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Production of D-psicose from D-fructose by whole recombinant cells with high-level expression of D-psicose 3-epimerase from *Agrobacterium tumefaciens*

Chang-Su Park,[1,‡] Chul-Soon Park,[2,‡] Kyung-Chul Shin,[2] and Deok-Kun Oh[2,*]

Department of Food Science and Technology, Catholic University of Daegu, Gyeongbuk, Gyeongsan 712-702, Republic of Korea[1] and Department of Bioscience and Biotechnology, Konkuk University, 1 Hwayang-dong, Gwangjin-gu, Seoul 143-701, Republic of Korea[2]

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The specific activity of recombinant *Escherichia coli* cells expressing the double-site variant (I33L-S213C) D-psicose 3-epimerase (DPEase) from *Agrobacterium tumefaciens* was highest at 24 h of cultivation time in Terrific Broth (TB) medium among the media tested. The contents of crude protein and DPEase in recombinant cells at 24 h were 37.0 and 8.6% (w/w), respectively, indicating that the enzyme was highly expressed. The reaction conditions for the production of D-psicose from D-fructose by whole recombinant cells with the highest specific activity were optimal at 60°C, pH 8.5, 4 g/l cells, and 700 g/l D-fructose. Under these conditions, whole recombinant cells produced 230 g/l D-psicose after 40 min, with a conversion yield of 33% (w/w), a volumetric productivity of 345 g/l/h, and a specific productivity of 86.2 g/g/h. These are the highest conversion yield and volumetric and specific productivities of D-psicose from D-fructose by cells reported thus far.

[**Key words:** *Agrobacterium tumefaciens*; High-level expression; D-Psicose; D-Psicose 3-epimerase; Recombinant *Escherichia coli*; Whole cell reaction]

The International Society of Rare Sugars (ISRS) defines rare sugars as monosaccharides and their derivatives that rarely occur in nature. Rare sugar such as D-psicose has recently receive much attention owing to their many uses, including uses as low-calorie sweeteners and bulking agents (1–5), as immunosuppressants in allogeneic liver transplantation in rat (6), as potential inhibitors of various glycosidases (7), in ischemia reperfusion injury of the rat liver (8), and in segmented neutrophil production without other detrimental clinical effects (9). D-Psicose (D-allulose), a carbon-3 epimer of D-fructose, is present in small quantities as a non-fermentable constituent of cane molasses. This rare sugar is absorbed poorly in the digestive tract (10), has zero energy for growth, is a helpful sweetener used as an aid for weight reduction (5), suppresses hepatic lipogenic enzyme activity (11), and is nontoxic (1).

D-Psicose has mainly been produced from D-fructose via the reactions of enzymes, including D-tagatose 3-epimerases (DTEases) from *Pseudomonas cichorii* (12) and *Rhodobacter sphaeroides* (13) and D-psicose 3-epimerases (DPEases) from *Agrobacterium tumefaciens* (14), *Ruminococcus* sp. (15), *Clostridium bolteae* (16), *Clostridium cellulolyticum* (17), *Clostridium scindens* (18), *Clostridium* sp. (19), and *Desmospora* sp. (20). As an alternative method, D-psicose can be produced by whole cell reactions, which have some advantages over enzyme reactions. Whole cells show greater stability and resistance to environmental perturbations than enzymes. Moreover, cells eliminate the need for purification steps, such as cell lysis, precipitation, and dialysis, and therefore the reactions are more commercially feasible (21,22). D-Psicose production by whole cell reactions has been reported for only two recombinant *Escherichia coli* cells. DPEases from *C. bolteae* (16) and *C. cellulolyticum* (17) are expressed in these cells. However, the productivity of D-psicose by these cells is low for industrial applications. Moreover, to the best of our knowledge, the optimization of reaction conditions for D-psicose production using cells has not yet been attempted.

In the present study, the expression of the double-site (I33L-S213C) variant of *A. tumefaciens* DPEase in *E. coli* was maximized by determining medium and cultivation time. The reaction conditions of whole recombinant cells with maximal expression, including pH, temperature, and cell and substrate concentrations, were optimized for the production of D-psicose from D-fructose. The optimized reaction conditions resulted in increased productivity of D-psicose.

MATERIALS AND METHODS

Bacterial strains and plasmid *E. coli* ER2566 (New England Biolabs, Hertfordshire, UK) strain was used as host cells, and an expression vector was derived from the pET-24a(+) plasmid (Novagen, Madison, WI, USA) and a double-site mutant gene of *A. tumefaciens* ATCC 33970 DPEase. The mutant gene was cloned between the *Nco*I and *Pst*I restriction sites as described previously (23). The expressed enzyme was a double-site variant (I33L-S213C) of *A. tumefaciens* DPEase.

Culture media and conditions Luria-Bertani (LB) contained 10 g/l tryptone, 5 g/l yeast extract, and 10 g/l NaCl. Terrific Broth (TB) medium consisted of 5 g/l glycerol, 12 g/l tryptone, 24 g/l yeast extract, 23.1 g/l KH_2PO_4, and 125.4 g/l K_2HPO_4. Super Optimal Broth (SOC) medium comprised 3.6 g/l glucose, 20 g/l tryptone, 5 g/l yeast extract, 0.5 g/l yeast extract, 0.18 g/l KCl, 2 g/l $MgCl_2$, and 2.4 g/l $MgSO_4$. Riesenberg medium contained 20 g/l glucose, 13.5 g/l KH_2PO_4, 4 g/l $(NH_4)_2HPO_4$, 1.7 g/l

* Corresponding author. Tel.: +82 2 454 3118; fax: +82 2 444 5518.
E-mail address: deokkun@konkuk.ac.kr (D.-K. Oh).
‡ The first two authors contributed equally to this study.

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citric acid, 1.4 g/l $MgSO_4 \cdot 7H_2O$, and 10 ml trace metal solution (10 mg/l $FeSO_4 \cdot 7H_2O$, 2.25 mg/L $ZnSO_4 \cdot 7H_2O$, 1 mg/L $CuSO_4 \cdot 7H_2O$, 0.5 mg/l $MnSO_4 \cdot 5H_2O$, 0.23 mg/l $Na_2B_4O_7 \cdot 10H_2O$, 2 mg/l $CaCl_2 \cdot 2H_2O$, and 0.1 g/l $(NH_4) \cdot 6Mo_7O_{24}$) supplemented with 0.2 mg/l biotin, and 4 g/l urea. Antibiotic (20 μg/ml kanamycin) was added to all media.

The recombinant *E. coli* cells for protein expression were cultivated with shaking at 200 rpm in a 2000-ml flask containing 500 ml of culture medium at 37°C with 20 μg/ml kanamycin. When the optical density at 600 nm of the bacterial culture reached 0.6, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.1 mM to induce enzyme expression. The culture was incubated at 16°C with shaking at 150 rpm for 28 h to express the enzyme.

Preparation of crude and purified enzymes The recombinant cells expressing the double-site variant (I33L-S213C) of *A. tumefaciens* DPEase were harvested from the culture broth by centrifugation at 6000 ×*g* for 30 min at 4°C, washed twice with 0.85% NaCl. The washed recombinant cells were resuspended in 50 mM phosphate buffer (pH 7.0) containing 300 mM NaCl and 1 mg/ml lysozyme. The resuspended cells were disrupted by sonication on ice for 2 min. The unbroken cells and cell debris were removed by centrifugation at 13,000 ×*g* for 20 min at 4°C, and the supernatant was filtered through a 0.45-μm filter. The filtrate was used as the crude enzyme of *A. tumefaciens* DPEase. The crude was applied to an immobilized metal ion affinity chromatography (IMAC) cartridge (Bio-Rad, Hercules, CA, USA) equilibrated with 50 mM phosphate buffer (pH 8.0) in a cold room at 4°C. The cartridge was washed extensively with the same buffer, and the bound protein was eluted with a linear gradient between 10 and 500 mM imidazole with a flow rate of 1 ml/min. The eluent was collected and loaded immediately onto a Bio-Gel P-6 desalting cartridge (Bio-Rad), previously equilibrated with 50 mM 4-(2-hydroxyethyl)-1-piperazinepropanesulfonic acid buffer (HEPPS) (pH 8.0). The loaded protein was eluted with 50 mM HEPPS buffer (pH 8.0) at a flow rate of 1 ml/min. The active fractions were collected and dialyzed against 50 mM HEPPS buffer (pH 8.0) at 4°C for 16 h. After dialysis, the resulting solution was used as the purified enzyme. All purification steps using the cartridges were carried out in a cold room at 4°C with a Profinia protein purification system (Bio-Rad).

Determination of crude protein and DPEase concentrations The concentrations of crude protein and purified DPEase were determined by the Bradford method using bovine serum albumin as a standard protein. The concentrations of recombinant cells were determined using a linear calibration curve of optical density at 600 nm versus dry cell weight. To determine the concentration of purified DPEase, the activity of recombinant cells for D-psicose production was converted to the corresponding concentration of purified DPEase.

Determination of specific and total activities for D-psicose production by recombinant cells Recombinant *E. coli* cells were cultivated in the TB medium for 28 h. Samples were withdrawn at several intervals, and the activity for D-psicose production was determined. To determine specific and total activities for D-psicose production by recombinant cells during cultivation, the reactions were performed in 50 mM HEPPS (pH 8.0) buffer containing 9 g/l D-fructose and 0.375 g/l cells at 50°C for 10 min.

Optimization of reaction conditions Unless otherwise stated, all reactions were performed in 50 mM HEPPS (pH 8.5) buffer containing 9 g/l D-fructose and 0.375 g/l cells at 60°C for 10 min. The effects of temperature and pH on D-psicose production were examined from 40 to 70°C while the pH was held constant at 8.5; and the pH was varied from 6.5 to 9.5 using 50 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES) buffer (pH 6.5–7.5), 50 mM HEPPS buffer (pH 7.5–8.5), and 50 mM *N*-cyclohexyl-2-aminoethanesulfonic acid (CHES) buffer (pH 8.5–9.5) while the temperature was held constant at 60°C. The concentrations of cells and substrate were varied from 0.5 to 15 g/l cells at 500 g/l D-fructose and from 100 to 700 g/l D-fructose at 4 g/l cells, respectively. All reactions were performed for 10 min. Time-course reactions for the production of D-psicose from D-fructose by whole recombinant cells and its crude enzyme were conducted in 50 mM HEPPS (pH 8.5) buffer containing 700 g/l D-fructose, 4 g/l cells or 2.8 g/l crude enzyme at 60°C for 60 min. The crude enzyme was obtained from the same concentration of whole recombinant cells.

Homology modeling and substrate docking Homology modeling of double-site variant (I33L-S213C) DPEase from *A. tumefaciens* was performed using Build Homology Models module in the MODELER application of Discovery Studio (DS) 4.0 (Accerlys, San Diego, CA, USA) based on the crystal structure of DPEase from *A. tumefaciens* (Protein data bank [PDB] entry, 2HK0) as a template. Comparative modeling was used to generate the most probable structure of the query protein by aligning it with the template sequence, simultaneously considering spatial restraints, and local molecular geometry. The generated structure was improved by subsequent refinement of the loop conformations by assessing the compatibility of amino acid sequences with the known PDB structures using Protein Health module in DS 4.0. The geometry of the loop region was corrected using the Refine Loop/MODELER, and the best models were selected. The quality of the models was analyzed by PROCHECK (24). Hydrogen atoms were added to the model; these atoms were minimized to have stable energy conformations and to relax the conformation away from close contacts. D-fructose as a substrate was docked into the active-site pocket in the models of double-site variant (I33L-S213C) DPEase from *A. tumefaciens* and DPEase from *C. cellulolyticum* using C-DOCKER module, and a sphere with a radius of 5 Å around the ligand-binding pocket of the enzyme was defined as the active site. Candidate poses were created using random rigid-body rotations, followed by simulated annealing. The structures of the protein, substrate, and their complexes were subjected to energy minimization using the CHARMM force field in DS 4.0 (25). Full-potential final minimization was used to refine the substrate poses. The energy-docked conformation of the substrate was retrieved for postdocking analysis using C-DOCKER module. The substrate orientation giving the lowest interaction energy was chosen for subsequent rounds of docking. An estimation of the binding energy between receptor and ligand was calculated using the equation EnergyBinding = EnergyComplex − EnergyLigand − EnergyReceptor (26).

Analytical methods The concentrations of D-fructose and D-psicose were determined using a Bio-LC system (Dionex ICS-3000, Sunnyvale, CA, USA) with an electrochemical detector and a CarboPac PA1 column. The column was eluted at 30°C with 200 mM sodium hydroxide at a flow rate of 1 ml/min. The gradient was increased to 100 mM between 0 and 10 min, to 200 mM between 10 and 15 min, and then decreased to 100 mM between 15 and 20 min.

RESULTS AND DISCUSSION

Determination of the medium and cultivation time that resulted in the maximal expression of double-site variant DPEase from *A. tumefaciens* in *E. coli* The double-site mutant gene of *A. tumefaciens* DPEase was used instead of the wild-type gene because of its higher thermostability (23). Whole recombinant cells expressing the double-site variant (I33L-S213C) DPEase from *A. tumefaciens* were cultivated in LB, TB, SOC, and Riesenberg media (Figs. 1 and S1). TB, SOC, and Riesenberg media have been used previously to obtain high-level expression and cell density (27–29). TB medium showed the highest cell mass and total activity, followed by LB, SOC, and Riesenberg media. TB medium showed the highest specific activity, followed by LB, Riesenberg, and SOC media. Thus, TB medium was used in whole cell reactions for D-psicose production. The concentrations of recombinant *E. coli* cells and D-psicose showed also highest in TB medium among LB, TB, SOC, SB, and SOB media (28).

The concentrations of cells, total crude protein, and DPEase were measured during cultivation (Fig. 2A). The concentration of cells increased with increasing cultivation time. However, the concentrations of total crude protein and DPEase were maximal at 20 h and 24 h, respectively. The expression level of *A. tumefaciens* DPEase in *E. coli* cells was investigated using SDS-PAGE (Fig. 2B). The molecular mass of DPEase was 33 kDa, and the expression level of

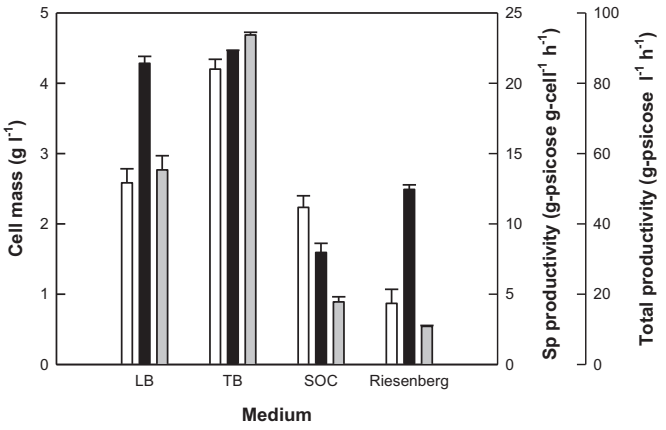

FIG. 1. Cell mass and specific and total activities for D-psicose production of whole recombinant cells expressing the double-site variant (I33L-S213C) DPEase from *A. tumefaciens* grown on LB, TB, SOC, and Riesenberg media for 24 h. Cell mass (open bars), specific activity (closed bars), and total activity (shaded bar). The reactions were performed in 50 mM HEPPS (pH 8.0) buffer containing 9 g/l D-fructose, 0.375 g/l cells at 50°C for 10 min. Data represent the means of three distinct experiments and error bars represent standard deviation.