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Two-step biosynthesis of D-allulose via a multienzyme cascade for the bioconversion of fruit juices

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ABSTRACT

D-Allulose, a low-calorie rare sugar with potential as sucrose substitute for diabetics, can be produced using D-allulose 3-epimerase (DAE). Here, we characterized a putative thermostable DAE from *Pirellula* sp. SH-Sr6A (PsDAE), with a half-life of 6 h at 60 °C. Bioconversion of 500 g/L D-fructose using immobilized PsDAE on epoxy support yielded 152.7 g/L D-allulose, which maintained 80% of the initial activity after 11 reuse cycles. A multienzyme cascade system was developed to convert sucrose to D-allulose comprising sucrose invertase, D-glucose isomerase and PsDAE. Fruit juices were treated using this system to convert the high-calorie sugars, such as sucrose, D-glucose, and D-fructose, into D-allulose. The content of D-allulose among total monosaccharides in the treated fruit juice remained between 16 and 19% during 15 reaction cycles. This study provides an efficient strategy for the development of functional fruit juices containing D-allulose for diabetics and other special consumer categories.

1. Introduction

D-Allulose is a C-3 epimer of D-fructose that is rarely found in nature, but it has aroused significant interest due to its suitability as an ultra-low-calorie functional sweetener (Matsuo, Suzuki, Hashiguchi, & Izumori, 2002). D-Allulose is an attractive sucrose substitute for food production due to its significantly reduced caloric content of about 0.2 kcal/g, while maintaining approximately 70% of the sweetness of sucrose (Mooradian, 2019). Furthermore, D-allulose improves the gelling properties of food and reduces oxidation via the Maillard reaction in food processing, better preserving the food flavor (Zeng, Zhang, Guan, & Sun, 2011). Notably, it has been granted the generally recognized as safe (GRAS) status by the US Food and Drug Administration (FDA) (Mooradian, 2019). In addition to potential uses as a food additive, studies have demonstrated that D-allulose also has notable physiological effects associated with blood glucose suppression, neuroprotective effects, reactive oxygen species (ROS) scavenging activity, and therapeutic effects against atherosclerosis (Zhang, Fang, Xing, Zhou, Jiang, & Mu, 2013; Zhang, Yu, Zhang, Jiang, & Mu, 2016). Accordingly, D-allulose is an attractive natural functional ingredient due to its health-promoting attributes such as mitigation of obesity, reducing lipidemia, and fighting diabetes (Shintani et al., 2017). The production of D-allulose by extraction from natural resources is not practical, and the chemical synthesis of D-allulose is difficult due to the complex multi-step reactions and functional group protection-deprotection steps. Moreover, chemical synthesis can produce toxic byproducts that render the final product unsuitable for food use (Emmadi & Kulkarni, 2014). Instead, a sustainable bioprocess based on Izumoring strategy has been established to biosynthesize D-allulose from D-fructose via the enzymatic and microbiological synthesis (Mu, Yu, Zhang, Zhang, & Jiang, 2015).

The biosynthesis of D-allulose via the enzymatic Izumoring strategy is more efficient, with higher substrate specificity and mild reaction conditions offering increased sustainability (Izumori, 2006). Biosynthesis of D-allulose can be realized via the epimerization of D-fructose by ketose 3-epimerases (KEases), which can be classified as D-tagatose 3-epimerases (DTEs, EC 5.1.3.-) and D-allulose 3-epimerase (DAEs, also named DPEs, EC 5.3.1) based on substrate specificity (Zhang, Mu, Jiang, & Zhang, 2009; Zhang et al., 2013). To date, at least 20 KEases from various bacterial species and strains have been characterized, such as the DTEs from *Sinorhizobium* sp. (Zhu, et al., 2019), as well as the DAEs from *Dorea* sp. CAG317 (Zhang et al., 2015) and *Arthrobacter globiformis* M30 (Yoshihara et al., 2017). Enzymatic catalysis offers many advantages with few or no side reactions, facile control and optimization of reaction conditions, and avoidance of rate-limiting diffusion across the cell

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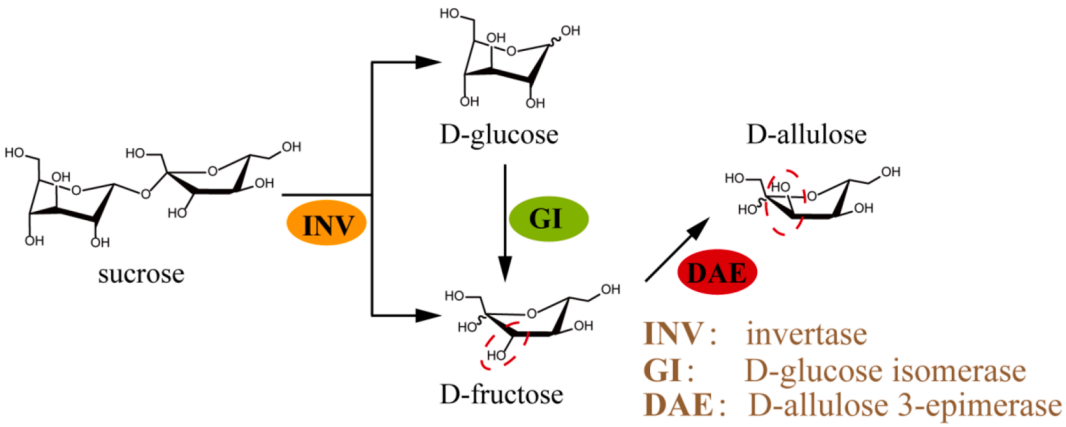

Fig. 1. Schematic representation of D-allulose synthesis from sucrose via the multienzyme cascade catalysis system developed in this study. The enzymes used are invertase (INV), D-glucose isomerase (GI), and D-allulose 3-epimerase (DAE).

membrane (Bujara, Schuemperli, Billerbeek, Heinemann, & Panke, 2010; Finnigan, Cutlan, Snajdrova, Adams, Littlechild, & Harmer, 2019; Hold & Panke, 2009; Xue & Woodley, 2012). Although enzymatic synthesis is considered a green and sustainable approach, the large-scale industrial application of enzymes to produce D-allulose is restricted by expensive unrecyclable biocatalysts, poor operational stability, and high production cost (Choi, Han, & Kim, 2015; DiCosimo, McAuliffe, Poulose, & Bohlmann, 2013; Klein-Marcuschamer, Oleskowicz-Popiel, Simmons, & Blanch, 2012). Notably, enzyme immobilization has been demonstrated as an efficient approach for improving the physical properties, recovery and reusability of enzymes to reduce process costs (Nawani, Singh, & Kaur, 2006; Su, Zhang, Zhu, Xu, Yang, & Li, 2013).

As a functional rare sugar, D-allulose has significant commercial potential as a low-calorie sweetener in fruit juices. Some high-calorie sugars in fruit juices, such as sucrose, D-glucose, and D-fructose, which are considered to be equivalent to high-calorie sugars in sugar-sweetened beverages can be utilized as substrates for multienzyme cascade systems (Zolot, 2017). Studies have demonstrated that excessive consumption of high-calorie sugars increases the risk of diabetes, obesity, and metabolic disease (Imamura et al., 2015; Yang et al., 2019). Notably, D-allulose can reduce food calorie intake and promote glucose tolerance in healthy persons as well as obese diabetics, since it induces glucagon-like peptide-1 (GLP-1) release and activates vagal afferent signaling following oral administration (Iwasaki et al., 2018). The conversion of high-calorie sugars into D-allulose can be achieved via enzymatic biocatalysis or fermentation technology, which are increasingly being viewed as attractive technologies for promoting better health in recent years (Men, Zhu, Zeng, Izumori, Sun, & Ma, 2014). Yang *et al.* reported that an engineered *Corynebacterium glutamicum* strain expressing DAE and a thermostable invertase (INV) can produce D-allulose using inexpensive sugarcane molasses as feedstock (Yang et al., 2019). Enzymatic catalysis pathways using glucose isomerase (GI) and DAE have enabled the conversion of D-glucose and D-fructose in high-fructose corn syrup into D-allulose (Men et al., 2014).

In this study, a thermostable D-allulose 3-epimerase from *Pirellula* sp. SH-Sr6A (PsDAE) has been characterized. Additionally, PsDAE was immobilized and used as a recyclable biocatalyst to biosynthesize D-allulose from D-fructose with high bioconversion efficiency. Furthermore, a multienzyme strategy was developed to convert high-calorie sugars in fruit juice into D-allulose via two-step reaction strategies. The reaction system consisted of free INV, immobilized GI, and immobilized PsDAE (Fig. 1). INV was firstly used to catalyze the hydrolysis of sucrose into D-glucose and D-fructose. D-Glucose was then converted into D-fructose by GI, while D-fructose was transformed into D-allulose by PsDAE. A variety of fruit juices were subjected to treatment using this multienzyme cascade catalysis system to convert high-calorie sugars into D-allulose with high efficiency.

2. Materials and methods

2.1. Strains, plasmids, and reagents

The strains and plasmids used in this study are listed in Table S1. The pET22b vector was used for protein expression in *Escherichia coli* BL21 (DE3). The sucrose, D-glucose, D-fructose and D-allulose standard were purchased from Sigma-Aldrich (Shanghai, China). The epoxy support ES-103B was purchased from Tianjin Nankai HECHENG (Tianjin, China). INV and immobilized GI were acquired from Novozymes Biotechnology Inc. (Suzhou, China). Oligonucleotides were synthesized by GENEWIZ (Suzhou, China). All other chemicals were of analytical grade and were obtained from Sigma-Aldrich (Shanghai, China) or Sangon Biotech (Shanghai, China).

2.2. Cloning and expression of the PsDAE gene

The coding sequence of PsDAE (GenBank: WP_146677337.1) was codon-optimized for *E. coli,* ordered from Genewiz (Suzhou, China) as synthetic DNA, and subcloned into the pET22b vector (Novagen, Madison, WI, USA) between the *Nde*I and *Eco*RI restriction sites. The recombinant pET22b plasmid containing the PsDAE gene and a His_6 tag at the C-terminus (pET-PsDAE) was introduced into *E. coli* BL21 (DE3), which was cultured in lysogeny broth (LB) containing ampicillin (100 μg/mL) at 37 °C and 220 rpm until the OD_{600} reached 0.6–0.8. Then, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.5 mM to induce the expression of PsDAE, which was continued at 16 °C for 16–18 h.

2.3. Purification of PsDAE

The recombinant cells expressing the protein were collected by centrifugation at 5000×*g* and 4 °C for 15 min, resuspended in lysis buffer (20 mM Tris-HCl, 10 mM imidazole, 500 mM NaCl, 1 mM dithiothreitol (DTT), 1 mg/mL lysozyme, and 1 mM phenylmethane sulfonyl fluoride (PMSF), pH 8.0), disrupted by sonication, and the resulting crude lysate cleared by centrifugation at 40,000×*g* and 4 °C for 30 min. The cleared supernatant containing His-tagged-PsDAE was loaded onto a column containing Ni-NTA Superflow resin (Qiagen, Hilden, Germany) equilibrated with lysis buffer. The recombinant His-tagged PsDAE was eluted using 10 mL of elution buffer (20 mM Tris-HCl, 300 mM imidazole, 100 mM NaCl, and 1 mM DTT, pH 8.0) and dialyzed against 20 mM Tris-HCl pH 8.0 with 1 mM DTT to remove the imidazole, and used directly for the activity assays and biosynthesis of D-allulose. The protein concentration was determined using a BCA assay kit (Solarbio, China) with bovine serum albumin as the standard. The purity of the target protein was assessed by sodium dodecyl sulfate

polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie Brilliant Blue staining.

2.4. Preparation of immobilized PsDAE enzyme

To immobilize PsDAE, the epoxy support ES-103B was first washed with 0.1 M potassium phosphate buffer pH 8.0, followed by distilled water. Then, 1 g of epoxy resin ES-103B and 30 mg PsDAE were mixed in 15 mL 1.5 M sodium phosphate buffer pH 6.5 and gently shaken for 24 h at 25 °C. The immobilized PsDAE was washed non-covalently bound enzyme with phosphate buffer saline (PBS) buffer. The amount of enzyme covalently immobilized onto the epoxy support was determined by calculating the difference between the initial PsDAE concentration in the solution and the amount of protein in the supernatant and washing fractions.

2.5. Identification of sugars by HPLC

The qualitative and quantitative analysis of D-allulose, D-fructose, D-glucose, and sucrose was conducted using high-performance liquid chromatography (HPLC) on an Agilent 1260 instrument (USA) equipped with a prevail Carbohydrate ES column-W (5 μm, 4.6 × 250 mm, Agela Technologies, China), an evaporative light-scattering detector (ELSD) (Agilent 1260 Infinity, USA), an Agilent (USA) multichannel interface, and a XWK-III pump. Acetonitrile (75%) was used as the mobile phase at a flow rate of 0.8 mL/min and the column was kept at 40 °C. All measurements were repeated three times.

2.6. Enzyme activity assay

The catalytic activity of free and immobilized PsDAE was determined by measuring the formation of D-allulose from D-fructose. The reaction mixtures in PBS (pH 7.4) contained 10 g/L D-fructose, 1 mM Mg^{2+}, and 0.5 g/L free PsDAE or 20 g/L immobilized PsDAE. The reaction was conducted at 60 °C for 10 min in a final volume of 1 mL. The reactions were stopped by boiling for 5 min, and the amount of the D-allulose produced by PsDAE was assayed using HPLC as described above. All measurements were conducted in triplicate, and the results were expressed as the means ± standard deviation (SD).

To determine the optimal pH of PsDAE for D-allulose production, the reactions were conducted at 60 °C for 10 min across a pH range of 5.5 to 11.0 in different buffers (NaAc-HAc: 4.5–5.5, 4-morpholineethanesulfonic acid (MES): pH 5.5–6.5, PBS: 7.0–8.0, Tris-HCl: 8.5–9.0, 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS): pH 9.5–11.0). The optimal temperature of PsDAE was determined by conducting the enzyme activity assay at temperatures from 30 to 90 °C. The effect of temperature on the stability of free and immobilized enzyme was tested by incubating the enzyme at different temperatures (40–70 °C) in 20 mM PBS buffer (pH 7.4) for 12 h, and analyzing the residual activity every 2 h. To investigate the effects of various metal ions on the activity of PsDAE, the activity assay was supplemented with 1 mM Ca^{2+}, Mg^{2+}, Zn^{2+}, Cu^{2+}, Mn^{2+}, Co^{2+}, or Fe^{2+}. In addition, 1 mM EDTA was added to test the metal-dependence of the enzyme. The activity without adding metal ions was defined as 100%.

The substrate specificity of PsDAE was measured under standard

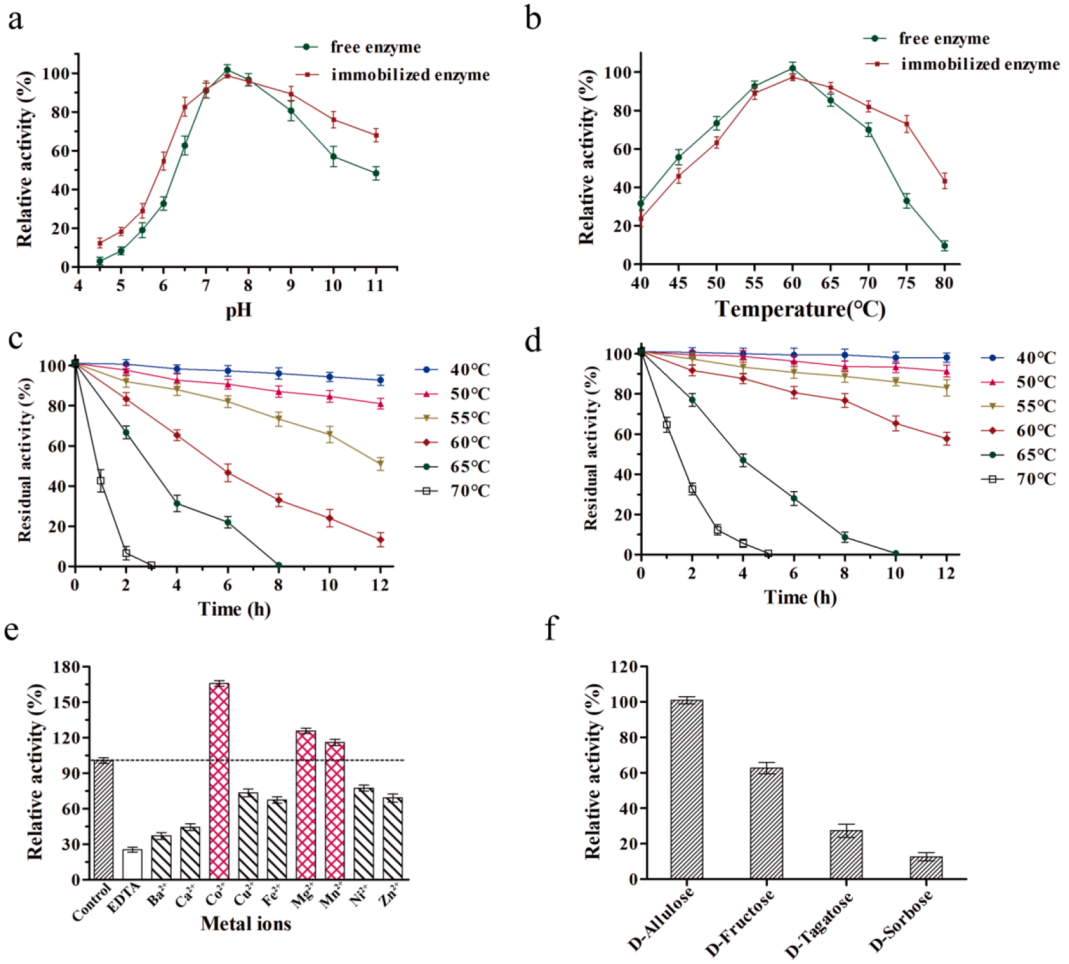

Fig. 2. Biochemical prosperities of free and immobilized PsDAE. pH dependence (a), temperature dependence (b), thermostability analysis of free PsDAE (c), thermostability analysis of immobilized PsDAE (d), the effect of mental ions on activity of PsDAE (e), substrate specificity of PsDAE (f).

reaction conditions using 100 mM D-allulose, D-fructose, D-tagatose, or D-sorbose as the substrate, respectively. One unit of enzyme activity was defined as the amount of the enzyme required to catalyze 1 μmol substrate per minute under standard assay conditions. The kinetic parameters of free and immobilized PsDAE were determined under standard conditions using a range of D-fructose concentrations (10–500 mM). The Michaelis-Menten constant (K_m), turnover number (k_{cat}), and catalytic efficiency (k_{cat}/K_m), were calculated using a Lineweaver Burk plot.

2.7. Production of D-allulose using free and immobilized PsDAE

For the biocatalytic production of D-allulose, solutions containing 50 g/L, 100 g/L, and 500 g/L of D-fructose were treated with 205U isolated PsDAE or 0.2 g epoxy resin ES-103 with immobilized PsDAE (containing 205U PsDAE) in a 10.0 mL reaction mixture comprising 1.0 mM Mg^{2+} in 20 mM PBS buffer (pH 7.4). The reaction mixtures were incubated at 60 °C and samples were drawn at regular time intervals to measure the D-allulose production by HPLC. At the end of the reaction, immobilized PsDAE was washed three times with 20 mM PBS buffer (pH 7.4) and reused for the next cycle of reaction. The reaction procedure was carried out in 15 sequential batches. The reusability of immobilized PsDAE was analyzed based on the residual activity after every cycle, whereby the activity during the initial cycle was defined as 100%.

2.8. Biocatalytic production of D-allulose via a multienzyme cascade

Biosynthetic production of D-allulose from sucrose was performed using a multienzyme cascade system with 500 g/L sucrose in a 10.0 mL reaction mixture. The reaction was conducted at 55 °C for 2 h with 500 g/L sucrose and 0.5 g/L INV (200000 U/g) in NaAc-HAc buffer (pH 4.5) in the first step. Subsequently, the pH of the reaction mixture was adjusted to 7.5 using 1 M NaOH and then 50–200 g/L immobilized GI (50000 U/g) and 20 g/L immobilized PsDAE were added into the reaction system. The reaction was implemented for another 1.5 h, whereby the substrate consumption and product formation were analyzed by HPLC every 30 min.

2.9. Conversion of native high-calorie sugars in fruit juices into D-allulose in situ

Pure mango juice, orange juice, and sugar cane juice were individually treated with INV, GI, and PsDAE via the two-step reaction strategy. In the initial step, 0.5 g/L INV was directly added into 10 mL of the 100% fruit juice. The reaction was carried out at 55 °C for 1 h. In the next step, the pH of the fruit juices was adjusted to 7.5 using 1 M NaOH and then 150 g/L immobilized GI and 20 g/L immobilized PsDAE were added to the fruit juices. The reaction was performed for another 1 h, and D-allulose formation was followed using HPLC every 10 min.

3. Results and discussion

3.1. Multiple sequence alignment

A search in the NCBI database for DAE sequences yielded an unusual epimerase from *Pirellula* sp. SH-Sr6A (PsDAE; GenBank Accession No. AMV33274.1). A phylogenetic tree was constructed based on the protein sequence of PsDAE, together with characterized DAEs and DTEs from other organisms. The phylogenetic tree revealed that PsDAE was closely related to homologs from *Rhodobacter sphaeroides* (GenBank: ACO59490.1) and *Sinorhizobium* sp. (GenBank: WP_069063284.1). Moreover, PsDAE exhibited the highest amino acid sequence identity with DAE from *Sinorhizobium* sp. (26.95%), and DTE from *Rhodobacter sphaeroides* (26.88%) (Fig. S1). This percentage was still low and indicated the uniqueness of PsDAE.

Table 1
Kinetic parameters of free and immobilized PsDAE for D-fructose and D-allulose.

Substrate	Kinetic parameters	Free enzyme	Immobilized enzyme
D-Fructose	K_m (mM)	74.7 ± 2.5	122.4 ± 3.2
	k_{cat} (s^{-1})	47.2 ± 1.3	42.2 ± 1.5
	k_{cat}/K_m ($s^{-1}mM^{-1}$)	0.63	0.34
D-Allulose	K_m (mM)	41.7 ± 1.2	71.1 ± 1.8
	k_{cat} (s^{-1})	63.6 ± 1.7	58.0 ± 1.4
	k_{cat}/K_m ($s^{-1}mM^{-1}$)	1.53	0.82

3.2. Characterization of PsDAE

The coding sequence of PsDAE was codon-optimized, overexpressed in *E. coli* BL21 (DE3) with a His_6 tag, and purified using Ni-affinity chromatography. The molecular weight of PsDAE was estimated based on SDS-PAGE, indicating a size of approximately 32 kDa (Fig. S2).

The biochemical properties of free and immobilized PsDAE were characterized using D-fructose as the substrate (Fig. 2). The HPLC retention times of D-allulose and D-fructose were 8.4 and 10.5 min, respectively (Fig. S3a). PsDAE was optimally active in PBS buffer pH 7.5, and was active in a pH range from 5.0 to 11.0. Notably, immobilized PsDAE showed high activity within a wider pH range (pH 6.5–11.0) than the free enzyme (pH 7.0–9.0) (Fig. 2a). The temperature-activity profile for both the free and immobilized PsDAE revealed an optimal temperature of 60 °C, with more than 80% relative activity between 55 and 65 °C. Moreover, the immobilized PsDAE exhibited higher activities at 60–80 °C than the free enzyme (Fig. 2b). The free enzyme was stable for 12 h at temperatures ranging from 40 to 50 °C. Moreover, PsDAE retained over 50% residual activity after 6 h at 60 °C. However, the enzyme was completely inactivated after 3 h at 70 °C (Fig. 2c). By contrast, other DAEs from the same family, including those from *A. tumefaciens* (Kim, Hyun, Kim, Lee, & Oh, 2006), *C. cellulolyticum* (Mu, Chu, Xing, Yu, Zhou, & Jiang, 2011a), and *C. scindens* (Zhang et al., 2013) were relatively stable below 45–50 °C, and their relative activity decreased significantly at 55 °C. Furthermore, the DAE from *Desmospora* sp. was inactive at temperatures over 60 °C (Xing, 2013). Therefore, PsDAE exhibited higher thermostability than other characterized DAEs. Notably, immobilization of PsDAE onto the epoxy support ES-103B greatly improved its thermal stability, reaching a half-life of 12 h at 60 °C (Fig. 2d). The effects of different divalent metal ions on the activity of free PsDAE are shown in Fig. 2e. PsDAE displayed activity in the absence of a metal cofactor, but its activity was increased in the presence of Co^{2+}, Mg^{2+}, and Mn^{2+} by 1.75-, 1.28-, and 1.17-fold, respectively. These results indicated that PsDAE is a metalloenzyme, similar to other homologous members of the same family (Kim et al., 2006; Mu, Chu, Xing, Yu, Zhou, & Jiang, 2011).

The substrate specificity of PsDAE was investigated using D-allulose, D-fructose D-tagatose, and D-sorbose as substrates. The maximal activity of PsDAE was observed with D-allulose, while the activity decreased for other substrates in the order D-fructose, D-tagatose, D-sorbose (Fig. 2f). This result was similar to the DAEs from *C. cellulolyticum* and *Ruminococcus* sp. (Mu et al., 2011; Zhu et al., 2012). Due to the high specificity of PsDAE for D-allulose, it can be classified as a D-allulose 3-epimerase. The kinetic parameters of the free and immobilized PsDAE for D-allulose and D-fructose were also investigated. For D-allulose, the free and immobilized PsDAE showed K_m 41.7 vs 71.1 mM and k_{cat}/K_m 1.53 vs 0.82 $s^{-1}mM^{-1}$. As for D-fructose, they showed K_m 74.7 vs 122.4 mM and k_{cat}/K_m 0.63 vs 0.34 $s^{-1}mM^{-1}$. These indicated that both free and immobilized PsDAE exhibited higher affinity and catalytic efficiency with D-allulose than D-fructose (Table 1). Notably, the immobilized PsDAE enzyme exhibited a k_{cat} that was nearly the same as that of its free form, revealed that immobilization did not significantly alter the enzymatic properties of PsDAE (Table 1 and Fig. 2).

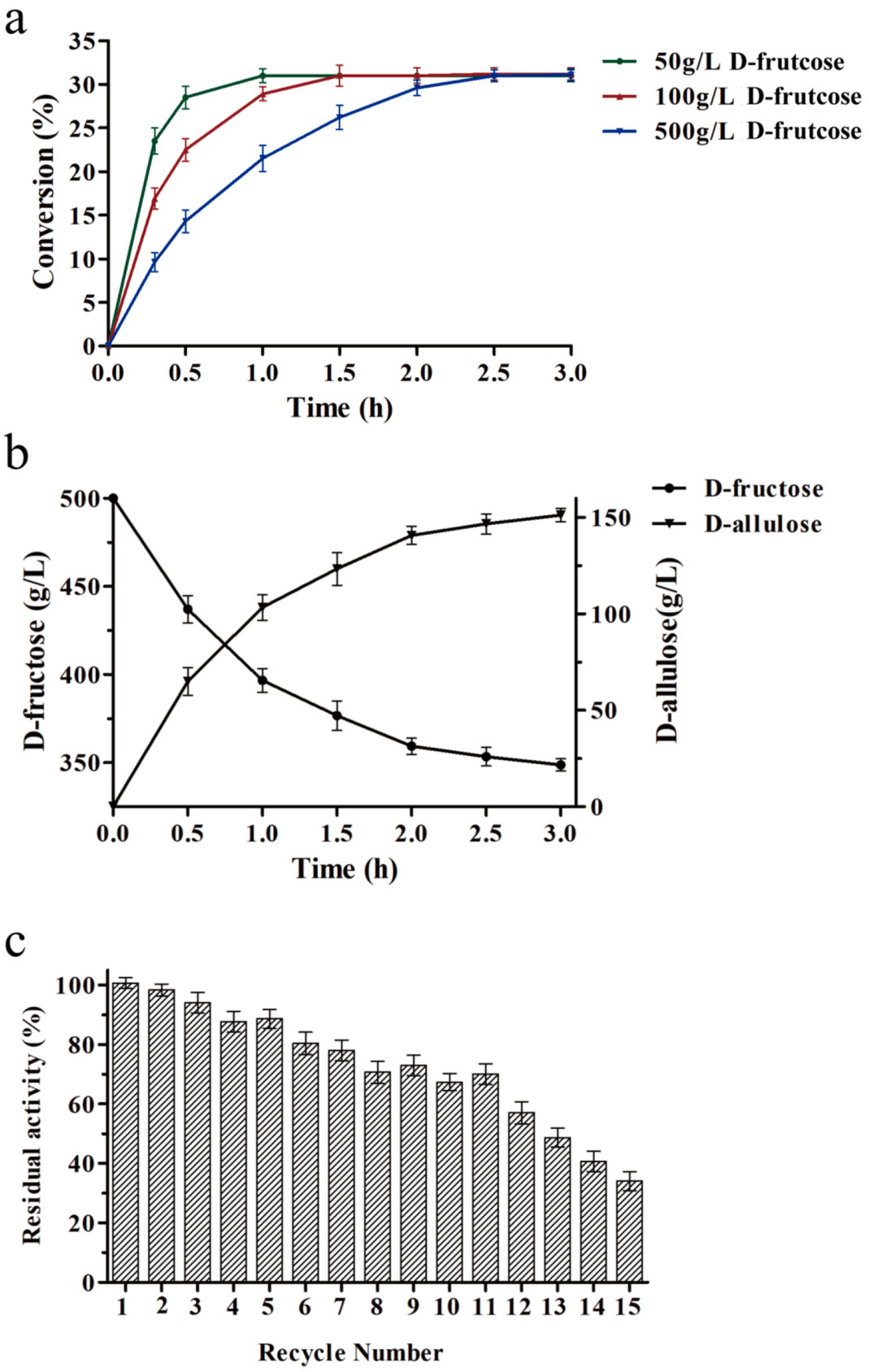

Fig. 3. (a) Bioconversion of D-allulose via isolated PsDTE enzyme with 50, 100, and 500 g/L D-fructose as substrate. Mean and error bars were calculated based on biological triplicate experiments; (b) Bioconversion of D-allulose via immobilized PsDTE enzyme with 500 g/L as substrate; (c) Residual activity of immobilized PsDAE during 15 reuses cycles.

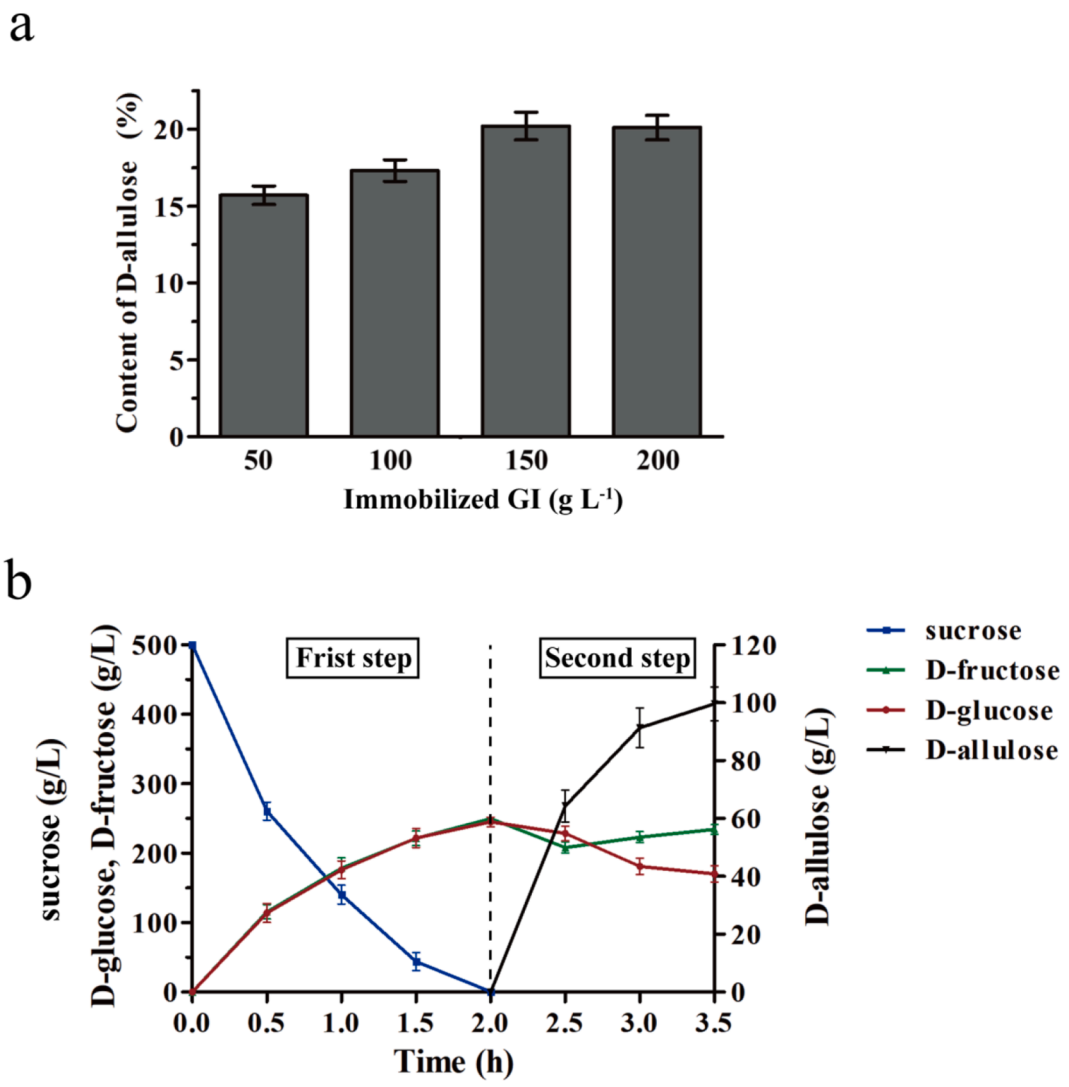

Fig. 4. (a) Effect of the ammount of immobilized glucose isomerase on D-allulose yield. (b) The conversion of sucrose to D-allulose catalyzed by 0.5 g/L INV, 150 g/L immobilized GI, and 20 g/L immobilized PsDAE with two-step strategy.

3.3. D-Allulose production using free PsDAE enzyme

Biocatalytic production of D-allulose was assessed with 50, 100, and 500 g/L D-fructose as the substrate, along with 0.5 g/L PsDAE in a 10.0 mL reaction volume. Under the optimal reaction conditions, PsDAE produced 15.3, 30.4, and 152.7 g/L D-allulose from 50, 100, and 500 g/L D-fructose, respectively. This corresponds to an equilibrium ratio of 30:70 between D-allulose and D-fructose (Fig. 3a), which was higher than what was reported for other enzymes from the same family, i.e. *P. cichorii* DTE (20:80) (Itoh, Okaya, Khan, Tajima, & Izumori, 1994) and *R. sphaeroides* DTE (23:77) (Zhang et al., 2009), but lower than that of *A. tumefaciens DAE* (32.9%) (Kim et al., 2006) and *Staphylococcus aureus* (38.9%) (Zhu et al., 2019).

3.4. D-Allulose production using immobilized PsDAE

Enzyme immobilization on covalent supports with epoxy groups provides a good strategy to prevent the shedding and leakage of enzymes in biocatalytic processes (Ai, Yang, Li, Shi, Wang, & Jiang, 2014), in which the epoxy groups of polymer resins can covalently bind with the amino groups of the enzymes. In this study, we immobilized PsDAE using epoxy resin ES-103B and determined its catalytic activity in the conversion of D-fructose into D-allulose. The immobilized PsDAE generated 150 g/L D-allulose from 500 g/L D-fructose, corresponding to a conversion ratio of about 30% after 3 h. This result indicated that PsDAE immobilized onto epoxy resin ES-103B had a similar bioconversion rate to free enzyme (Fig. 3b). Next, the operational stability and reusability of immobilized PsDAE were investigated via consecutive cycles of repeated reactions. The relative activity of immobilized DAE barely declined during 5 consecutive cycles, and it retained 80% of its initial activity after 11 cycles of repeated reactions (Fig. 3c). Jung *et al.* reported that a DAE enzyme immobilized onto graphene oxide retained more than 50% of the initial activity after 5 continuous cycles, but lost more than 80% of the initial activity after 10 cycles (Samir R. Dedania, Patel, Patel, Akhani, & Patel, 2017). Dedania *et al.* reported that DAE immobilized onto titanium dioxide lost 80% of its initial activity after 9 consecutive cycles of epimerization reactions (Dedania, Patel, Soni, & Patel, 2020). Yang *et al.* reported that the catalytic rate of immobilized cells of DAE barely decreased in 25 consecutive cycles and still retained 80% after 36 cycles of repeated reactions (Yang et al., 2019). Compared to these earlier reports, the PsDAE enzyme immobilized onto the epoxy support ES-103B showed excellent reusability, indicating its great application potential for industrial D-allulose production.

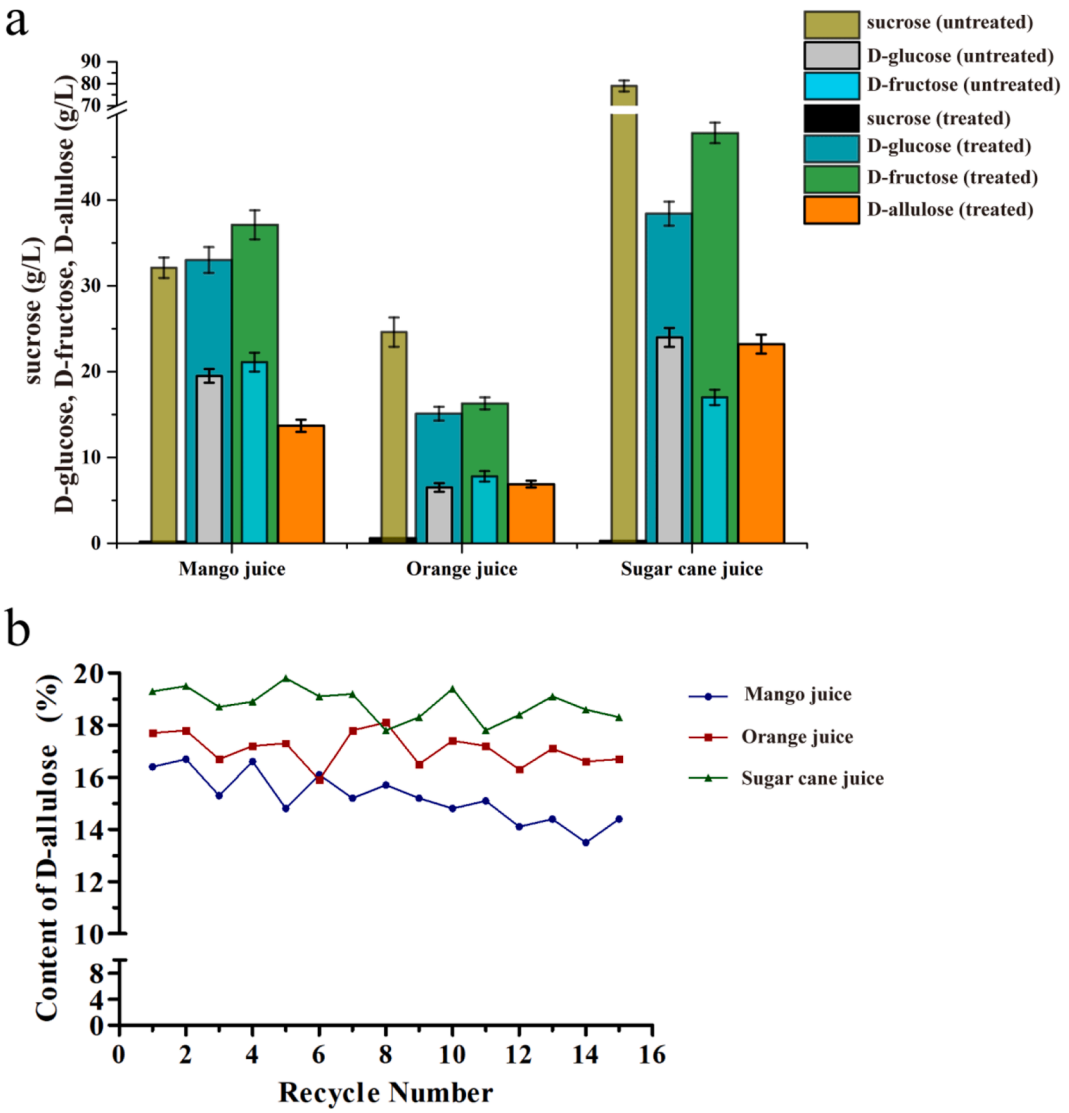

Fig. 5. D-Allulose synthesis in fruit juices catalyzed by INV and immobilized GI and PsDAE with a two-step strategy. (a) The content of sucrose, D-frucose and D-allulose in untreated and treated fruit juices, respectively; (b) The effect of consecutive cycle reaction on the conversion of high-calorie sugar to D-allulose via immobilized GI and PsDAE within 15 reuses cycles.

3.5. Production of D-allulose from sucrose via a multienzyme cascade

We designed a multienzyme cascade combining invertase, D-glucose isomerase, and PsDAE to produce D-allulose from sucrose. The reaction was analyzed by HPLC, and the retention times of D-glucose and sucrose were 13.9 and 19.7 min, respectively (Fig. S3b). Notably, INV showed high catalytic activity under acidic conditions at pH 4.2–4.5, while GI and PsDAE were inactivated under these conditions, having maximal catalytic activity at slightly alkaline pH of 7.5. Initially, sucrose hydrolysis was performed simultaneously with epimerization of D-fructose using INV, GI, and PsDAE. However, only a low yield (32.5 g/L) of D-allulose was obtained due to discordant reaction conditions (Fig. S4). Therefore, the one-pot strategy was not a viable approach to produce D-allulose.

Consequently, we developed a two-step strategy in which sucrose was initially hydrolyzed by INV to produce D-fructose and D-glucose. This reaction was completed within 2 h, yielding 250 g/L of D-fructose and D-glucose. Subsequently, the pH of the reaction mixture was adjusted to 7.5, and 50–200 g/L of immobilized GI and 20 g/L immobilized PsDAE were added to convert D-glucose and D-fructose to D-allulose. The highest yield of D-allulose from sucrose was obtained using 150 g/L immobilized GI (Fig. 4a). This reaction system yielded 101 g/L D-allulose, and the D-allulose content among total monosaccharides reached 20.2% after 1.5 h (Fig. 4b). This was a significantly higher yield than that reported in a previous study using immobilized cells expressing DAE and INV, which produced 75 g/L D-allulose from 500 g/L sucrose (Yang et al., 2019). Thus, the highest yield of D-allulose was obtained via a two-step strategy catalyzed by INV followed by immobilized GI and PsDAE.

3.6. Application of the two-step strategy for D-allulose production in fruit juices in situ

Fruit juices often contain high concentrations of sucrose, D-glucose, and D-fructose, which are considered high-calorie sugars that promote obesity and associated negative health consequences (Wojcicki & Heyman, 2012). In the present study, to convert high-calorie sugar of fruit juices into the functional alternative D-allulose, a variety of fruit juice containing sucrose, D-glucose, and D-fructose, such as mango juice, orange juice, and sugar cane juice were subjected to treatment using the two-step approach utilizing INV, GI, and DAE.

The sugar content in the initial fruit juices (untreated sample) and

fruit juices subjected to treatment using the multienzyme cascade system (treated sample) was determined by HPLC. Under the optimal reaction conditions, the INV combined with immobilized GI and PsDAE enzymes was able to effectively transform the high-calorie sugars in the different juices into D-allulose. The percentage of D-allulose among total monosaccharides in mango juice, orange juice, and sugar cane juice reached 16.4% (11.9 g/L), 17.7% (6.9 g/L), and 19.3% (23.2 g/L), respectively (Fig. 5a). The concentrations of D-fructose and D-glucose were increased due to the hydrolysis of sucrose in juice. D-Fructose was further converted to D-allulose (low-calorie) and thus the total kcal value of fruit juices was thought to be decreased. Furthermore, after hydrolysis of sucrose in the first step, the immobilized GI and PsDAE enzymes were reused for up to 15 cycles without exhibiting an unacceptable loss of activity. As shown in Fig. 5b, after fifteen successive use cycles, the content of D-allulose among total monosaccharides in mango juice, orange juice, and sugar cane juice remained at approximately 16, 17, and 19%, respectively. Considering the food safety, no metals have been added in reaction system for bioconversion of D-allulose in furit juice, although both GI and PsDAE are metal dependent enzymes. These results confirmed the high application potential of the immobilized PsDAE for converting high-calorie sugars in fruit juices into D-allulose, offering functional products for diabetics and other special consumers. Future work will be focused on the directed evolution of PsDAE to improve its thermostability and bioconversion efficiency for the production of D-allulose.

4. Conclusions

A novel thermostable D-allulose 3-epimerase from *Pirellula* sp. SH-Sr6A was successfully overexpressed, purified and characterized. Furthermore, PsDAE was immobilized onto an epoxy support as a reusable biocatalyst for the production of D-allulose from D-fructose with high bioconversion efficiency. Finally, multienzyme cascade system was established to efficiently produce D-allulose from sucrose via a two-step strategy, and was applied to produce 16–19% D-allulose among total monosaccharides in various fruit juices in situ. This study offers a viable approach for the industrial production of functional fruit juices containing D-allulose to satisfy the increasing health consciousness of consumers.

CRediT authorship contribution statement

Chao Li: Conceptualization, Methodology, Investigation, Data curation, Writing - original draft. **Lei Li:** Conceptualization, Methodology, Investigation. **Zhiyuan Feng:** Data curation, Writing - original draft. **Lijun Guan:** . **Fuping Lu:** Supervision, Funding acquisition, Supervision, Funding acquisition. **Hui-Min Qin:** Supervision, Writing - review & editing, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Availability of data and materials

The data collected upon which this article is based upon are all included in this manuscript and the Additional files associated with it.

Ethics approval and consent to participate

No animals or human subjects were used in the above research.

Consent for publication

Our manuscript does not contain any individual data in any form.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2021.129746.

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