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Recent advances in D-allulose: Physiological functionalities, applications, and biological production

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ABSTRACT

Background: D-Allulose, an epimer of D-fructose, is a rare monosaccharide that exists in extremely small quantities in nature. It is an ideal substitute for sucrose, because it has 70% of the sweetness of sucrose and ultra-low the energy. In addition, it has received sustained attention because of its unique physiological functions and potential health benefits. However, it is scarce in nature, and difficult to chemically synthesize.

Scope and approach: Because of its scarcity, bioconversion of D-allulose is attractive to researchers. It has been demonstrated that ketose 3-epimerase plays an irreplaceable role in the bioconversion of D-fructose to D-allulose. Herein, an overview of recent advances regarding the physiological functions as well as the biological production of D-allulose is provided. Additionally, a comparison of the biochemical properties and a structural analysis of ketose 3-epimerases are also reviewed in detail in this paper.

Key findings and conclusions: Up to now, ketose 3-epimerase has been experimentally identified and characterized from only twelve types of microorganisms. In addition, 4 types of crystal structures from ketose 3-epimerases have been already solved, and the catalytic mechanism has also been proposed. However, the researches on molecular modification of ketose 3-epimerase are very few. In the future, molecular modification to improve the enzyme activity and thermostability through site-directed mutagenesis or directed evolution must be the research focus.

1. Introduction

Currently, the prevalence and incidence of diseases related to excessive weight gain, such as obesity, diabetes, hypertension and hyperlipidemia, have dramatically increased throughout the world. The main explanation given for this increase is the excessive consumption of high-fat and high-sugar foods. In 2014, the World Health Organization (WHO) released a guideline on the intake of sugars, suggesting that daily intake should be controlled below 5% for additional benefits. However, this target is difficult to achieve (Hossain, Yamaguchi, Matsuo, et al., 2015). As a result, low-calorie rare sugars, which are defined as "monosaccharides and their derivatives that are rare in nature" by the International Society of Rare Sugars in 2002 (Granstrom, Takata, Tokuda, & Izumori, 2004), have attracted the attention of many researchers.

D-Allulose (D-ribo-2-hexulose or D-psicose) is a novel low-calorie functional rare sugar and the epimerization product of D-fructose at the C-3 position. D-Allulose is also called D-psicose, and the name "psicose" is derived from the antibiotic psicofuranine, from which it was isolated (Eble, Hoeksema, Boyack, & Savage, 1959). It is an ultra-low calorie sweetener and ideal substitute for sucrose with 70% of the sweetness. Most of D-allulose is absorbed in the small intestine and excreted from the body via the urine. Moreover, it is likely to be inert in terms of energy metabolism (Iida et al., 2010).

D-Allulose has distinct physiological functions, such as anti-hyperglycemic effects (Hayashi et al., 2010), anti-hyperlipidemic effects (Matsuo et al., 2001), anti-inflammatory effects (Moller & Berger, 2003), neuroprotective effects (Takata et al., 2005), reactive oxygen species (ROS) scavenging activity (Suna, Yamaguchi, Kimura, Tokuda, & Jitsunari, 2007), and therapeutic effects against atherosclerosis (Murao et al., 2007). Moreover, it also improves the gelling properties of food, increases the pleasantness of the flavor of food, and reduces the extent of oxidation occurring through the Maillard reaction during food processing (Oshima,

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Kimura, Kitakubo, Hayakawa, & Izumori, 2014; Zeng, Zhang, Guan, Zhang, & Sun, 2013). In 2014, D-allulose was designated as generally regarded as safe (GRAS) by the US Food and Drug Administration (FDA, GRN No. 498). An application for the use of D-allulose as a food product for Specified Health Uses (FOSHU) has been submitted to the Japanese Ministry of Health, Labour, and Welfare (Hossain, Yamaguchi, Hirose, et al., 2015).

D-Allulose is rarely encountered in nature, and is difficult to chemically synthesize. Prof. Ken Izumori (Kagawa University Rare Sugar Research Centre) developed a strategy for the preparation of all hexoses using cheap and widely available substrates (Izumori, 2006). According to the strategy, D-allulose could be converted from D-fructose via ketose 3-epimerase. In this review, we focus on the recent advances relating to the physiological function of D-allulose, as well as biochemical properties and structural information of ketose 3-epimerase. The production of D-allulose by fermentation as well as immobilization and modifications and are also reviewed in this paper.

2. Brief introduction of D-allulose

2.1. Chemical structure

D-Allulose (or D-psicose) is a rare monosaccharide and a bioactive D-fructose epimer, which is the epimerization product of D-fructose at the C-3 position. It is systematically named as D-ribo-2-hexulose by the International Union of Pure and Applied Chemistry (IUPAC). The molecular formula and molecular weight of D-allulose (CAS No. 551-68-8) are $C_6H_{12}O_6$ and 180.16 g/mol, respectively. It is prepared as a white, odorless, crystalline powder via the ortho-rhombic system, and crystallizes solely as β-D-pyranose with the 1C ($^1C_4$ (D)) conformation (Fukada et al., 2010). D-Allulose, which contains one ketone group, acts as a reducing D-ketose.

2.2. Existing sources

D-Allulose is very rare in nature, and is found in small quantities in Itea and wheat, which are its few plant source (Ayers et al., 2014; Miller & Swain, 1960), and in certain bacteria (Zhang, Mu, Jiang, & Zhang, 2009). D-Allulose is not present in animals. However, it has been found in various foodstuffs, such as commercial mixtures of D-glucose and D-fructose (Cree & Perlin, 1968), steam-treated coffee (Luger & Steinhart, 1995), processed cane and beet molasses, (Binkley & Wolfrom, 1952; Thacker & Toyoda, 2008), and fruit juice with a long-term heating process (Oshima, Kimura, & Izumori, 2006). The psicose contents in various foodstuffs were closely related to the sugar concentration, temperature and heating time during manufacturing processes (Oshima et al., 2006).

2.3. Chemical synthesis

In previous reports, D-allulose could be produced by chemical synthesis, including synthesis from 1,2:4,5-di-*O*-isopropylidene-b-D-fructopyranose (McDonald, 1967), synthesis from D-fructose through a molybdate ion catalyst (Bilik & Tihlarik, 1973) or boiling in ethanol and trimethylamine (Doner, 1979). In 2005, Soengas et al. reported a new method of D-Allulose synthesis by the Kiliani-acetonation sequence, which could achieve an overall yield of 41% (Soengas et al., 2005). However, all these chemical syntheses have disadvantages, including the sophisticated purification processes required, and the formation of chemical waste and byproduct. As a result, the biological production of D-allulose has gained attention, and the separation of D-allulose and D-fructose was achieved using simulated moving bed chromatography. According to the simulation of the simulated moving bed (SMB) process, the complete separation purity of D-allulose could reach 99.36% (Long, Le, Kim, Lee, & Koo, 2009).

3. Excellent physiological functions

3.1. Absorption and metabolism in organisms

The absorption and metabolism of D-allulose was investigated by rat experiments using ^{14}C-labelled D-allulose. It was found that, approximately 70% of the D-allulose was absorbed in the small intestine without being metabolized into energy and then eliminated via urine within 24 h (Hossain, Yamaguchi, Matsuo, et al., 2015; Matsuo, Tanaka, Hashiguchi, Izumori, & Suzuki, 2003). Moreover, a small part of D-allulose was transferred to the large intestine, and seldom fermented in the appendix of rats and humans (Iida et al., 2010). Following single-dose oral administration of 100 mg/kg, D-allulose easily moved to the blood, and 19% and 37% of the administered dose was determined in rats at 1 and 2 h. The residual D-allulose was almost entirely excreted within 7 days. In contrast, following intravenous administration, the excretion to urine was almost 50% within 1 h. Interestingly, D-allulose accumulation could be detected in the liver, kidney, and urinary bladder, but not in brain (Tsukamoto et al., 2014).

Monosaccharide uptake and efflux in enterocytes of the human intestinal are mediated by different sugar transporters, which separately located on the brush border membrane and in the basolateral membrane. D-glucose uptake is mediated by the active transporter SGLT1 (sodium-dependent glucose co-transporter 1), while D-allulose as well as the D-fructose, enters into enterocytes via the passive transporter GLUT5 (glucose transporter 5) (Hishiike et al., 2013). It is interesting that D-glucose, D-fructose and D-allulose are effluxed by the same transporter GLUT2 (glucose transporter 2).

3.2. Health benefits

It was reported that the absorption rate of D-allulose is lower than that of other sweeteners, especially D-glucose. In addition, D-allulose could reduce the absorption of dietary D-fructose and D-glucose by competitive inhibition of the influx and efflux transporter (Hossain, Yamaguchi, Matsuo, et al., 2015). GLUT2 and GLUT5 are distributed not only in the intestine but also in other tissues and cells. The suppressive uptake of D-glucose and D-fructose by D-allulose contributed to health benefits, such as enhancing insulin resistance, reducing body fat accumulation and potentially producing anti-diabetic effects (Hossain, Kitagaki, et al., 2011; Ochiai, Nakanishi, Yamada, Iida, & Matsuo, 2013).

In the oral glucose tolerance test (OGTT), D-allulose obviously inhibited the postprandial glycemic responses by reducing intestinal α-glucosidase and α-amylase activities in vitro, which could delay carbohydrate digestion (Matsuo & Izumori, 2009). Matsuo et al. also found that dietary D-allulose only increased liver glycogen, while muscle glycogen did not change as the result of glucose oxidation in skeletal muscles (Matsuo, Shirai, & Izumori, 2006).

Researchers have discovered the anti-obesity activity of D-allulose by the reducing weight of the adipose tissue weight in animals and humans (Chung, Lee, et al., 2012; Iida et al., 2013; Yagi & Matsuo, 2009). The anti-obesity functions are not simply due to reducing food intake but also related to decreasing adipose tissue weight and fat mass. A potential mechanism was that a D-allulose diet might enhance fat oxidation and reduce carbohydrate oxidation (Nagata, Kanasaki, Tamaru, & Tanaka, 2015). Additionally, a variety of studies have already confirmed the anti-hyperlipidemic effects of D-allulose; however, the anti-hyperlipidemic

mechanism is still unclear and needs to be studied further studies. It is speculated that D-allulose affects the activities of lipogenic and lipolytic enzymes (Matsuo et al., 2001; Ochiai, Onishi, Yamada, Iida, & Matsuo, 2014). Furthermore, D-allulose treatments show beneficial actions on T2DM and β-cell for both short-term and long-term feeding (Hossain, Yamaguchi, Hirose, et al., 2015; Hossain et al., 2012).

D-Allulose has other physiological function, such as anti-inflammatory effects by suppressing proinflammatory cytokines (Moller & Berger, 2003), neuroprotective effects by elevating the intracellular glutathione level (Takata et al., 2005), reactive oxygen species (ROS) scavenging activity (Suna et al., 2007), and therapeutic effects against atherosclerosis by inhibiting the expression of monocyte chemoattractant protein-1 (MCP-1) (Murao et al., 2007).

In recent years, several clinical trials on D-allulose were carried out on normal and borderline diabetic individuals (Hayashi et al., 2010; Iida et al., 2008). Iida et al. reported that the addition of D-allulose at a ratio of approximately 1:15 (D-allulose: carbohydrate) could effectively suppress blood glucose elevation. Additionally, a single ingestion of D-allulose exhibited no impact on blood glucose and insulin levels, suggesting that D-allulose did not induce hypoglycemia (Iida et al., 2008).

D-Allulose could not only stimulate translocation of glucokinase (GK) in the liver but also suppress the increase of plasma glucose levels which led to the increase of hepatic glucose utilization (Toyoda et al., 2010). By feeding rats a pellet diet containing D-allulose, Nagata et al. found that glucose-6-phosphate dehydrogenase (G6PDH) activity was significantly reduced, thereby probably exerting hypolipidemic affects (Nagata et al., 2015). Besides, accumulating clinical and animal studies indicated that D-allulose had hypoglycemic activity mediated by increasing GK translocation, suppressing intestinal α-glucosidase, and protecting against pancreatic β-cell failure (Chung, Oh, & Lee, 2012).

4. Application of D-allulose

Due to its excellent physiological functions mentioned above, D-allulose has potential as a pharmaceutical agent in clinical applications such as the treatment of obesity, diabetes, hypertension, hyperlipidemia and atherosclerotic diseases. Moreover, D-allulose could be spray-dried by the addition of excipients, which would be helpful for pulmonary drug delivery (Kawakami et al., 2013; Kawakami et al., 2014). Additionally, it is reported that D-allulose is the precursor of other hexoses and plays an extremely important role in the production of D-allose (Feng, Mu, & Jiang, 2013; Yeom, Seo, Kim, & Oh, 2011), D-allitol (Han et al., 2014), D-talitol (Sasahara, Mine, & Izumori, 1998), D-tagatose (Yoshihara, Shinohara, Hirotsu, & Izumori, 2006) and D-altrose (Menavuvu et al., 2006).

Recently, D-allulose has been approved as GRAS by FDA in USA, and is permitted to be served as the food ingredient and dietary supplement (Mu, Zhang, Feng, Jiang, & Zhou, 2012). According to the toxicity rating chart, it belongs to the “relatively harmless” category, which is the lowest toxicity rating (Mu et al., 2012). Hossain et al. also obtained the maximum D-allulose intake of 0.55 g/kg body weight, without causing diarrhea in humans (Hossain, Yamaguchi, Matsuo, et al., 2015). Because of its food safety and unique physiochemical properties (low-calorie and high-sweetness), D-allulose has promising market potential in the food industry. Moreover, D-allulose is a reducing sugar, and it could improve food quality through the Maillard reactions. Among all of the D-ketoses (D-tagatose, D-sorbose, D-allulose and D-fructose), D-allulose offered the best overall improvement in the food properties of egg white protein through the Maillard reactions, such as excellent gel strength, emulsifying stability, foaming properties, and antioxidant activities (O'Charoen, Hayakawa, & Ogawa, 2015; Sun, Hayakawa, Ogawa, Fukada, & Izumori, 2008; Zeng et al., 2013). For all we know, industrial production of D-allulose has been achieved in Japan and Korea. Recently, a rare sugar syrup containing D-allulose has been used as a functional food, and can be commercially obtained from Matsutani Chemical Industry Co. Ltd. (Hyogo, Japan).

Furthermore, D-allulose shows many other unique applications. Like D-tagatose, the addition of D-allulose could cause an elevation of chitosan synthesis (Yoshihara, Shinohara, Hirotsu, & Izumori, 2003). Plant-based materials derived from D-allulose have recently been developed and used as permanent, water-repellent, eco-friendly, light-transparent films for optical devices and liquid crystal displays (Takei & Hanabata, 2015). D-allulose has also been identified as the first anthelmintic sugar, and exerts some positive effects on the inhibition of parasite growth (Harada et al., 2012; Sato, Kurose, Yamasaki, & Izumori, 2008).

5. Biotechnological production of D-allulose

5.1. Properties of various ketose 3-epimerase

As a result of its extreme rarity in nature and difficult chemical synthesis, the method of D-allulose biotransformation has attracted many attentions (Izumori, 2002). Ketose 3-epimerase is placed at the crucial position in the Izumoring strategy, which has been schematized for the biological production of all rare sugars. Moreover, it plays an irreplaceable role in the biological production of D-allulose, which catalyzes the reversible epimerization at the C-3 position between D-fructose and D-allulose (Granstrom et al., 2004; Mu, Yu, Zhang, Zhang, & Jiang, 2015). Currently, ketose 3-epimerase has been isolated and identified from various microorganisms, in crude, partially purified, completely purified, recombinant, or immobilized forms.

5.2. Novel enzyme sources

Although the genes of ketose 3-epimerase are presumed in a wide range of microorganisms based on the GenBank database from NCBI, so far, the enzyme has been experimentally identified and characterized from only twelve types of bacteria (Table 1). Based on the different optimal substrate, theses twelve ketose 3-epimerases were divided into D-tagatose 3-epimerase (DTEase), D-psicose 3-epimerase (DPEase) and L-Ribulose 3-epimerase (LREase). In 1993, Izumori et al. reported the first characterized DTEase from *Pseudomonas cichorii* ST-24, which catalyzed the reversible epimerization of free ketose sugars and showed the best substrate specificity for D-tagatose (Itoh et al., 1994; Izumori, Khan, Okaya, & Tsumura, 1993). The second ketose 3-epimerase was not determined until Kim et al. reported a novel DPEase from *Agrobacterium tumefaciens*, which could effectively catalyze the isomerization of D-allulose from D-fructose (Kim, Hyun, Kim, Lee, & Oh, 2006). In the past ten years, research on ketose 3-epimerases has been accelerated, and several ketose 3-epimerases have been isolated and identified one after another. The isomerization capacity of different ketose 3-epimerases varied greatly depending on its microbial source and reaction conditions, such as reaction temperature, optimum pH and metal ionic strength.

5.3. Comparison of ketose 3-epimerase

To enhance the catalytic efficiency of ketose 3-epimerase, the reaction conditions have been optimized, including reaction temperature, pH, and metal ions. A summary of the biochemical properties from the aforementioned ketose 3-epimerases is shown

Table 1
Comparison of biochemical properties of various ketose 3-epimerases.

Ketose 3-epimerase	Optimum temperature (°C)	Optimum pH	Optimum metal ion	Substrate optimum	Metal dependence	Bacterium source	References
A. tumefaciens DPEase	50	8.0	Mn^{2+}	D-Allulose	No	*Agrobacterium tumefaciens*	(Kim, Hyun, Kim, Lee, & Oh, 2006)
C. cellulolyticum DPEase	55	8.0	Co^{2+}	D-Allulose	Yes	*Clostridium cellulolyticum* H10	(Mu et al., 2011)
Ruminococcus sp. DPEase	60	7.5–8.0	Mn^{2+}	D-Allulose	No	*Ruminococcus* sp. 5_1_39BFAA	(Zhu et al., 2012)
C. scindens DPEase	60	7.5	Mn^{2+}	D-Allulose	Yes	*Clostridium scindens* ATCC 35704	(Zhang, Fang, Xing, et al., 2013)
Clostridium sp. DPEase	65	8.0	Co^{2+}	D-Allulose	Yes	*Clostridium* sp. BNL1100	(Mu et al., 2013)
Desmospora sp. DPEase	60	7.5	Co^{2+}	D-Allulose	Yes	*Desmospora* sp. 8437	(Zhang, Fang, Zhang, et al., 2013)
C. bolteae DPEase	55	7.0	Co^{2+}	D-Allulose	Yes	*Clostridium bolteae* ATCC BAA-613	(Jia et al., 2014)
T. primitia DPEase	70	8.0	Co^{2+}	D-Allulose	Yes	*Treponema primitia* ZAS-1	(Zhang et al., 2016)
Dorea sp. DPEase	70	6.0	Co^{2+}	D-Allulose	Yes	*Dorea* sp. CAG317	(Zhang et al., 2015)
P. cichorii DTEase	60	7.5	None	D-Tagatose	No	*Pseudomonas cichorii* ST-24	(Izumori et al., 1993)
R. sphaeroides DTEase	40	9.0	Mn^{2+}	D-Fructose	No	*Rhodobacter sphaeroides*	(Zhang et al., 2009)
M. loti LREase	60	8.0	Mn^{2+}	L-Ribulose	No	*Mesorhizobium loti* MAFF303099	(Uechi, Takata, Fukai, Yoshihara, & Morimoto, 2013)

in Table 1 and is discussed below.

5.3.1. Temperature

The optimal temperature for ketose 3-epimerase catalysis varied from 40 to 70 °C (Table 1). Among all ketose 3-epimerases reported, DPEase from *Treponema primitia* as well as DPEase from *Dorea* sp., exhibited the highest optimum temperature of 70 °C (Zhang, Zhang, Jiang, & Mu, 2016; Zhang et al., 2015), while DTEase from *Rhodobacter sphaeroides* showed the lowest optimum temperature of 40 °C (Zhang et al., 2009). It is generally known that an elevated operating temperature is required for the industrial production of rare sugars, because high reaction temperatures could induce higher reactivity (a higher reaction rate and lower diffusional restrictions), lower viscosity, higher stability, higher process yield (increased solubility of substrates and products and a favorable equilibrium displacement during endothermic reactions), and less contamination (Mozhaev, 1993). Nevertheless, operation at temperatures over 70 °C accelerates the non-enzymatic browning reaction and formation of by-products. Similar to L-arabinose, the most suitable reaction temperature for D-allulose is 60–70 °C (Xu, Li, Feng, Liang, & Xu, 2014).

Thermostability is a crucial property of ketose 3-epimerase to realize the practical production of D-allulose; however, all the ketose 3-epimerases reported show poor thermostability. Most of them exhibit relatively high thermostability below 50 °C, and drastically inactivate at a higher temperature, except for *Mesorhizobium loti* LREase (Uechi, Takata, Fukai, Yoshihara, & Morimoto, 2013). In contrast, the half-life of *M. loti* LREase at 60 and 70 °C could reach more than 24 h and 2.3 h, respectively (Uechi, Sakuraba, Yoshihara, Morimoto, & Takata, 2013). As concluded through previous reports, the thermostability of ketose 3-epimerases is related to metal ions. It is noteworthy that the thermostability of some ketose 3-epimerases could be significantly enhanced by the addition of metal ion cofactor, such as *Clostridium cellulolyticum* DPEase, *Ruminococcus* sp. DPEase, or *Clostridium bolteae* DPEase (Jia et al., 2014; Mu et al., 2011; Zhu et al., 2012). In contrast, adding metal ion (1 mM Co^{2+}) during the incubation barely changed the thermostability of *Desmospora* sp. DPEase, *T. primitia* DPEase and *Dorea* sp. DPEase (Zhang et al., 2016; Zhang, Fang, Zhang, et al., 2013; Zhang et al., 2015). In addition, the thermostability might be increased through molecular modification. For example, when compared with the wild-type enzyme, the I33L/S213C double-site variant of *A. tumefaciens* DPEase showed not only remarkably improvement in thermostability but also an optimal temperature, half-life, and melting temperature (Choi, Ju, Yeom, & Oh, 2011).

5.3.2. pH

Most often, commercial production of rare sugars is best carried out in acidic conditions. It is well known that, under alkaline conditions, non-enzymatic browning of carbohydrates is extremely accelerated, while in slightly acidic conditions the browning effect and unwanted by-product formation are notably suppressed and reduced, which need to be excluded at the end of the reaction. Several acidic L-arabinose and D-xylose isomerases have already been isolated and characterized using screening and molecular-modification techniques (Mu et al., 2015). Nevertheless, there are few similar studies on ketose 3-epimerases, and most of the reported ketose 3-epimerases show optimal activity and a relatively wide pH spectrum under weakly alkaline conditions (pH 7.5 to 9.0). In the past two years, the neutral *C. bolteae* DPEase (pH 7.0) and the acidic *Desmospora* sp. DPEase (pH 6.0) have been characterized in our research group through a screening method (Jia et al., 2014; Zhang et al., 2015). *Dorea* sp. DPEase displayed significantly higher productivity (803 U/mg) of D-allulose in acidic conditions compared to other reported DPEases, suggesting that it has tremendous potential in industrial applications of D-allulose.

5.3.3. Metal ions

Previous findings have found that metal ions play a critical role in isomerization of ketose 3-epimerases. Different types of metal ions have various impacts on the enzyme activity of ketose 3-epimerases. Some ketose 3-epimerases are strictly metal-dependent, which displayed null enzyme activity in the absence of any metal ions. For *C. cellulolyticum* DPEase, *C. bolteae* DPEase, *Clostridium* sp. DPEase, *Desmospora* sp. DPEase, *T. primitia* DPEase and *Dorea* sp. DPEase, metal ion is an essential cofactor for catalysis (Jia et al., 2014; Mu et al., 2011; Mu et al., 2013; Zhang, Fang, Zhang, et al., 2013; Zhang et al., 2015; Zhang et al., 2016); they required Co^{2+} as the optimum metal ion. *Clostridium scindens* DPEase is also metal-dependent, but the optimum metal ion is Mn^{2+} (Zhang, Fang, Xing, et al., 2013). In contrast, other ketose 3-epimerases do not

require any metal ions for their activity, but the presence of Co^{2+} or Mn^{2+} could remarkably improve their catalytic efficiency. Cu^{2+}, Zn^{2+}, as well as EDTA, which exists as a metal chelator, could significantly inhibit the catalytic activity of most ketose 3-epimerases, except for *P. cichorii* DTEase and *M. loti* LREase.

The concentration of metal ions can also influence the enzyme activity. Previous studies have investigated the precise effects of the metal ion concentration on the catalysis activity of ketose 3-epimerases such as *C. scindens* DPEase, *C. bolteae* DPEase, *Clostridium* sp. DPEase and *T. primitia* DPEase. The overall results were visualized as an S-shaped curve. At extremely low concentrations of metal ions, the enzyme activity was undetectable, most likely because the enzymes required a certain amount of metal ions to bind the metal cofactor effectively. After that, the enzyme activity enhanced steeply with the increase in concentration of metal ions, in agreement with a first order reaction; as the concentration increased, the activity tended to be stationary. A similar result appeared in another metal-dependent *Geobacillus stearothermophilus* L-arabinose isomerase (Lee et al., 2005).

Metal ions might be related to the stability of ketose 3-epimerases, including thermostability and structural stability. As mentioned above, the thermostability of *C. cellulolyticum* DPEase, *Ruminococcus* sp. DPEase, and *C. bolteae* DPEase could be significantly enhanced by the addition of metal ions, with half-life ($t_{1/2}$) values increasing at least to 2.6-fold. In addition, Mn^{2+} improved the structural stability of *C. scindens* DPEase during both heat- and urea-induced unfolding processes, with the apparent melting temperature (T_m) and the urea mid-transition concentration (C_m) increasing by 6.1 °C and 2.68 mM, respectively (Zhang, Fang, Xing, et al., 2013).

5.3.4. Substrate specificity and kinetic parameters

For almost all ketose 3-epimerases, except *M. loti* LREase, the optimal substrate was ketohexose. Nine D-psicose 3-epimerases displayed optimum substrate specificity for D-allulose, while *P. cichorii* DTEase and *R. sphaeroides* DTEase showed the best substrate specificity for D-tagatose and D-fructose, respectively. Interestingly, for *M. loti* LREase, L-ribulose is the optimal substrate, this is the only report on a ketose 3-epimerase showing the highest activity towards ketopentose. The kinetic parameters of ketose 3-epimerases are also important parameters for D-allulose biotransformation. The catalytic efficiency (k_{cat}/K_m) values of the above-mentioned ketose 3-epimerases have been compared in Table 2. Although the amino acid sequence homology of DPEases is considerably high, significant differences are shown in the Michaelis constant (K_m). The K_m values for D-fructose and D-allulose are estimated in the range from 24 to 549 mM, and from 12 to 228 mM, respectively. As for the k_{cat}/K_m values, *Dorea* sp. DPEase shows the maximal k_{cat}/K_m values for D-allulose and D-fructose, with 412 and 199 mM^{-1} min^{-1}.

5.4. Structural analysis and molecular modification

5.4.1. Overall structures

Multiple sequence alignment of amino acids from ketose 3-epimerases was performed and shown in Fig. 1. Although all these enzymes can produce D-allulose from D-fructose, the amino acid sequence homology among them varied considerably from 23.9 to 94.2% (Table 3). To date, 4 types of crystal structures from ketose 3-epimerases were already solved, including *A. tumefaciens* DPEase (Protein Data Bank, PDB: 2HK0), *P. cichorii* DTEase (PDB: 2QUL), *C. cellulolyticum* DPEase (PDB: 3VNI) and *M. loti* LREase (PDB: 3VYL). *A. tumefaciens* DPEase, *C. cellulolyticum* DPEase and *M. loti* LREase were confirmed to be tetrameric structures, while *P. cichorii* DTEase assembled into a dimeric structure (Fig. 2). Nevertheless, as demonstrated in Fig. 3, their monomer structures were extremely similar. Each subunit showed a formation as a TIM-like $(\beta/\alpha)_8$ barrel fold with a metal ion in the active site. The metal-binding sites are shown as an octahedral coordination with two water molecules and four residues which were completely conserved across all ketose 3-epimerases. Notably, the volume size of the α8 helix and the C-terminal tail was much bigger in *M. loti* LREase than those in other ketose 3-epimerases, leading to the unique formation of further intermolecular interactions. Through mutation experiments deleting some C-terminal residues, it was found that the existence of a long C-terminal tail was integrant for the dramaticlly thermostability of *M. loti* LREase (Uechi, Sakuraba, et al., 2013).

Table 2
Comparison of kinetic parameters of various ketose 3-epimerases.

Ketose 3-epimerase	Equilibrium ratio between D-allulose and D-fructose	Specific activity (U mg^{-1})[a]	k_{cat} (min^{-1})		K_m (mmol L^{-1})		k_{cat}/K_m (min^{-1} L mmol $^{-1}$)		References
			D-allulose	D-fructose	D-allulose	D-fructose	D-allulose	D-fructose	
A. tumefaciens DPEase	33:67 (40 °C)	8.9	2381	2068	12	24	205	85	(Kim, Hyun, Kim, Lee, & Oh, 2006)
C. cellulolyticum DPEase	32:68 (55 °C)	287.0	3243.5	3354.5	17.4	53.5	186.4	62.7	(Mu et al., 2011)
Ruminococcus sp. DPEase	28:72 (60 °C)	8.9	2427	3562	48	216	51	16	(Zhu et al., 2012)
C. scindens DPEase	28:72 (50 °C)	171.0	1827	350	28.3	40.1	64.5	8.72	(Zhang, Fang, Xing, et al., 2013)
Clostridium sp. DPEase	28:72 (65 °C)	249.5	32,185	16,372	227.6	279	141	58.7	(Mu et al., 2013)
Desmospora sp. DPEase	30:70 (60 °C)	252.1	5157.8	63,573	81.3	549	327	116	(Zhang, Fang, Zhang, et al., 2013)
C. bolteae DPEase	32:68 (60 °C)	150.7	2490	3540	27.4	59.8	107	59	(Jia et al., 2014)
T. primitia DPEase	28:72 (70 °C)	234.9	30,166	17,573	209	279	144	63	(Zhang et al., 2016)
Dorea sp. DPEase	30:70 (70 °C)	803.5	78,692	30,447	191	153	412	199	(Zhang et al., 2015)
P. cichorii DTEase	20:80 (30 °C)	NR[b]	NR	NR	NR	NR	NR	NR	(Izumori et al., 1993)
R. sphaeroides DTEase	23:77 (40 °C)	NR	NR	NR	NR	NR	NR	NR	(Zhang et al., 2009)
M. loti LREase	NR	4.0	0.34	NR	58	NR	5.4	NR	(Uechi, Takata, Fukai, Yoshihara, & Morimoto, 2013)

[a] The specific activity values were determined with D-fructose as substrate.
[b] NR, not reported.

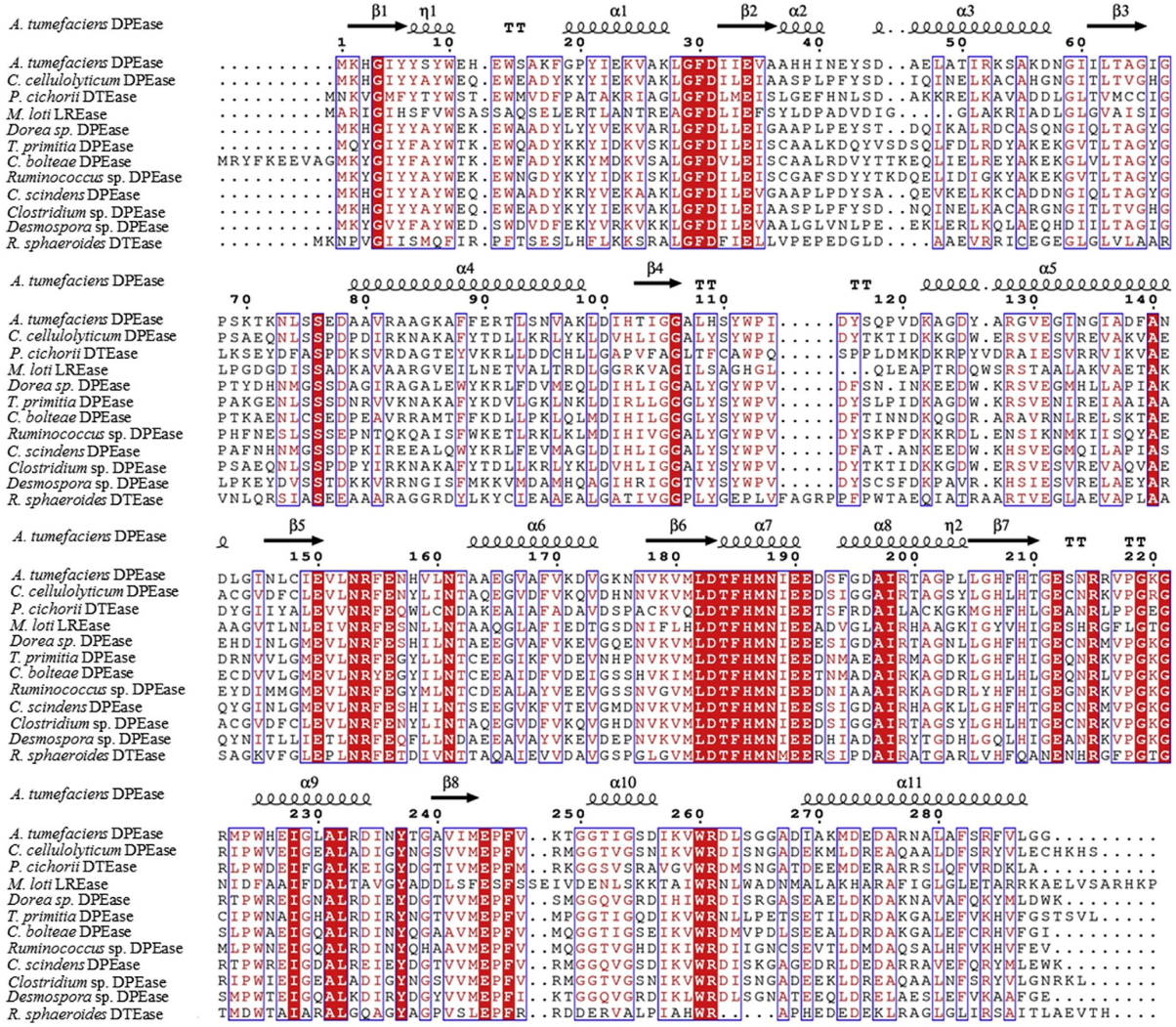

Fig. 1. Multiple sequence alignment of amino acids from ketose 3-epimerases. The microorganism origins of ketose 3-epimerases with the GenBank accession numbers as follows: *A. tumefaciens* DPEase (AAK88700.1), *C. cellulolyticum* DPEase (ACL75304), *P. cichorii* DTEase (BAA24429), *M. loti* LREase (BAB50456), *Dorea* sp. DPEase (CDD07088), *T. primitia* DPEase (WP_010256447), *C. bolteae* DPEase (EDP19602), *Ruminococcus* sp. DPEase (ZP04858451), *C. scindens* DPEase (EDS06411.1), *Clostridium* sp. DPEase (WP_014314767), *Desmospora* sp. DPEase (WP_009711885.1), and *R. sphaeroides* DTEase (ACO59490). The alignment was prepared using the program ESPript.

Table 3
Comparison of amino acid sequence homology of ketose 3-epimerases.[a]

Enzyme	Amino acid sequence homology (100%)											
	Agtu	Clce	Rusp	Clsc	Clsp	Desp	Clbo	Trpr	Dosp	Psci	Rhsp	Melo
Agtu	100											
Clce	60.9	100										
Rusp	50.5	52.3	100									
Clsc	59.0	61.9	52.8	100								
Clsp	61.3	94.2	52.9	62.6	100							
Desp	49.8	49.8	48.8	50.4	50.9	100						
Clbo	49.5	52.9	62.5	51.7	54.0	47.4	100					
Trpr	55.4	58.4	62.5	55.0	59.3	52.3	68.0	100				
Dosp	59.0	60.6	52.1	78.2	61.3	50.4	52.4	56.1	100			
Psci	39.6	42.4	33.7	39.4	42.40	42.0	36.0	35.1	38.0	100		
Rhsp	31.6	29.4	27.7	31.6	29.8	27.7	30.6	29.0	31.2	29.7	100	
Melo	28.2	26.0	23.9	26.0	26.1	25.0	24.6	27.1	26.3	30.3	32.6	100

[a] Agtu (*A. tumefaciens* DPEase, Genbank no. AAL45544), Clce (*C. cellulolyticum* DPEase, ACL75304), Rusp (*Ruminococcus* sp. DPEase, ZP_04858451), Clsc (*C. scindens* DPEase, EDS06411.1), Clsp (*Clostridium* sp. DPEase, WP_014314767), Desp (*Desmospora* sp. DPEase, WP_009711885.1), Clbo (*C. bolteae* DPEase, EDP19602), Trpr (*T. primitia* DPEase, WP_010256447), Dosp (*Dorea* sp. DPEase, CDD07088), Psci (*P. cichorii* DTEase, BAA24429), Rhsp (*R. sphaeroides* DTEase, ACO59490), Melo (*M. loti* LREase, BAB50456).

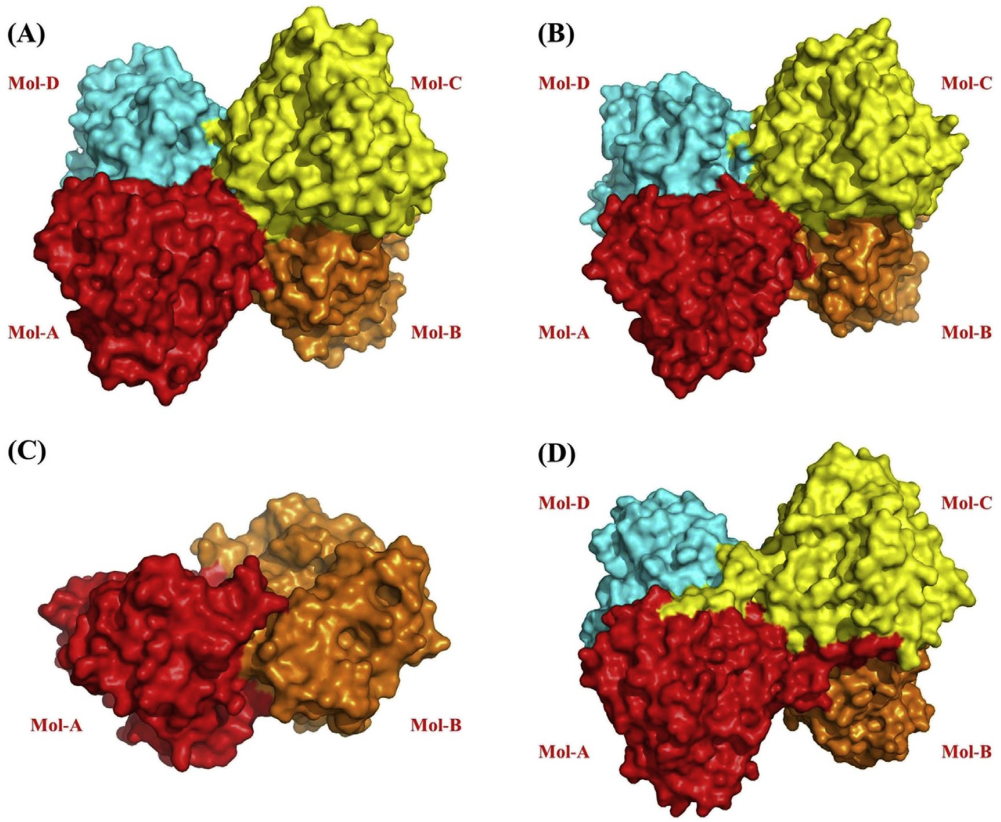

Fig. 2. Stereo views of the overall structures of reported apo-form ketose 3-epimerases. (A) *A. tumefaciens* DPEase (PDB: 2HK0); (B) *C. cellulolyticum* DPEase (PDB: 3VNI); (C) *P. cichorii* DTEase (PDB: 2QUL); (D) *M. loti* LREase (PDB: 3VYL). Subunits Mol A, Mol B, Mol C and Mol D were shown in red, orange, yellow and cyan, respectively. The stereo views were illustrated by the program PyMol. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Interestingly, two D-tagatose 3-epimerase-related proteins from *Thermotoga maritima* and *Methanocaldococcus jannaschii* were already determined (Sakuraba, Yoneda, Satomura, Kawakami, & Ohshima, 2009; Uechi, Takata, Yoneda, Ohshima, & Sakuraba, 2014). The main-chain coordinates of the subunit from both of the D-tagatose 3-epimerase-like proteins were found to be similar to those of the ketose 3-epimerases. However, the active-site architectures of the two enzymes differed completely from the ketose 3-epimerases.

5.4.2. Catalytic mechanism

Based on the crystal structure and mutant analysis for the two Glu residues of *A. tumefaciens* DPEase, Kim et al. proposed a catalytic mechanism where one of the two Glu residues coordinated with Mn^{2+} removed a proton from C-3 to form a *cis*-enediolate intermediate firstly, and immediately another Glu residue protonated C-3 in the opposite direction (Kim, Kim, Oh, Cha, & Rhee, 2006). After that, Yoshida et al. solved the crystal structure of *P. cichorii* DTEase, and proposed a C3—O3 proton-exchange mechanism. During substrate binding, the Glu246 residue removed a proton from C-3 to generate a *cis*-enediolate intermediate with the plane structure of O2—C2—C3—O3. Then, proton possibly transferred from Glu246 to Glu152 via O3, which was in the *cis*-enediolate intermediate. At last, the Glu152 protonated C-3, and the two Glu residues reached the negatively ionized state (Yoshida et al., 2007). In 2012, Chan et al. successfully crystallized both substrate-bound and product-bound complex structures of *C. cellulolyticum* DPEase, which gave more evidence of the C3—O3 proton-exchange mechanism and offered a clear method for the deprotonation/protonation effects of Glu150 and Glu 244 in the isomerization process (Chan et al., 2012).

5.4.3. Active site and substrate binding

The active sites of these ketose 3-epimerases were shown in Fig. 4. The Mn^{2+} ion was octahedrally coordinated by two water molecules and four residues (Glu, Asp, His and Glu) that were strictly conserved in all of the other ketose 3-epimerases. In addition, three residues (Glu, His and Arg), which were related with interactions between the enzymes and O-1, O-2 and O-3 of D-fructose, were also strictly conserved. In contrast, as displayed in Table 4, the amino-acid residues providing a hydrophobic environment around the O-4, O-5 and O-6 positions of D-fructose were significantly different in the these enzymes and led to distinction in specificity and affinity for substrates binding. The electron density for the bound D-fructose at the C-1 to C-3 positions was explicit, and the discovered eclipsed configuration of O-2 and O-3 suggested a rotation of the C2—C3. However, the electron density for the C-4 to C-6 region of the bound D-fructose, especially for the hydroxyl groups O-4, O-5, and O-6, maintained relatively disordered. According to the mutant analysis of *A. tumefaciens* DPEase, Kim et al. proposed that three residues were essential elements for catalytic activity and D-fructose binding as well as D-allulose binding, including Trp112 with a ring, Glu156 with the negative charge, and Arg215 with the positive charge. Furthermore, Ile66 was involved

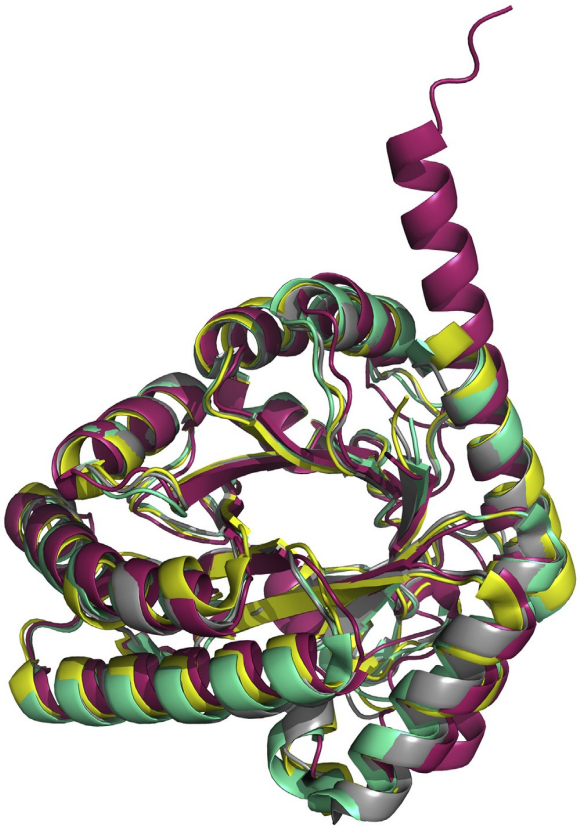

Fig. 3. The superimposition of subunits from *A. tumefaciens* DPEase (gray), (B) *C. cellulolyticum* DPEase (cyan), (C) *P. cichorii* DTEase (yellow), and (D) *M. loti* LREase (warmpink). The stereo views were illustrated by the program PyMol. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

in substrate recognition, and Ala107 located in the β-turn region was be related to the thermostability (Kim, Lim, et al., 2010; Kim, Yeom, et al., 2010).

Upon substrate binding with *A. tumefaciens* DPEase, a significant ligand-induced conformational change was discovered. Particularly, the side-chains of Trp112 located in the loop β4-α4 moved towards the substrate, which served as a lid and shut down the active site (Kim et al., 2006). In contrary, no ligand-induced conformational change was observed in substrate binding with *P. cichorii* DTEase and *C. cellulolyticum* DPEase. In *P. cichorii* DTEase, Pro117 located in the loop was stuck in the middle of Trp160 and Trp262 of the neighboring subunit, leading to efficient hydrophobic interactions in the intersubunit contact area that could hinder the conformational changes of the loop (Yoshida et al., 2007). At the present stage, the structure of the *M. loti* LREase substrate complex was not yet determined. It was speculated that the substrate-induced shift of Tyr39 might occur. Moreover, the mutant analysis of Try39 suggested that Tyr39 was not necessary for maintaining the substrate affinity, but was actually important for proper isomerization. The relatively small size of hydrophobic pocket around the substrate probably was the major reason for the unique specificity for ketopentoses by *M. loti* LREase (Uechi, Sakuraba, et al., 2013).

5.4.4. Molecular modification and directed evolution

In recent years, molecular modification technology had been widely applied to L-arabinose isomerase and D-glucose isomerase, and some desirable mutants had already been obtained. However, there were very few similar studies on the molecular modification of ketose 3-epimerases. In 2011, by utilizing the random and site-directed mutagenesis, the double-site I33L/S213C variant of *A. tumefaciens* was gained, which displayed excellent improvement in the thermostability, including optimal temperature, half-life and melting temperature (Choi et al., 2011). Recently, Bosshart et al. reported the directed divergent evolution of the thermostable variant (Var8) of *P. cichorii* DTEase into two efficient epimerizations at the C-3 position, such as D-fructose to D-allulose and L-sorbose to L-tagatose. By iterative randomization and screening around the substrate-binding site, the eight-site mutant IDF8 and the six-site mutant ILS6 were achieved. Compared to Var8, IDF8 showed 9-fold improved k_{cat} for the epimerization of D-fructose, while ILS6 14-fold improved k_{cat} for the epimerization of L-sorbose (Bosshart, Hee, Bechtold, Schirmer, & Panke, 2015). All these mutants mentioned above might have a great potential in the industrial production of D-allulose.

5.4.5. Bioproduction of D-allulose

Ketose epimerization is generally regarded as reversible and equilibrium reactions. As depicted in Table 2, for most ketose 3-epimerases, the maximum equilibrium ratio between D-fructose

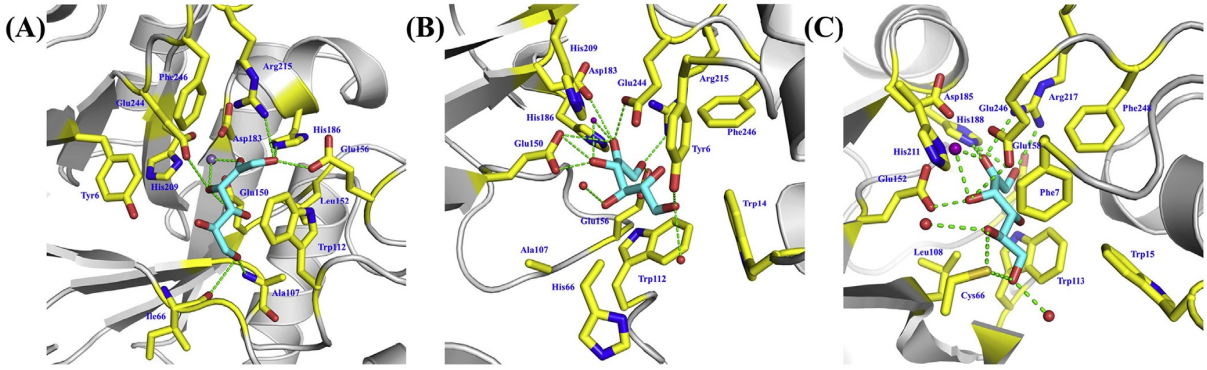

Fig. 4. The active sites in ketose 3-epimerases in complex with D-fructose. (A) *A. tumefaciens* DPEase (PDB: 2HK1); (B) *C. cellulolyticum* DPEase (PDB: 3VNK) (C) *P. cichorii* DTEase (PDB: 2QUN); Mn^{2+} ions are shown as purple spheres and the water molecules are shown as red spheres. The stereo views were illustrated by the program PyMol. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 4
Comparison of the active sites in ketose 3-epimerases bound with D-fructose.

The active sites	*A. tumefaciens* DPEase	*C. cellulolyticum* DPEase	*P. cichorii* DTEase	*M. loti* LREase
Residues involved in the metal coordinating site	Glu150 Asp183 His209 Glu244	Glu150 Asp183 His209 Glu244	Glu152 Asp185 His211 Glu246	Glu147 Asp180 His206 Glu241
Residues responsible for the interaction between the enzyme and O-1, O-2, and O-3 of D-fructose	Glu156 His186 Arg215	Glu156 His186 Arg215	Glu158 His188 Arg217	Glu153 His183 Arg212
Residues providing a hydrophobic environment around the substrate around the O-4, O-5, and O-6 of D-fructose	Tyr6 Trp14 Ile66 Ala107 Trp112 Phe246	Tyr6 Trp14 His66 Ala107 Trp112 Phe246	Phe7 Trp15 Cys66 Leu108 Trp113 Phe248	His7 Leu252 Ser64 Ile106 His111 Phe243
References	(Kim et al., 2006)	(Chan et al., 2012)	(Yoshida et al., 2007)	(Uechi, Sakuraba, et al., 2013)

and D-allulose was approximately 30:70, except for *R. sphaeroides* (23:77) DTEase, *P. cichorii* DTEase (20:80) and *M. loti* LREase (not reported). The reaction temperature might change the equilibrium ratio between substrate and product, such as the isomerization process by D-xylose isomerase and L-arabinose isomerase. Similar effects appeared in ketose 3-epimerase. For *C. bolteae* DPEase, the equilibrium ratio between D-fructose and D-allulose was shifted from 23:77 to 32:68, when the temperature increased from 30 °C to 60 °C. However, the reaction temperature showed no obvious effect of *Dorea* sp. DPEase on the equilibrium ratio, which was maintained in the range of 29:71 to 32:68. Similar to D-tagatose and L-ribulose, the conversion rate of D-allulose could be greatly enhanced by a borate supplement. The maximum conversion rate was shifted to 64% by *A. tumefaciens* DPEase, with a borate-to-fructose ratio of 0.6 (Kim et al., 2008), because the borate binding affinity with D-allulose was stronger than with D-fructose. Moreover, *C. cellulolyticum* DPEase was overexpressed in *Bacillus subtilis*, producing 120 g/L D-allulose from 500 g/L of D-fructose. This is the sole report on food-grade overexpression of ketose 3-epimerase, making the industrial D-allulose production possible (Li, Zhu, Zeng, Zhang, & Sun, 2013).

It is generally known that, immobilization of free enzymes is an effective method for enzyme recycling and cost-efficiency increasing. In a previous study, Itoh et al. first reported the immobilization of *P. cichorii* DTEase, using the *Chltopearl* beads of BCW 2503 as the carrier. By this means, 90 g of D-allulose were produced from 500 g of D-fructose at pH 7.5 and 45 °C for 10 d (Itoh, Sato, & Izumori, 1995). After that, Takeshita et al. improved the mass production of D-allulose by immobilizing *P. cichorii* DTEase on a continuous bioreactor, which could reach a conversion rate of 25%. Through treatment with baker's yeast and crystallization with ethanol, approximately 20 kg of pure D-allulose was achieved after 60 d (Takeshita, Suga, Takada, & Izumori, 2000). In 2009, Lim et al. immobilized another *A. tumefaciens* DPEase on Duolite A568 beads with borate, producing 441 g/L D-allulose from 700 g/L D-fructose (Lim, Kim, & Oh, 2009). Moreover, the I33L/S213C variant of *A. tumefaciens* DPEase with and without borate was immobilized as well, and showed no decreased activity during the operation time of 30 d (Choi et al., 2011). Recently, *C. cellulolyticum* DPEase immobilized on Artificial Oil Bodies (AOBs) retained more than 50% of its initial activity after five cycles (Tseng et al., 2014).

According to the Izumoring strategy, D-allulose could be produced from D-glucose via the intermediate D-fructose by coupling D-glucose isomerase (GIase) and DPEase. In 1995, Itoh et al. carried out this project by coupling immobilized *P. cichorii* DTEase with GIase (Itoh et al., 1995). Moreover, by constructing the co-expression of *Bacillus* sp. GIase and *Ruminococcus* sp. DPEase in *Escherichia coli*, Men et al. presented a one-step process for D-allulose production from D-glucose with a conversion yield of 16% (Men et al., 2014). Furthermore, *Thermus thermophilus* GIase and *Agrobacterium tumefacienswere* (I33L/S213C variant) DPEase were successfully immobilized on the wall of *Saccharomyces cerevisiaespore* spores with a 12.0% yield of D-allulose from D-glucose by continuously repeating the two-step process (Li et al., 2015). Additionally, Yang et al. designed and constructed a novel recombination pathway in *Corynebacterium glutamicum*, which contained dihydroxyacetone phosphate (DHAP)-dependent rhamnulose-1-phosphate aldolase (RhaD) and fructose-1-phosphatase (YqaB). Using a fed-batch culture mode, this strain could accumulate 13.4 g/L of D-allulose and 19.5 g/L of D-sorbose (Yang et al., 2015).

Nevertheless, to achieve the large-scale D-allulose production, the high cell density cultivation (HCDC) technique should be introduced, because all of the methods mentioned above need large quantities of cells or enzymes.

6. Conclusion and future scope

Although ketose 3-epimerases from various microorganism sources have been identified and characterized, until now, no ketose 3-epimerases were regarded as completely appropriate for industrial applications. The major restrictions are poor thermostability, alkaline optimal pH, catalytic inefficiency, and low turnover for the substrate. To solve these problems, further screening of novel ketose 3-epimerases is needed. In addition, reported ketose 3-epimerases need molecular modification through site-directed mutagenesis or directed evolution to improve the enzyme activity and thermostability. Moreover, mutants with higher catalytic properties could be screened, by using a high-throughput screening (HTS) method. To avoid food safety problems, expression in *E. coli* should be urgently substituted with the food-grade expression in *Bacillus subtilis*, *Pichia pastoris*, *Saccharomyces cerevisiae* or *Lactococcus lactis* in the future.

Acknowledgements

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