23.5	23.5	[73]
	αGP, PGM, PGI, GatZ, and PGP from *E. coli* BL21	50	20 g/L maltodextrin	9.2	46.0	[94]
Whole-cell catalysis	*E. coli* BLT	60	10 g/L maltodextrin	1.88	18.8	[109]
	L. brevis sp. D-tag 1	55	9 g/L D-galactose	3.916	43.5	[105]
	L-AI from *Lactobacillus parabuchneri*	45	140 g/L D-galactose	54	39.0	[104]
	E. coli/pETDuet-αgp-pgm and pCDFDuet-pgi-gatz-pgp	60	20 g/L maltodextrin	3.2	16.0	[110]

4. Conclusions and Prospects

D-tagatose is a low-calorie sweetener with physiological functions, such as hypoglycemia, obesity prevention, anti-caries, and probiotics, and it is gaining popularity among consumers and is widely employed in the food and biomedical fields [24]. The production of D-tagatose by biological methods has the advantages of green environmental protection, safety, and efficiency, and it can be achieved by in vitro enzymes and whole-cell catalysis using D-galactose as substrates. However, D-galactose has a high cost, and traditional manufacturing methods can only convert about 30% of D-galactose, with the additional high cost of separating D-tagatose, leading to a persistently high retail price for D-tagatose. The development of synthetic biology and other technologies has brought more solutions to the future production of D-tagatose, such as using inexpensive substrates like D-fructose, D-lactose, maltodextrin, and starch as raw materials to reduce production costs. A multi-enzyme catalytic system based on the Izumoring strategy and a phosphorylation–dephosphorylation multi-enzyme catalytic system has been shown to produce D-tagatose using fructose as a substrate. Although in vitro multi-enzyme catalytic systems simplify the biotransformation process and reduce production costs, they are susceptible to interference from external conditions, and product isolation and purification can be challenging. An alternative approach for industrial production is the whole-cell catalytic method, which involves the construction of a de novo D-tagatose synthesis pathway in *E. coli* and other cells to catalyze the synthesis of D-fructose or D-lactose into D-tagatose. This method offers high conversion rates and a stable production process, and does not require additional ATP.

Overall, the whole-cell catalytic method is the most promising approach for the industrial production of D-tagatose.

By optimizing the whole-cell catalytic conditions and strengthening the conversion system, safe and efficient production of D-tagatose was achieved, laying a good research foundation for the industrial production of D-tagatose. Various efforts have been made to reduce the cost of D-tagatose production by using inexpensive raw materials as substrates; however, there are still some shortcomings at the current stage of research. For example, the enzyme has poor thermal stability, poor substrate specificity, low catalytic efficiency, and other shortcomings. In subsequent research, the protein's crystal structure has been analyzed and resolved by combining it with molecular simulation tools, and the target protein was modified using directed evolution and rational design methods to obtain a recombinant enzyme with stronger substrate specificity and catalytic conditions that meet the requirements of industrial production. The popular host in D-tagatose production is *E. coli*. However, the use of *E. coli*, a conditional pathogenic bacterium, as a host for producing D-tagatose, a functional food additive, is controversial. From the perspective of food safety, subsequent experiments should select food-safe strains or adopt extracellular expression and other means to realize the function of D-tagatose synthesis so that the safety of the whole-cell D-tagatose catalyst meets the requirements of industrial food production.

Fortunately, the development of synthetic biology and other technologies has brought more solutions to the future production of D-tagatose [121]. Currently, there are mainly three approaches to attempt to expand the production capacity of D-tagatose. Some companies, like Roquette and Bonumose, use starch as a raw material to produce D-tagatose on a large scale through enzymatic conversion processes. Another approach involves utilizing genetic engineering and synthetic biology technologies to produce D-tagatose on a large scale through yeast-based fermentation [113]. The third approach employs bacteria such as *E. coli* as miniature bioreactors that encapsulate enzymes and reactants, using substrates like maltodextrin to produce D-tagatose. We believe that with the continuous development of synthetic biology and enzyme engineering technology, the technology of producing D-tagatose by biological methods will become more mature. By optimizing enzyme preparations, improving conversion rates, and reducing energy consumption, the production of D-tagatose by biological methods will be safer and more efficient, and the cost will gradually decrease, thereby enhancing its market competitiveness.

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