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# Cloning, Expression, and Characterization of a D-Psicose 3-Epimerase from *Clostridium cellulolyticum* H10

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**ABSTRACT:** The noncharacterized protein ACL75304 encoded by the gene Ccel_0941 from *Clostridium cellulolyticum* H10 (ATCC 35319), previously proposed as the xylose isomerase domain protein TIM barrel, was cloned and expressed in *Escherichia coli*. The expressed enzyme was purified by nickel-affinity chromatography with electrophoretic homogeneity and then characterized as D-psicose 3-epimerase. The enzyme was strictly metal-dependent and showed a maximal activity in the presence of $Co^{2+}$. The optimum pH and temperature for enzyme activity were 55 °C and pH 8.0. The half-lives for the enzyme at 60 °C were 6.8 h and 10 min when incubated with and without $Co^{2+}$, respectively, suggesting that this enzyme was extremely thermostable in the presence of $Co^{2+}$ but readily inactivated without metal ion. The Michaelis−Menten constant ($K_m$), turnover number ($k_{cat}$), and catalytic efficiency ($k_{cat}/K_m$) values of the enzyme for substrate D-psicose were estimated to be 17.4 mM, 3243.4 $min^{-1}$, and 186.4 mM $min^{-1}$, respectively. The enzyme carried out the epimerization of D-fructose to D-psicose with a conversion yield of 32% under optimal conditions, suggesting that the enzyme is a potential D-psicose producer.

**KEYWORDS:** D-psicose 3-epimerase, *Clostridium cellulolyticum*, D-psicose, purification, characterization

## INTRODUCTION

Rare sugars were defined by the International Society of Rare Sugars (ISIR) as monosaccharides and their derivatives that exist in nature but are present in only limited quantities (First International Symposium of ISRS, Takamatsu, Japan, 2002). D-Psicose (D-ribo-2-hexulose or D-allulose), an epimer of D-fructose at the C3 position, is considered to be a rare sugar.[1] This rare sugar is absorbed poorly in the digestive tract,[2] has zero energy for growth, and is a useful low-calorie sweetener.[1,3,4] It can be also used as an inhibitor of hepatic lipogenic enzyme[5] and intestinal α-glycosidase for reducing body fat accumulation.[6] It exists in extremely small quantities in commercial carbohydrate or agricultural products and is difficult to chemically synthesize. Interconversion between D-fructose and D-psicose by epimerization using D-tagatose 3-epimerase (DTEase) family enzymes has been focused on as a commercially attractive enzymatic reaction for the production of D-psicose.[7−10]

The genes of DTEase family enzymes are widely presumed in various microorganisms and show relative sequence similarity, indicating that the enzymes possibly have a common catalytic mechanism; however, there are only several studies that characterize the enzymes. DTEase was first purified and characterized from *Pseudomonas cichorii*,[11,12] showing that it can efficiently catalyze the epimerization of ketohexoses, and *P. cichorii* DTEase has been successfully used in D-psicose mass bioproduction.[7,8] Kim et al. reported another characterization study of the putative DTEase from *Agrobacterium tumefaciens* that can specifically catalyze the interconversion of D-fructose and D-psicose.[13] Due to its high substrate specificity for D-psicose, it was named D-psicose 3-epimerase (DPEase). Soon after, the three-dimensional structures of *A. tumefaciens* DPEase[14] and *P. cichorii* DTEase[15,16] were solved one after another, and the active sites were well identified. Recently, we have characterized a third DTEase family enzyme, the DTEase from *Rhodobacter sphaeroides* SK011,[17] and deposited the enzyme as GenBank accession no. ACO59490.[18] Although these three DTEase family enzymes can all produce D-psicose from D-fructose, a low homology in amino acid sequence was found among them.[18]

In this study, the noncharacterized protein previously proposed as xylose isomerase domain protein TIM barrel, which is encoded by a hypothetical open reading frame Ccel_0941 in the genome of the *Clostridium cellulolyticum* H10 (ATCC 35319), was expressed in *Escherichia coli* and then characterized as DPEase. The enzymatic properties, substrate specificity, and kinetic parameters were also investigated and compared to other DTEase family enzymes.

## MATERIALS AND METHODS

**Chemicals and Reagents.** Taq DNA polymerase, deoxynucleoside triphosphate (dNTP), chemicals for PCR, T4 DNA ligase, and plasmid miniprep kit were obtained from Takara (Dalian, China). The resin for protein purification, the Chelating Sepharose Fast Flow, was obtained from GE (Uppsala, Sweden). The pET-22b(+) expression vector was obtained from Novagen (Darmstadt, Germany). Electrophoresis reagents were purchased from Bio-Rad. Isopropyl β-D-1-thiogalactopyranoside (IPTG) and all chemicals used for enzyme assays and characterization were at least of analytical grade obtained from Sigma (St. Louis, MO) and Sinopharm Chemical Reagent (Shanghai, China). Oligonucleotides were synthesized by Sangon Biological Engineering Technology and Services (Shanghai, China).

**Bacterial Strains and Culture Conditions.** *C. cellulolyticum* H10 (ATCC 35319) was obtained from American Type Culture Collection (ATCC, USA) and cultivated as described previously.[19]

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The *E. coli* strains DH5α and BL21(DE3) were purchased from Sangon Biological Engineering Technology and Services (Shanghai, China) and used as host strains for transformation of plasmid and expression. They were routinely cultured in Luria−Bertani (LB) medium supplemented with ampicillin (50 μg/mL) at 37 °C with rotary shaking at 200 rpm.

**Gene Cloning and Protein Expression.** The genomic DNA of *C. cellulolyticum* H10 was extracted by using the DNAzol reagent according to the manufacturer's protocol. The whole genome of this strain was sequenced and released in GenBank (NCBI accession no. NC_011898), which revealed that there is a putative gene, Ccel_0941, encoded the putative protein, ACL75304. The protein was proposed as the product xylose isomerase domain protein TIM barrel. According to the nucleotide sequence of the putative gene, forward (5′-CGGG-TGCATATGAAACATGGTATATACTACG-3′) and reverse primer (5′-GCTGGTCTCGAGGGAGTGTTTATGACATTCT-3′) were designed to introduce the *Nde*I and *Xho*I restriction sites (underlined).

**Table 1. Amino Acid Sequence Homology of DTEase Family Enzymes**

| sequence A | length (Aa) | sequence B | length (Aa) | amino acid homology (%) |
|---|---|---|---|---|
| CC-DPEase[a] | 293 | AT-DPEase[b] | 289 | 60 |
| CC-DPEase | 293 | PC-DTEase[c] | 290 | 41 |
| CC-DPEase | 293 | RS-DTEase[d] | 295 | 28 |
| CC-DPEase | 293 | TM0416p[e] | 270 | 23 |
| AT-DPEase | 289 | PC-DTEase | 290 | 38 |
| AT-DPEase | 289 | RS-DTEase | 295 | 30 |
| AT-DPEase | 289 | TM0416p | 270 | 17 |
| PC-DTEase | 290 | RS-DTEase | 295 | 31 |
| PC-DTEase | 290 | TM0416p | 270 | 20 |
| RS-DTEase | 295 | TM0416p | 270 | 18 |

[a] *C. cellulolyticum* DPEase, GenBank accession no. ACL75304. [b] *A. tumefaciens* DPEase, GenBank accession no. AAL45544. [c] *P. cichorii* DTEase, GenBank accession no. BAA24429. [d] *R. sphaeroides* DTEase, GenBank accession no. ACO59490. [e] *T. maritima* TM0416p.

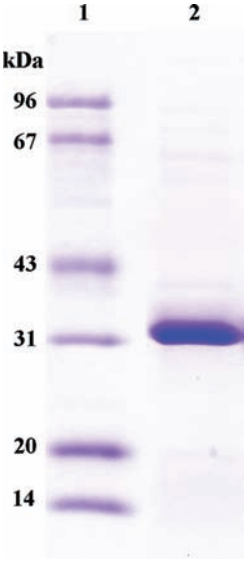


**Figure 2.** SDS-PAGE analysis of the purified *C. cellulolyticum* DPEase (lane 2) and protein markers stained with Coomassie blue.

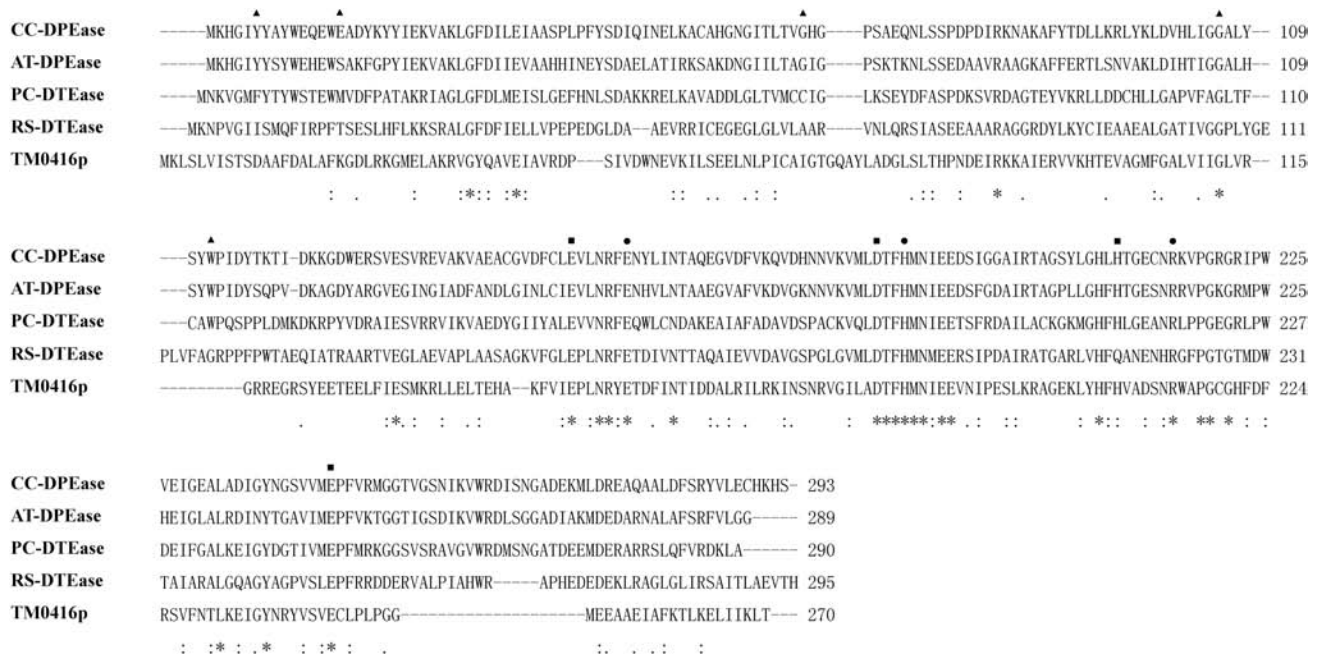


**Figure 1.** Multiple sequence alignment of DTEase family enzymes and their homologues. The amino acid sequence for DPEase from *C. cellulolyticum* H10 (CC-DPEase; GenBank accession no. ACL75304) was aligned with those of *A. tumefaciens* DPEase (AT-DPEase; AAL45544), *P. cichorii* DTEase (PC-DTEase; BAA24429), *R. sphaeroides* DTEase (RS-DTEase; ACO59490), and *T. maritima* TM0416p. The alignment was performed using the ClustalW2 program (http://www.ebi.ac.uk/Tools/clustalw2/index.html). Amino acid residues that are identical in all of the displayed sequences are marked by asterisks (*); strongly conserved or weakly conserved residues are indicated by colons (:) or dots (.), respectively. The residues involved in the metal coordinating site (■), the residues responsible for the interaction between the enzyme and O1, O2, and O3 of D-fructose (●), and the residues providing a hydrophobic environment around the substrate around the 4-, 5-, and 6-position of D-fructose (▲) are symbolized according to the crystal structures of *A. tumefaciens* DPEase[14] and *P. cichorii* DTEase.[15]

PCR amplification was performed by Taq Plus DNA polymerase for 35 cycles consisting of 94 °C for 1 min, 60 °C for 1 min, and 72 °C for 1 min, followed by a final extension step of 10 min at 72 °C. The 0.9 kb PCR-amplified fragment was purified and then digested with *Nde*I and *Xho*I. The digested fragment was inserted into the pET-22b(+) vector with *Nde*I and *Xho*I sites; therefore, an in-frame fusion 6×histidine-tag sequence at the C-terminus was provided in the reconstructed plasmid, named pET-*Cc*-dpe, and the pET-*Cc*-dpe was transformed into *E. coli* BL21(DE3).

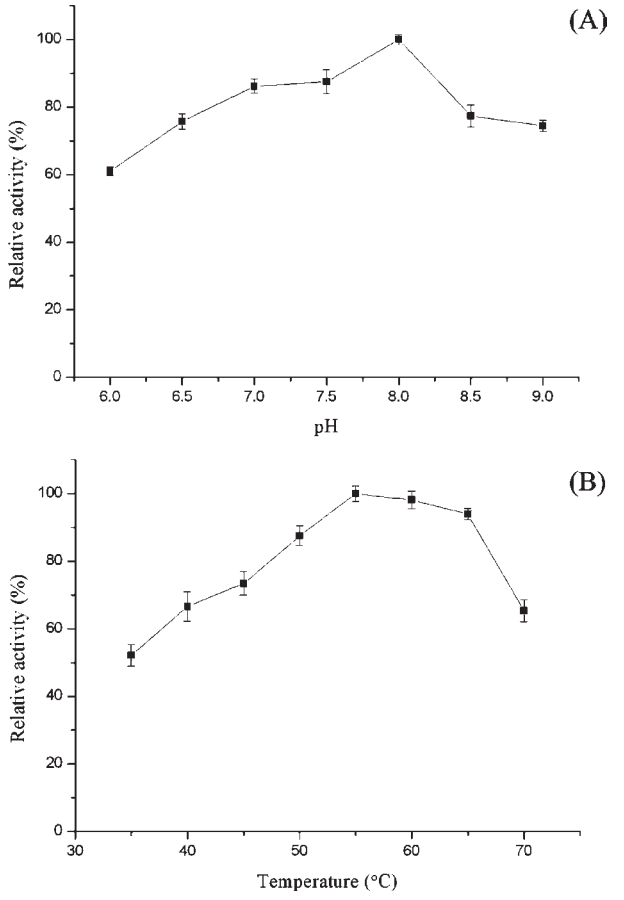


**Figure 3.** Effect of pH (A) and temperature (B) on *C. cellulolyticum* DPEase. Values are the mean of three replications ± standard deviation.

The recombinant *E. coli* for protein expression was cultivated with shaking (200 rpm) in 400 mL of LB medium containing 50 μg/mL ampicillin at 37 °C. When the $OD_{600}$ reached 0.6, IPTG was added at 1 mM, and DPEase was induced and overexpressed at 30 °C for 5 h and then harvested by centrifugation at 4 °C for 10 min at 10000*g*. The enzyme was expressed as 6×histidine-tagged fusion protein, which was available for affinity chromatography.

**Protein Purification.** To purify the recombinant DPEase, the centrifuged cell pellets were resuspended in lysis buffer (50 mM Tris-HCl, 100 mM NaCl, pH 7.5) and disrupted by sonication at 4 °C for 6 min (pulsations of 3 s, amplify 90) using a Vibra-Cell 72405 sonicator, and cell debris was removed by centrifugation (20000*g*, 20 min, 4 °C). The cell-free extract was applied onto a Chelating Sepharose Fast Flow resin column (1.0 cm × 10.0 cm), previously chelating $Ni^{2+}$, and equilibrated with a binding buffer (50 mM Tris-HCl, 500 mM NaCl, pH 7.5). Unbound proteins were eluted from the column with a washing buffer (50 mM Tris-HCl, 500 mM NaCl, 50 mM imidazole, pH 7.5). Then, the DPEase was eluted from the column with an elution buffer (50 mM Tris-HCl, 500 mM NaCl, 500 mM imidazole, pH 7.5). The active fractions were pooled and dialyzed overnight against 50 mM Tris-HCl buffer (pH 7.5) containing 10 mM ethylenediaminetetraacetic acid (EDTA) for 48 h at 4 °C. Subsequently, the enzyme was dialyzed against 50 mM EDTA-free Tris-HCl buffer (pH 7.5).

**Enzyme Assay.** The activity was measured by the determination of the amount of produced D-psicose from D-fructose. The reaction mixture of 1 mL contained D-fructose (50 g/L), Tris-HCl buffer (50 mM, pH 8.0), 0.1 mM $Co^{2+}$, and 0.5 μM enzyme. The reaction mixture were incubated at 55 °C for 2 min, and the reaction was stopped after 10 min by boiling. The generated D-psicose was determined by the HPLC method. One unit of enzyme activity was defined as the amount of enzyme catalyzing the formation of 1 μmol of D-psicose/min at pH 8.0 and 55 °C.

**Effect of Temperature and pH.** The optimum temperature of enzyme activity was measured by assaying the enzyme samples over the range of 35−70 °C for 2 min. Two buffer systems, sodium phosphate (50 mM, pH 6.0−7.0) and Tris-HCl (50 mM, pH 7.5−9.0), were used for measuring the optimum pH of enzyme activity. The thermal stability of the enzyme was studied by incubating the enzyme in Tris-HCl buffer (50 mM, pH 8.0) at various temperatures. At given time intervals, samples were withdrawn and the residual activity was measured under standard assay conditions. To determine the pH stability, the enzyme was incubated at pH 6.0−9.0 at 4 °C for up to 2 h, and the remaining enzyme activity was measured at time intervals under standard assay conditions.

**Table 2. Comparison of Biochemical Properties of DTEase Family Enzymes**

| | strain for enzyme source | | | |
|---|---|---|---|---|
| | *C. cellulolyticum* | *A. tumefaciens*[13] | *P. cichorii*[12] | *R. sphaeroides*[18] |
| optimal temperature (°C) | 55 | 50 | 60 | 40 |
| optimal pH | 8.0 | 8.0 | 7.5 | 9.0 |
| metal ion required | $Co^{2+}$ | $Mn^{2+}$ | no | $Mn^{2+}$ |
| substrate with highest specificity | D-psicose | D-psicose | D-tagatose | D-fructose |
| half-life | 24 min (55 °C, without metal ion)<br>10 min (60 °C, without metal ion)<br>9.5 h (55 °C, with 0.1 mM $Co^{2+}$)<br>6.8 h (60 °C, with 0.1 mM $Co^{2+}$) | 63.5 min (50 °C)<br>8.90 min (55 °C)<br>3.99 min (60 °C) | NR[a] | approximately 60 min (50 °C) |
| equilibrium ratio between D-psicose and D-fructose | 32:68 (55 °C) | 32:68 (30 °C)<br>33:67 (40 °C) | 20:80 (30 °C) | 23:77 (40 °C) |

[a] NR, not reported.

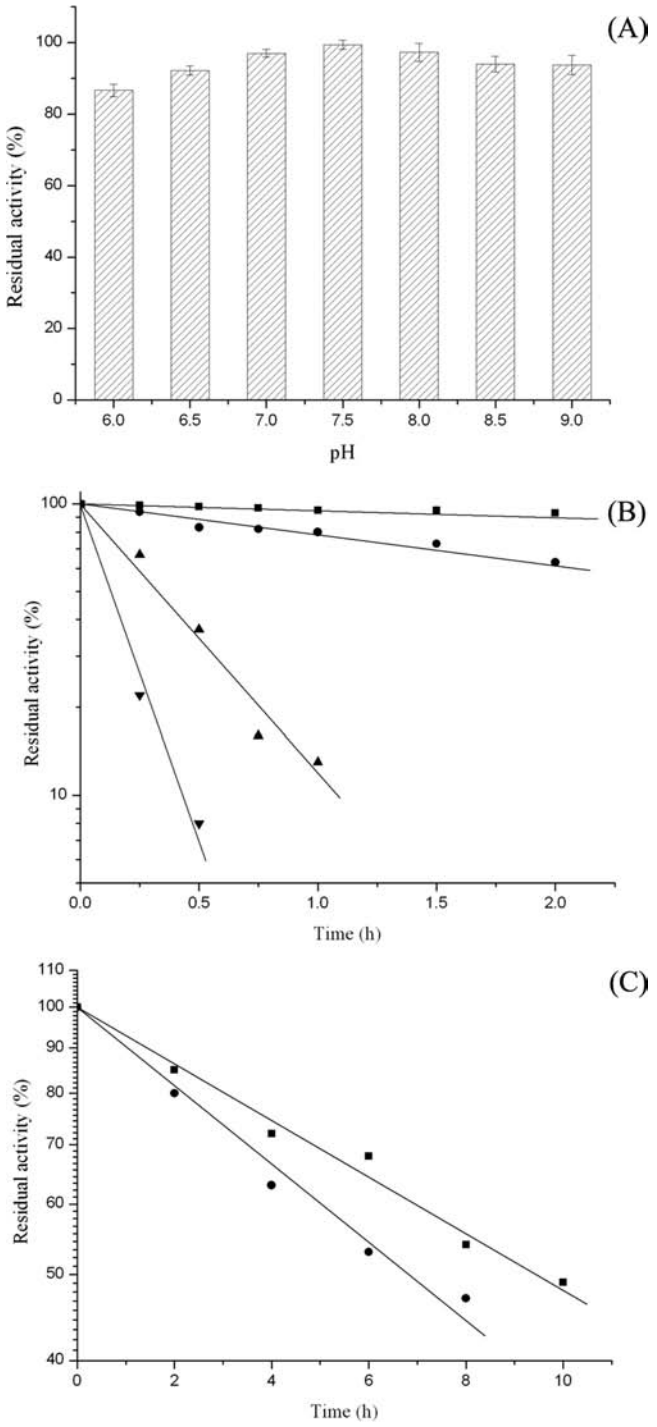


**Figure 4.** Effect of pH and temperature on the stability of *C. cellulolyticum* DPEase. (A) Effect of pH. The pH stability was investigated by preincubating the enzyme buffers of different pH values at 4 °C for 2 h and measuring the remaining activity at 55 °C and pH 8.0. (B) Effect of temperature on enzyme stability without metal ion. The temperature stability was investigated by exposing the enzyme at 45 °C (■), 50 °C (●), 55 °C (▲), and 60 °C (▼) for different time intervals at pH 8.0. (C) Effect of temperature on enzyme stability with 0.1 mM $Co^{2+}$. The temperature stability was investigated by exposing the enzyme at 55 °C (■) and 60 °C (●) for different time intervals at pH 8.0 in the presence of 0.1 mM $Co^{2+}$.

**Effect of Various Metallic Ions.** Before the effects of various metallic ions on *C. cellulolyticum* DPEase activity were studied, the enzyme solution purified by affinity chromatography was dialyzed against 50 mM Tris-HCl buffer (pH 7.5) containing 10 mM EDTA for 48 h at 4 °C. Subsequently, the enzyme was dialyzed against 50 mM EDTA-free Tris-HCl buffer (pH 7.5). Enzyme activity was then assessed as described above in the presence of the following ions at 1 mM: $Co^{2+}$, $Mn^{2+}$, $Mg^{2+}$, $Fe^{2+}$, $Ni^{2+}$, $Zn^{2+}$, and $Cu^{2+}$, respectively.

**Determination of Kinetic Parameters.** Kinetic parameters of *C. cellulolyticum* DPEase were determined in 50 mM Tris-HCl buffer (pH 8.0) containing 0.1 mM $Co^{2+}$ and 5−200 mM substrate for reaction at 55 °C. The enzyme reactions were stopped after 10 min by boiling, and the amount of D-psicose was determined by the HPLC assay. Kinetic parameters, such as the Michaelis−Menten constant ($K_m$) and turnover number ($k_{cat}$) values for substrates, were obtained using the Lineweaver−Burk equation and quantification of enzyme concentration.

**Analytical Methods.** The concentrations of D-fructose and D-psicose were analyzed by HPLC equipped with a refractive index detector and a $Ca^{2+}$-carbohydrate column (Waters Sugar-Pak 1, Waters Corp., Milford, MA), which was eluted with water at 85 °C and 0.4 mL/min. Protein concentration was determined according to the method of Bradford using bovine serum albumin as a standard. SDS-PAGE was carried out according to the method of Laemmli. Gels (12% w/v polyacrylamide) were stained with Coomassie Brilliant Blue and destained with an aqueous mixture of 10% (v/v) methanol/10% (v/v) acetic acid.

## RESULTS AND DISCUSSION

**Amino Acid Sequence Alignment of DTEase Family Enzymes.** DTEase family enzymes catalyze C3 epimerization of various ketohexoses, including rare sugars that exhibit physiological functions and promote beneficial health for humans. They have been confirmed as the key enzymes for the bioproduction of valuable rare sugars and, in particular, commercially used for the production of D-psicose from D-fructose. Although many genes of the DTEase family enzymes have been predicted from various sources (GeneBank accession no. ACO59490, BAA24429, F72381, AL939126, BAB50266, AAL45542, NP_865388, BX294153, NC_005126, and NP_435986), to date, however, only three enzymes have been conclusively characterized as DTEase family enzymes, including *P. cichorii* DTEase,[12] *A. tumefaciens* DPEase,[13] and *R. sphaeroides* DTEase.[18] A DTEase-related protein (TM0416p) from the hyperthermophilic bacterium *Thermotoga maritima* was purified and its crystal structure resolved; however, it was suggested that the substrate specificity of TM0416p was likely to differ substantially from those of DTEase family enzymes, and it was also not confirmed whether the protein could convert D-fructose to D-psicose.[20] The amino acid sequence alignment among *P. cichorii* DTEase, *A. tumefaciens* DPEase, *R. sphaeroides* DTEase, and *T. maritima* TM0416p was developed, and the results revealed that the protein sequence homology of DTEase family enzymes previously characterized was not significant, with the highest value of 38% similarity between *P. cichorii* DTEase and *A. tumefaciens* DPEase, as shown in Table 1 and Figure 1.

The *C. cellulolyticum* H10 genomic sequence was recently completed and deposited as GenBank accession no. NC_011898. The protein with ProteinID ACL75304 encoded by the hypothetical open reading frame Ccel_0941 was supposed as the product xylose isomerase domain protein TIM barrel in the *C. cellulolyticum* H10 genome. However, the ACL75304 was found to be highly homologous in amino acid sequence with *A. tumefaciens* DPEase (60% identity), as shown in Table 1 and Figure 1. Also, ACL75304 exhibited 41, 28, and 23% amino acid sequence similarities with *P. cichorii* DTEase, *R. sphaeroides* DTEase, and *T. maritima* TM0416p, respectively.

**Table 3. Comparison of the Epimerization Activities of DTEase Family Enzymes**

| | relative activity (%) | | | |
|---|---|---|---|---|
| ketose | *C. cellulolyticum* DPEase[a] | *A. tumefaciens* DPEase[13] | *P. cichorii* DTEase[12] | *R. sphaeroides* DTEase[18] |
| D-psicose | 100 ± 0.4 | 100 | 100 | 100 |
| D-tagatose | 4.9 ± 0.4 | 33.7 | 167 | 109.9 |
| D-fructose | 48.2 ± 2.3 | 50.7 | 33.3 | 181.3 |
| D-sorbose | 0.9 ± 0.2 | 0.7 | 33.3 | 34.1 |
| D-fructose-6-phosphate | 0 | 0 | 0 | 0 |
| D-ribulose-5-phosphate | 0 | 0 | 0 | 0 |

[a] Experiments were carried out at 55 °C and pH 8.0 in the presence of 0.1 mM $Co^{2+}$. Values are the mean of three replications ± standard deviation.

**Table 4. Comparison of the Kinetic Parameters of DTEase Family Enzymes**

| | *C. cellulolyticum* DPEase[a] | | | *A. tumefaciens* DPEase[13] | | | *P. cichorii* DTEase[12] | | |
|---|---|---|---|---|---|---|---|---|---|
| substrate | $k_{cat}$ ($min^{-1}$) | $K_m$ (mM) | $k_{cat}/K_m$ ($mM^{-1}$ $min^{-1}$) | $k_{cat}$ ($min^{-1}$) | $K_m$ (mM) | $k_{cat}/K_m$ ($mM^{-1}$ $min^{-1}$) | $k_{cat}$ ($min^{-1}$) | $K_m$ (mM) | $k_{cat}/K_m$ ($mM^{-1}$ $min^{-1}$) |
| D-psicose | 3243.5 ± 56.5 | 17.4 ± 0.2 | 186.4 ± 1.9 | 2381 | 12 | 205 | NR[b] | NR | NR |
| D-tagatose | 184.8 ± 9.6 | 244 ± 5 | 0.75 ± 0.01 | 270 | 762 | 0.35 | NR | 55 | NR |
| D-fructose | 3354.5 ± 47.2 | 53.5 ± 1.8 | 62.7 ± 1.5 | 2068 | 24 | 85 | NR | NR | NR |

[a] This study. Experiments were carried out at 55 °C and pH 8.0 in the presence of 0.1 mM $Co^{2+}$; values are the means of three replications ± standard deviation. [b] NR, not reported.

The crystal structures of *A. tumefaciens* DPEase[14] and *P. cichorii* DTEase[15] have both been determined. The $Mn^{2+}$ ion is coordinated by Glu150, Asp183, His209, and Glu244 in *A. tumefaciens* DPEase[14] or with Glu152, Asp185, His211, and Glu246 in *P. cichorii* DTEase,[15] and these residues are found to be strictly conserved in ACL75304 as Glu150, Asp183, His209, and Glu244, respectively. In addition, the amino acid residues responsible for the interaction between the enzyme and O1, O2, and O3 of D-fructose (Glu156, His186, and Arg215 in *A. tumefaciens* DPEase[14] or Glu158, His188, and Arg217 in *P. cichorii* DTEase[15]) are also conserved in ACL75304 as Glu156, His186, and Arg215, respectively. As for the amino acid residues providing a hydrophobic environment around the substrate, ACL75304 is completely conserved with *A. tumefaciens* DPEase,[14] in which Tyr6, Trp14, Gly65, Ala107, Trp112, and Phe246 create a hydrophobic pocket around the 4-, 5-, and 6-positions of D-fructose, but less well conserved with *P. cichorii* DTEase provided by Phe7, Trp15, Cys66, Leu108, Trp113, and Phe248.[15] Due to the high similarities with *A. tumefaciens* DPEase and *P. cichorii* DTEase in amino acid sequence, especially the key amino acid residues of the active site, metal coordinating site, and substrate combinant binding site, ACL75304 is suggested as a possible member of the DTEase family of enzymes.

In this study, we characterized this protein by experimental approaches and confirmed it as DTEase family enzyme with high substrate specificity for D-psicose. Therefore, we named ACL75304 as *C. cellulolyticum* DPEase.

**Expression and Purification of *C. cellulolyticum* DPEase.** To investigate the biochemical properties of *C. cellulolyticum* DPEase, we expressed the enzyme with a C-terminal 6×histidine-tag in *E. coli* BL21(DE3) and purified the protein to homogeneity. Bacteria transformed with the expression vector and induced with IPTG abundantly expressed the 6×histidine-tagged protein. SDS-PAGE analyses on the extracts of *E. coli* BL21(DE3) harboring pET-*Cc*-dpe induced by IPTG, compared to that of the control *E. coli* BL21(DE3) cells harboring plasmid pET-22b(+), revealed the presence of large amounts of protein around 31 kDa, which was in agreement with the predicted molecular mass for the DPEase protein. It was suggested that DPEase could be overexpressed by IPTG induction. The *E. coli* cells were harvested and disrupted by sonification. Cell debris was removed by centrifugation (20000*g*, 20 min, 4 °C). The supernatant was the crude extract of the enzyme, which was then further purified by $Ni^{2+}$-Chelating Sepharose Fast Flow resin. Recombinant DPEase with a C-terminal 6×histidine-tag was purified to electrophoretic homogeneity with a single band on SDS-PAGE by metal chelating affinity chromatography (Figure 2). The enzyme exhibited 32 and 130 kDa as apparent molecular mass through SDS-PAGE and gel filtration, respectively. It was indicated that the enzyme is a tetramer with four identical subunits.

**Effects of Metal Ions on *C. cellulolyticum* DPEase.** The relative activity of *C. cellulolyticum* DPEase was investigated in the presence of several divalent metal ions, which were added at the final concentration of 1 mM. In previous findings, the enzyme activities of *A. tumefaciens* DPEase and *R. sphaeroides* DTEase were enhanced in the presence of metal ions. Interestingly, the *P. cichorii* DTEase does not require any cofactor for its activity.[12] Opposite from *P. cichorii* DTEase, *C. cellulolyticum* DPEase showed null activity in the absence of any ions, which indicated that it is an enzyme with a strict divalent metal ion requirement. *C. cellulolyticum* DPEase showed a maximal activity in the presence of $Co^{2+}$; however, when $Co^{2+}$ was replaced with $Mn^{2+}$, $Fe^{2+}$, $Ni^{2+}$, or $Mg^{2+}$, the enzyme activity was reduced to 92, 67, 65, and 38% of that in presence of $Co^{2+}$, respectively. For $Zn^{2+}$ and $Cu^{2+}$, the enzyme activity was barely detected. Therefore, *C. cellulolyticum* DPEase is obviously different from other DTEase family enzymes in metal ion dependence. Every reported DTEase family enzyme could display activity without metal ion, but *C. cellulolyticum* DPEase is strictly metal-dependent. It is interesting that for aldose isomerase or ketose epimerase some

enzymes must require metal ions as cofactors to display activity,[21] some do not require metal ions,[12,22,23] and others could display activity without metal ion, but their activity could be remarkably enhanced by metal ions.[13,18]

**Effects of Temperature and pH on *C. cellulolyticum* DPEase.** Figure 3 shows pH and temperature profiles for *C. cellulolyticum* DPEase activity. The optimum pH and temperature for enzyme activity are 8.0 and 55 °C. Comparison of the biochemical properties of *C. cellulolyticum* DPEase and other reported DTEase family enzymes is shown in Table 2. All four of these enzymes' optimal pH values were found to be weakly alkaline, ranging from 7.5 to 9.0, and optimal temperatures ranged from 40 to 60 °C.

Furthermore, pH and thermal stabilities of the enzyme were investigated. More than 90% activity of the purified *C. cellulolyticum* DPEase was retained at pH 6.5−9.0 after incubation at 4 °C for 2 h (Figure 4A), indicating that the *C. cellulolyticum* DPEase was relatively stable under neutral and weakly alkaline conditions. In the absence of metal ion, the *C. cellulolyticum* DPEase was relatively stable below 45 °C but decreased above 50 °C with increasing incubation time (Figure 4B). The results were very similar to those of *A. tumefaciens* DPEase.[13]

In addition, we found that $Co^{2+}$ could remarkably enhance the thermostability of *C. cellulolyticum* DPEase (Figure 4C), and it was the first study about the effect of metal ion on the thermostability of DTEase family enzymes. When incubated with 0.1 mM $Co^{2+}$, the half-lives for the enzyme could extend to 9.5 and 6.8 h at 55 and 60 °C, respectively, which indicated that the enzyme is extremely thermostable in the presence of $Co^{2+}$. However, without metal ion, the half-lives for the enzyme were only 24 and 10 min at 55 and 60 °C, respectively, so *C. cellulolyticum* DPEase requires $Co^{2+}$ for both enzyme activity and thermostability.

**Substrate Specificity and Enzyme Kinetics.** Epimerization activities of *C. cellulolyticum* DPEase with various substrates were carried out (Table 3). It was concluded that the substrate specificity of *C. cellulolyticum* DPEase was prior to D-psicose and decreased for other substrates in the following order: D-fructose, D-tagatose, and D-sorbose. As reported for other DTEase family enzymes,[12,13,18] *C. cellulolyticum* DPEase also could not catalyze D-fructose-6-phosphate or D-ribulose-5-phosphate. The substrate specificity of *C. cellulolyticum* DPEase was similar to that of *A. tumefaciens* DPEase,[13] but different from that of *P. cichorii* DTEase and *R. sphaeroides* DTEase. The optimal substrates of *P. cichorii* DTEase[12] and *R. sphaeroides* DTEase[18] were D-tagatose and D-fructose, respectively. Interestingly, *C. cellulolyticum* DPEase activity on D-psicose was 10-fold higher than for D-tagatose, which showed a higher multiple than that of *A. tumefaciens* DPEase at 2.7-fold.[13] However, the epimerization activity on D-tagatose was 1.67- and 1.1-fold higher than that on D-psicose for *P. cichorii* DTEase[12] and *R. sphaeroides* DTEase,[18] respectively.

The kinetic parameters of *C. cellulolyticum* DPEase were determined (Table 4). The $K_m$, $k_{cat}$, and catalytic efficiency ($k_{cat}/K_m$) values of *C. cellulolyticum* DPEase for substrate D-psicose were estimated to be 17.4 mM, 3243.4 $min^{-1}$, and 186.4 mM $min^{-1}$, respectively. The $k_{cat}/K_m$ of *C. cellulolyticum* DPEase for D-psicose was 2.97- and 248-fold higher than those for D-fructose and D-tagatose, respectively, indicating that the enzyme was not DTEase, but DPEase. These results showed a relatively similar tendency with *A. tumefaciens* DPEase,[13] in which the $k_{cat}/K_m$ for D-psicose was 2.41- and 586-fold higher than for D-fructose and D-tagatose, respectively. It was concluded that we had characterized the second DPEase after *A. tumefaciens* DPEase. However, the catalytic efficiency of other DTEase family enzymes was not reported.

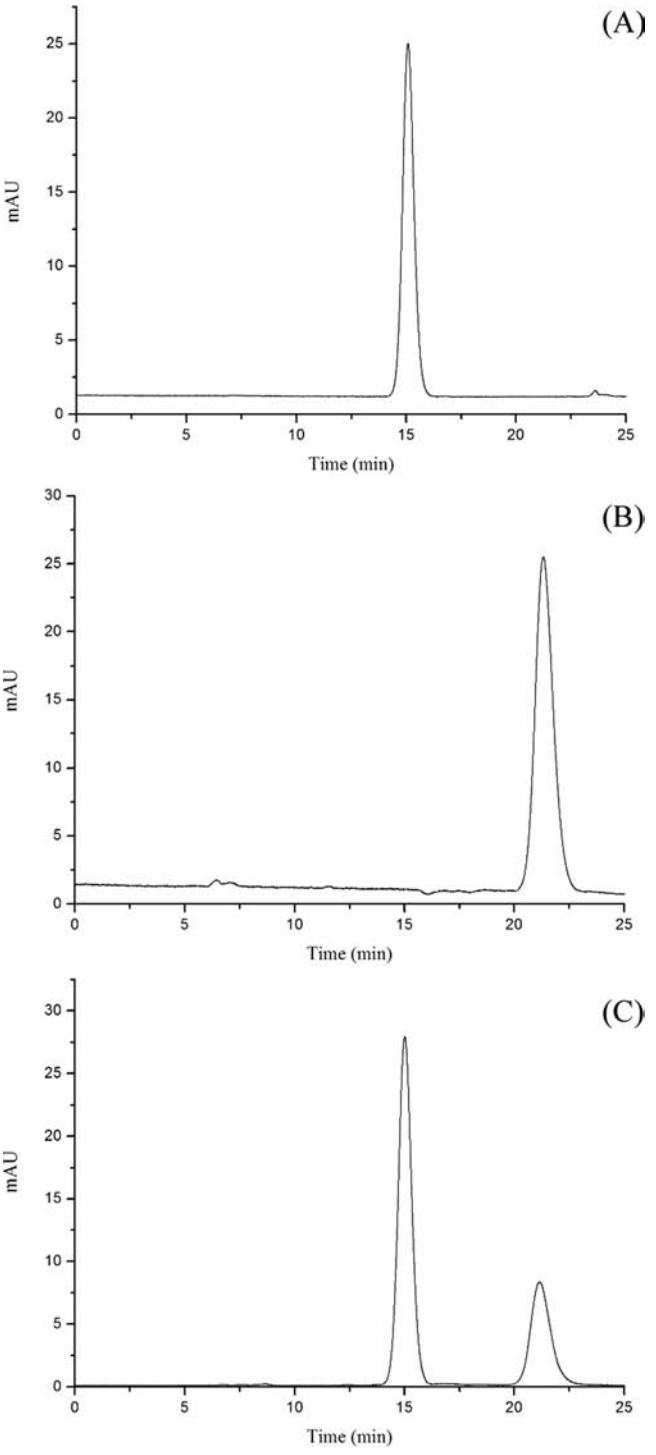


**Figure 5.** HPLC analysis profiles of epimerization products and equilibrium ratio between D-fructose and D-psicose: (A, B) standards of D-fructose and D-psicose, respectively; (C) reaction products after the reaction equilibrium.

**Bioconversion of D-Psicose by *C. cellulolyticum* DPEase.** The equilibrium ratio between D-psicose and D-fructose of *C. cellulolyticum* DPEase was determined to be 32:68 when D-psicose and D-fructose were at a total concentration of 50 g/L, with three

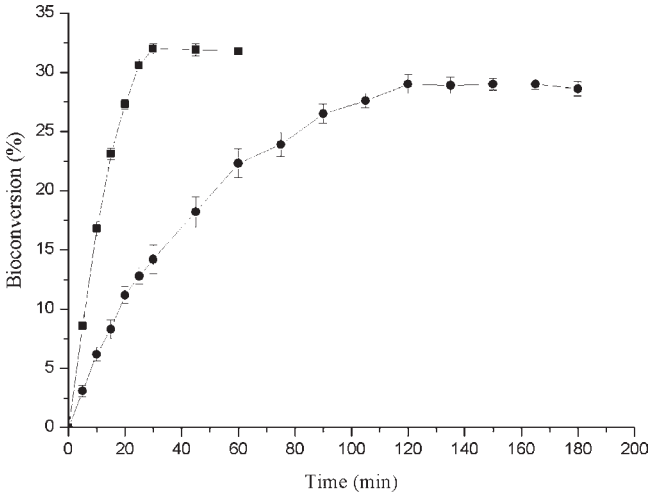


**Figure 6.** Bioconversion of D-fructose into D-psicose by *C. cellulolyticum* DPEase: (■) turnover ratio in the 5 mL reaction system containing 50 g/L D-fructose with 0.1 mM $Co^{2+}$ and 0.5 $\mu$M enzyme at 55 °C and pH 8.0; (●) ratio in the 1 L reaction system containing 750 g/L D-fructose with 0.1 mM $Co^{2+}$ and 2 g dry cell wt/L recombinant *E. coli* whole cells harboring *C. cellulolyticum* DPEase at 55 °C and pH 8.0. Values are the mean of three replications ± standard deviation.

initial ratios of 0:100, 50:50, and 100:0, and reacted at 55 °C and pH 8.0 with 0.5 $\mu$M enzyme.

The bioconversion from D-fructose into D-psicose was a conversion 32% at 55 °C after 30 min, without any byproducts (Figure 5). For high levels of production of D-psicose from D-fructose, recombinant *E. coli* whole cells harboring *C. cellulolyticum* DPEase were used as catalyst. The highest amount of D-psicose in 1 L of reaction system was obtained after incubation of 750 g/L D-fructose with 0.1 mM $Co^{2+}$ and 2 g dry cell wt/L of resting cells containing *C. cellulolyticum* DPEase at 55 °C and pH 8.0; 218 g/L D-psicose could be produced after 2 h, with 29% turnover yield (Figure 6).

In conclusion, the noncharacterized gene Ccel_0941 from *C. cellulolyticum* H10, previously proposed as the xylose isomerase domain protein TIM barrel, was cloned and expressed in *E. coli* and characterized as a member of the DTEase family of enzymes, which was the second DPEase reported after *A. tumefaciens* DPEase. The enzyme is strictly metal-dependent and requires $Co^{2+}$ as a cofactor for activity. In addition, the enzyme is extremely thermostable in the presence of $Co^{2+}$. The catalytic efficiencies of *C. cellulolyticum* DPEase for D-psicose and D-fructose were remarkably higher than for D-tagatose, suggesting that this enzyme was more beneficial to epimerization between D-fructose and D-psicose. These findings show that *C. cellulolyticum* DPEase could be a good candidate to produce D-psicose in ideal industrial conditions.

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## ABBREVIATIONS USED

DTEase, D-tagatose 3-epimerase; DPEase, D-psicose 3-epimerase; LB, Luria−Bertani; IPTG, isopropyl $\beta$-D-1-thiogalactopyranoside; HPLC, high-performance liquid chromatography; SDS-PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; EDTA, ethylenediaminetetraacetic acid.

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