Research Article
Overexpression of D-psicose 3-epimerase from *Clostridium cellulolyticum* H10 in *Bacillus subtilis* and its Prospect for D-psicose Production

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Abstract: The aim of this study was to overexpress the D-psicose 3-epimerase from *Clostridium cellulolyticum* H10 in food-grade microbe. This gene was cloned and expressed in *Bacillus subtilis*. The results showed that the recombinant protein was soluble, bioactive, and expressed at high levels. The optimum pH and temperature of the enzyme were 8.0 and 50°C, respectively. The activity of the enzyme was not dependent on metal ions; however, some metal ions, such as Co^{2+}, can make the enzyme more thermostable. The Michaelis-Menten constant (K_m) of the enzyme for D-psicose was much lower than that for D-tagatose, suggesting that the optimum substrate of the enzyme is D-psicose. D-Psicose 3-epimerase expressed in the food-grade *B. subtilis* may be used for the industrial production of D-psicose.

INTRODUCTION

D-psicose, a C-3 epimer of D-fructose, is an ultra-low-energy monosaccharide sugar. This rare sugar has great prospect for use as a food sweetener to aid in combating hyperglycemia, hyperlipidemia, diabetes, and obesity (Yagi and Matsuo, 2009). As a rare sugar, D-psicose is very expensive, because it is rarely found in nature and difficult to synthesize chemically. Interconversion between D-fructose and D-psicose by epimerization using enzymes is an efficient and attractive way for the production of D-psicose. Izumori *et al*. (1993) first reported that D-Tagatose 3-Epimerase (DTE) from *Pseudomonas cichorii* converts D-fructose to D-psicose (Ishida *et al*., 1997a; Itoh *et al*., 1994) and can thus be used for the enzymatic production of D-psicose. Then, the DTE gene from *Rhodobacter sphaeroides* and the D-Psicose 3-Epimerase (DPE) genes from *Agrobacterium tumefaciens* and *Clostridium cellulolyticum* have been cloned and expressed in *Escherichia coli* (Kim *et al*., 2006; Mu *et al*., 2011; Zhang *et al*., 2009). A number of studies have found that the DTE/DPE enzyme may be used in the production of D-psicose (Ishida *et al*., 1997b; Kim *et al*., 2008; Takeshita *et al*., 2000).

However, the recombinant DTE/DPE expressed in *E. coli* might not be suitable for the industrial production of D-psicose, particularly when used for food. Its high cost, low expression level, and endotoxin render the *E. coli* expression system unideal for industrial application, especially for the food industry. On the contrary, *Bacillus subtilis* is on the U.S. Food and Drug Administration's GRAS (Generally Regarded as Safe) list, because of its non-pathogenic, well-characterized biochemical and physiological properties (Kunst *et al*., 1997; Schallmey *et al*., 2004). Moreover, *Bacillus strains* are able to produce and secrete large quantities (20-25 g/L) of recombinant enzymes. As a result, *B. subtilis* is considered important in biotechnological applications and in the biotechnological industry (Zhang *et al*., 2007; Zhang *et al*., 2005).

In this study, the protein expression system of *B. subtilis* was established and compared with that of *E. coli*. Specifically, the DPE gene from *C. cellulolyticum* H10 was cloned and expressed in *B. subtilis*. This is the first report of DPE gene expression in a food-grade microorganism, with the properties of the recombinant DPE expressed in *B. subtilis* characterized in detail.

MATERIALS AND METHODS

Materials: D-Fructose, D-psicose, D-tagatose, and D-sorbose were purchased from Sigma Aldrich. Tryptone, yeast extract, agar, and the other chemicals and reagents used in this study were of analytical grade.

Bacterial strains, plasmids, and media: *E. coli* strain BL21 (DE3) and plasmid pET-21a (Novagen) were

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used as the host cell and expression vector, respectively, for DPE gene expression in *E. coli*, whereas *B. subtilis* strain WB600 and plasmid pMA5 were used as the host cell and expression vector, respectively, for DPE gene expression in *B. subtilis*. Both *E. coli* and *B. subtilis* cells were cultivated in Luria-Bertani (LB) medium.

Expression of DPE in *E. coli*: The full-length nucleotide sequence of the DPE gene from *C. cellulolyticum* H10 was synthesized according to the sequence of xylose isomerase domain protein TIM barrel (accession, CP001348; region, 1118193-1119074) by Takara Bio Inc., and the *Nde*I restriction site in the DPE gene was modified based on an *E. coli* codon usage database without changing the amino acid sequence. The *Nde*I and *Eco*RI restriction sites were added to the 5' and 3' termini of the DPE gene.

The pET-21a expression vector and DPE gene in pUC57 were digested with *Nde*I and *Eco*RI. The gene was then ligated with the expression vector to create pET-DPE. The recombinant plasmid was transformed into *E. coli* BL21 (DE3).

The recombinant cells of *E. coli* were cultivated in LB medium containing 100 μg/mL of ampicillin while being shaken at 37°C and 200 rpm. When the OD600 reached 0.8, IPTG was added to the culture medium at 0.5 mM (final concentration) to induce enzyme expression, and the culture was grown at 37°C and 200 rpm for 3 h as well as at 20°C and 100 rpm for 20 h.

Expression of DPE in *B. subtilis*: The full-length sequence of the DPE gene from *C. cellulolyticum* H10 was amplified by PCR using two oligonucleotide primers: DPEBSF (5'-GGAATTCCATATGAAACAT GGTATATAC-3') and DPEBSR (5'-CGGGA TCCTCAGTGGTGGTGGTGGTGGTGGGAGTGTTT ATGACA-3'). The *Nde*I and *Bam*HI restriction sites (underlined) were added to the primer sequences to aid in the construction of the expression vector, and the 6×H is tag was added to the C-terminus of the DPE protein to simplify its purification.

The pMA5 expression vector and PCR product of the DPE gene were digested with *Nde*I and *Bam*HI. The gene was then ligated with the expression vector to create pMA-DPE. The recombinant plasmid was transformed into *B. subtilis* WB600.

The recombinant cells of *B. subtilis* were cultivated in LB medium containing 50 μg/mL of kanamycin while being shaken at 37°C and 200 rpm for 16 h.

Purification of the recombinant DPE: The *B. subtilis* cells expressing DPE were harvested from culture broth by centrifugation at 6,000×g for 10 min at 4°C and then suspended in lysis buffer (25 mM Tris-HCl, 300 mM

NaCl, and 40 mM imidazole, pH 8.0). The suspended cells were disrupted using an APV 2000 high-pressure homogenizer (Denmark) at 900-1,000 bar. The supernatant was obtained by centrifugation at 15,000×g for 30 min at 4°C and filtered through a 0.45-μm filter. The crude extract was applied to a 5-mL HisTrap HP affinity chromatography column (GE Healthcare Biosciences) equilibrated with binding buffer (25 mM Tris-HCl, 300 mM NaCl, and 40 mM imidazole, pH 8.0). The column was washed with the same buffer, and the bound protein was eluted with a linear gradient between 40 and 250 mM imidazole at a flow rate of 1 mL/min. Highly active fractions were pooled and subsequently desalted and concentrated using Amicon Ultra 15 (Millipore). The resulting solution was then loaded onto a Source 15Q anion-exchange chromatography column (GE Healthcare Biosciences) equilibrated with 50 mM sodium phosphate buffer (pH 6.5) and eluted with a linear gradient between 0 and 500 mM NaCl at a flow rate of 0.5 mL/min. After desalting and concentration against 20 mM HEPES buffer (pH 8.0), the resulting solution was used as a purified enzyme. All purification steps were carried out with an ÄKTA Purifier TM 100 system (GE Healthcare Biosciences).

Effects of metal ions, pH and temperature on DPE: The activity of DPE was analyzed by determining the amount of D-psicose obtained from D-fructose. The standard reaction was performed by adding 2.5 μg of the purified DPE into 500 μL of reaction solution containing 20 mM HEPES (pH 8.0) and 50 mM D-fructose and then incubating the mixture at 50°C for 10 min. The reaction was terminated by heating the mixture in boiling water for 5 min. One unit of enzymatic activity was defined as the amount of enzyme that catalyzes the formation of 1 μmol of D-psicose per minute.

Enzymes incubated with 1 and 10 mM LiCl, NaCl, KCl, $CoCl_2$, $MnCl_2$, $NiCl_2$, $MgCl_2$, $BaCl_2$, $ZnCl_2$, $CuCl_2$, or $FeCl_3$ at 20°C for 1 h were used to determine the effects of metal ions on DPE activity. The enzyme reaction was carried out under standard reaction conditions.

The effects of pH on the enzymatic activity of DPE were determined at various pH levels, ranging from 6.5 to 8.5, using 20 mM HEPES buffer. Similarly, the effects of temperature on the enzymatic activity of DPE were determined at various temperatures, ranging from 10 to 70°C. DPE was incubated at various temperatures (35, 40, 45, and 50°C, respectively) for 30, 60, 120, 180, and 240 h, respectively and the residual activities were examined to determine the thermal stability of the enzyme.

Kinetic analysis of DPE: Kinetic analysis was performed at pH 8.0 and 50°C for 10 min in 20 mM HEPES buffer containing D-psicose, D-fructose and D-tagatose as substrates at various concentrations (10, 20, 30, 40, 50, and 100 mM, respectively). The Michaelis-Menten constant (K_m), turnover number (k_{cat}), and catalytic efficiency (k_{cat}/K_m) values were obtained using Lineweaver-Burk plots.

Analysis of carbohydrates: The products of all reactions were determined using High-Performance Liquid Chromatography (HPLC), which was carried out with an Agilent 1200 system (Agilent Technologies). The conditions of HPLC were as follows: one column of Sugar-Pak^{TM} (6.5×300 mm; Waters); deionized H_2O eluent; flow velocity, 0.4 mL/min; column temperature, 80°C; and refractive index detection.

The research was finished on April 2012 at Tianjin Institute of Industrial Biotechnology, CAS.

RESULTS AND DISCUSSION

Overexpression and purification of DPE: The expression level of the recombinant DPE in *E. coli* BL21 was very low. Almost all of the recombinant protein was also found in the form of inclusion bodies whether the cells were cultured at 37°C and 200 rpm or at 20°C and 100 rpm for induction (Fig. 1A).

Hence, the expression system of *B. subtilis* was used to enhance the expression level of DPE. The DPE gene was at the control of the HpaII promoter, which is constitutive and does not need to be induced. The recombinant strain containing DPE gene was cultured at 37°C and 200 rpm for approximately 16 h. Extra bands were found both in the total cell lysate and in the

soluble supernatant of the recombi *B. subtilis* transformed with pMA5-DPE, as revealed by SDS-PAGE (Fig. 1B). The results showed that the DPE gene was expressed at a larger scale and in the form of a soluble enzyme.

Compared with the expression system of *E. coli*, that of *B. subtilis* has many advantages and is more suitable for the expression of DPE, which is used in the industrial production of D-psicose. First, the DPE was controlled by a constitutive promoter, which does not need inducers, such as toxic IPTG. Second, the yield of recombinant DPE in *B. subtilis* was much higher than that in *E. coli*, and the protein was soluble. Importantly, *B. subtilis* is a food-grade bacterium.

The recombinant DPE was purified through a HisTrap HP affinity chromatography column and a Source 15Q anion-exchange chromatography column. Electrophoresis under reducing conditions (12% SDS-PAGE) revealed a highly pure enzyme of recombinant DPE, and its molecular mass was approximately 33 kDa (Fig. 2A). The molecular mass of the native enzyme was estimated as 132 kDa by 10% native PAGE (Fig. 2B), suggesting that the DPE was a 33-kDa tetramer, the same as that from *A. tumefaciens*.

Effects of metal ions, pH and temperature on DPE: The effects of metal ions on DPE activity are depicted in Fig. 3. The DPE from *C. cellulolyticum* H10 showed activity without any metal ions. On the other hand, the results indicated that Co^{2+} and Mn^{2+} increased enzymatic activity. The DPE activity in the presence of 1 mM Co^{2+} was approximately 160% of that without metal ions. When the reaction reached equilibrium, the conversion rate of D-fructose was 27.61%±0.53% in the absence of metal ions, but the value was
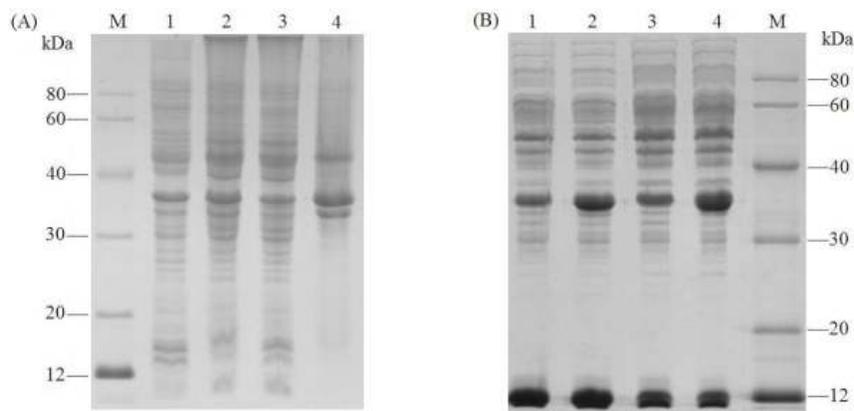
Fig. 1: (A) SDS-PAGE analysis of DPE expressed in *E. coli*. Lane M, protein markers; lane 1, total cell lysate of negative control; lane 2, total cell lysate of *E. coli* with DPE; lane 3, the soluble supernatant of negative control; lane 4, the inclusion body of *E. coli* with DPE (B) SDS-PAGE analysis of DPE expressed in *B. subtilis*. Lane M, protein markers; lane 1, total cell lysate of negative control; lane 2, total cell lysate of *B. subtilis* with DPE; lane 3, the soluble supernatant of negative control; lane 4, the soluble supernatant of *B. subtilis* with DPE
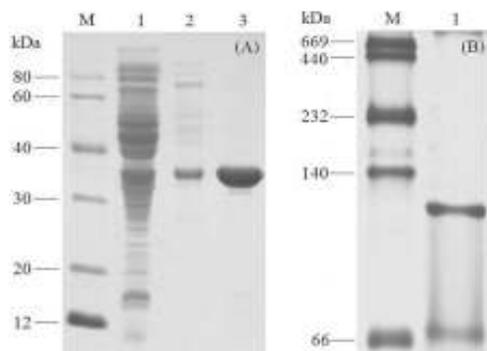
Fig. 2: (A) Purification of the recombinant DPE expressed by *B. subtilis*. Lane M, protein markers; lane 1, crude extract; lane 2, after Ni column; lane 3, after source 15Q column (B) Native PAGE analysis of the purified recombinant DPE. Lane M, protein markers; lane 1, the purified recombinant DPE
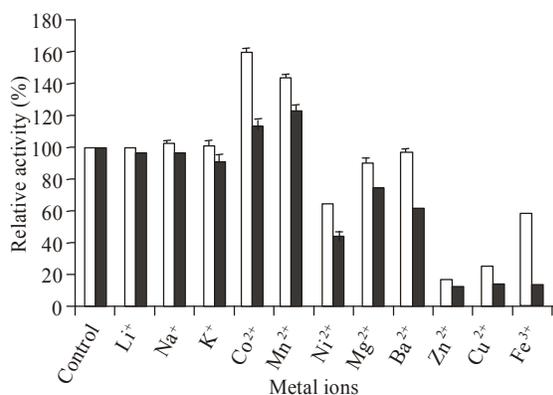
Fig. 3: Effect of metal ions on the activity of DPE
The blank bar shows the relative activity at 1 mM metal ions, whereas the gray bar shows the relative activity at 10 mM metal ions

Fig. 4: Effects of pH (A) and temperature (B) on the activity of DPE

28.18%±0.32% in the presence of 1 mM Co^{2+}. These data suggest that the activity of the DPE was not dependent on metal ions, similar to the case for *P. cichorii* DTE but not that for *A. tumefaciens* DPE. The metal ions Co^{2+} and Mn^{2+} likely increased the stability of the DPE, thereby enhancing enzymatic activity.

The optimum pH and temperature for the recombinant DPE were 8.0 and 50°C, respectively, as with previously identified DTEs and DPEs (Fig. 4).

On the other hand, the stability of the DPE from *C. cellulolyticum* H10 was enhanced by either Co^{2+} or Mn^{2+}. Without metal ions, the enzyme was very stable below 35°C, but its activity decreased by 50% after DPE was incubated at 50°C for 30 min (Fig. 5A). When 1 mM Co^{2+} or Mn^{2+} was added to the enzyme solution, the stability of the DPE significantly increased. The effect of Co^{2+} on the stability of the DPE was stronger than that of Mn^{2+} and the residual activity of the DPE

was greater than 50% after being incubated at 50°C for 4 h in the presence of 1 mM Co^{2+} (Fig. 5B). Moreover, the half-life of the DPE with 1 mM Co^{2+} at 50°C was 265 min, which was about 11-fold to that without any metal ions. These findings indicate that Co^{2+} was not required in the catalytic reaction, but it stabilized the enzyme.

Substrate specificity of DPE: The specificity of the DPE from *C. cellulolyticum* H10 was investigated in different substrates (D-psicose, D-fructose, D-tagatose and D-sorbose). Their relative activities are shown in Table 1, with the data indicating that D-psicose is the optimum substrate of the enzyme.

The results of the kinetic analysis performed on DPE are also shown in Table 1. The DPE showed the lowest K_m value and the highest k_{cat}/K_m values for D-psicose among the three substrates. The lowest K_m value for D-psicose indicates that this enzyme had the best affinity for D-psicose, suggesting that the enzyme from *C. cellulolyticum* H10 was a DPE. However, the kcat value for D-psicose was close to that for D-fructose and higher than that for D-tagatose, which suggests the mechanism of the reaction to D-fructose/D-psicose was a little different from that to
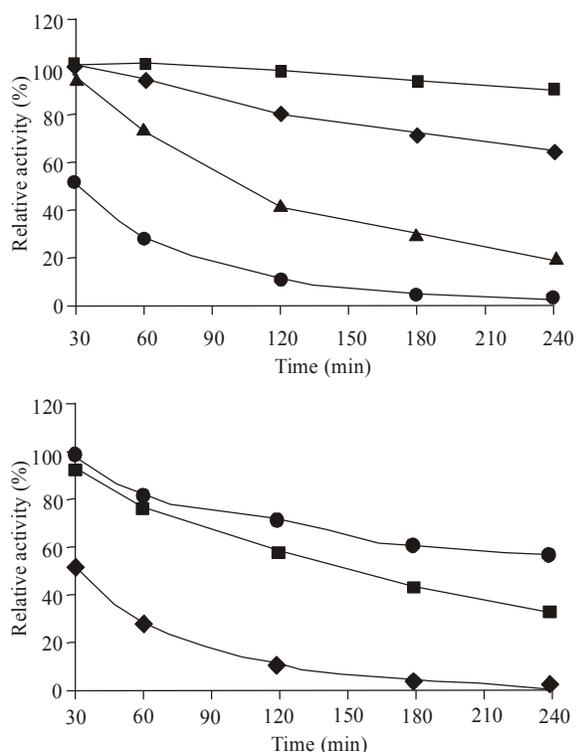
Fig. 5: (A) Effect of temperature on the enzyme stability of DPE. The reactions were performed at 35°C (▪), 40°C (♦), 45°C (▲) and 50°C (●) without any metal ions (B) Effect of metal ions on the enzyme stability of DPE. The reactions were performed at 50°C in the presence of 1 mM Co^{2+} (●) and 1 mM Mn^{2+} (▪)

Table 1: Activities and kinetic parameters of DPE from *C. cellulolyticum* for different ketoses

Substrate	Relative activity (%)	K_m (mM)	K_{cat} (min^{-1})	k_{cat}/k_m (min^{-1}/mM)
D-psicose	100	19.24±0.21	9854.63±63.19	512.18±8.76
D-fructose	38	106.44±1.84	9037.88±45.41	84.91± 1.62
D-tagatose	8.27	371.95±5.63	1610.12±12.36	4.33±0.25
D-sorbose	3.70	-	-	-

D-tagatose/D-sorbose. Chan *et al*. (2012) reported that the configuration of substrate and the number of hydrogen bonds formed by *C. cellulolyticum* DPE and substrate led to the different epimerization activities. D-Psicose formed more hydrogen bonds with DPE than D-fructose did; for the D-tagatose and D-sorbose binding, the hydrogen bond between Y6 of DPE and O6 of sugars could not be formed. So the enzyme has the highest relative activity to D-psicose.

The prospect of DPE for D-psicose production: The DPE from *C. cellulolyticum* is suitable for large-scale production of D-psicose. First, the activity of the DPE is high, and the reaction reaches equilibrium faster. For large-scale D-psicose production, 500 g/L D-fructose syrup was used as substrate and 24 μg/mL of enzyme was added; the conversion yield of D-psicose was 24.83%. Second, the DPE is expressed at high levels in the food-grade *B. subtilis*, and much of the recombinant protein is soluble. Third, the activity of the DPE does not require any metal ions, such as Co^{2+} or Mn^{2+}, which may be harmful to human health. Although the stability of the enzyme is not strong in the absence of Co^{2+}, it might be improved through enzyme immobilization.

CONCLUSION

The recombinant DPE was expressed in *B. subtilis* successfully, making the industrial production of D-psicose practicable. The expressed enzyme was purified and characterized. The activity of the DPE does not require any metal ions, but its stability can be improved by Co^{2+}. D-Psicose was produced at 120 g/L from 500 g/L of D-fructose using the new enzyme. These findings indicate that *C. cellulolyticum* DPE may be used for efficient conversion of D-fructose to D-psicose.

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