**Enhancement of isomerization activity and lactulose production of cellobiose 2-
epimerase from *Caldicellulosiruptor saccharolyticus***

**Qiuyun Shen ^{a, b, c}, Yuzhu Zhang ^c, Ruijin Yang ^{b, *}, Siyi Pan ^a, Juan Dong ^b,
Yuting Fan ^b, Liang Han ^b**

^a Key Laboratory of Environment Correlative Dietology, College of Food Science and
Technology, Huazhong Agricultural University, Wuhan, China 430070

^b School of Food Science and Technology, Jiangnan University, 1800 Lihu Avenue,
Wuxi, China 214122

^c United States Department of Agriculture, Agricultural Research Service, Western
Regional Research Center, USA 94710

* Corresponding authors:

Prof. Ruijin Yang (RJ Yang)

Email: yrj@jiangnan.edu.cn

TEL/FAX: 86-510-85919150

Abstract

Industrial application of *Caldicellulosiruptor saccharolyticus* cellobiose 2-epimerase (CsCE) for lactulose synthesis is limited by low enzyme activity and formation of epilactose as by-product. After four sequential rounds of random mutagenesis and screening, an optimal mutant G4-C5 was obtained. Compared with wild type (WT) enzyme, mutant G4-C5 demonstrated 2.8- and 3.0-fold increases in specific activity and k_{cat}/K_m for lactulose production, respectively, without compromising thermostability. DNA sequencing of mutant G4-C5 revealed five amino acid substitutions, namely, R5M, I52V, A12S, K328I and F231L, which were located on the protein surface, except for the mutation I52V. The yield of lactulose catalyzed by mutant G4-C5 increased to approximately 76% with no obvious epilactose detected, indicating that mutant G4-C5 was more suitable for lactulose production than the WT enzyme.

Keywords: lactulose; cellobiose 2-epimerase; activity improvement; random mutagenesis

1. Introduction

Lactulose is a non-digestible disaccharide widely used in food and pharmaceutical industries as prebiotic food additive and medicine against chronic constipation and hepatic encephalopathy (Paik, Lee, Han, Song, Kim, Moon, et al., 2005). Commercially available lactulose is mainly produced through chemical isomerization of lactose in alkaline media. This process requires a considerable amount of chemical catalysts, involves complicated separation and purification steps to remove catalysts, and produces high amount of undesirable by-products (Aider & de Halleux, 2007). β -galactosidases (EC 3.2.1.23) (Guerrero, Vera, Araya, Conejeros, & Illanes, 2015; Qiuyun Shen, Yang, Hua, Ye, Wang, Zhao, et al., 2012) and cellobiose 2-epimerases (CEs, EC 5.1.3.11) (J. E. Kim, Kim, Kang, & Oh, 2012; Y. S. Kim & Oh, 2012) have been used as biocatalysts for lactulose production over the last decade.

Lactulose production catalyzed by β -galactosidases requires fructose as co-substrate and exhibits a low conversion yield. As such, direct conversion of lactose into lactulose catalyzed by CsCE is more attractive. This process requires lactose as single substrate and achieves a high lactulose yield (Y. S. Kim & Oh, 2012). Cellobiose 2-epimerase, which belongs to the *N*-acyl-D-glucosamine 2-epimerase (AGE) superfamily, catalyzes epimerization at the C-2 position of the glucose moiety in the reducing end of cellobiose and lactose to generate 4-*O*- β -D-glucopyranosyl-D-glucose and epilactose (4-*O*- β -D-galactopyranosyl-D-mannose), respectively. CsCE and CE from *Dictyoglomus turgidum* (DtCE) can also catalyze isomerization reaction that convert lactose into lactulose (4-*O*- β -galactopyranosyl-D-fructose) (J. E. Kim, Kim, Kang, & Oh, 2012). Under the optimal condition, the yields of lactulose and epilactose from lactose catalyzed by CsCE was 58% and 15%, respectively (Y. S.

Kim & Oh, 2012). However, high amount of protein is required due to the low specific activity of CsCE, which limits the industrial applications of this enzyme for lactulose production. Therefore, an engineered CsCE with improved specific activity must be developed to attain economical lactulose production with biological catalysis.

Compared with rational design, the directed molecular evolution of proteins does not require knowledge on the detailed structure or accurate prediction on the active site or the binding pocket (Kaur & Sharma, 2006). This technique commonly relies on random mutagenesis and recombination, followed by screening or selection schemes. A number of mutagenesis methods developed with appropriate screening or selection procedures have successfully improved enzyme properties, including activity, stability, substrate specificity, and enantioselectivity, in artificial environments (Balci, Ozturk, Pijning, Ozturk, & Gumusel, 2014; Callanan, Russell, & Klaenhammer, 2007; Goldsmith & Tawfik, 2012; Zhu, Tee, Roccatano, Sonmez, Ni, Sun, et al., 2010). In this study, directed evolution was adopted to improve specific activity of CsCE because the structural basis and catalytic mechanism of CsCE are unavailable.

Usually, the success of directed protein evolution experiments depends on the efficiency of the methods used for screening libraries to identify mutants exhibiting properties of interest. Measurement of lactulose in enzymatic reactions was performed based on the chromatographic separation of lactulose and other sugars, such as high-performance liquid chromatography (HPLC) and gas chromatography (GC). These chromatographic methods require expensive apparatus, reagents, and long pretreatment duration and are time consuming, labor intensive, and impractical for high-throughput screening of lactulose producing enzymes (J. E. Kim, Kim, Kang, & Oh, 2012; Y. S. Kim & Oh, 2012). Colorimetric methods for lactulose detection are based on the colored products of the reactions of fructose following hydrolysis of

lactulose by strong acids or enzymes, which might be suitable for large library screening.

In this study, a 96-well microplates screening procedure was determined for CsCE mutant libraries evaluation based on colorimetric activity assay (Figure 1). Four sequential rounds of random mutagenesis and screening were applied to enhance the activity of CsCE. The enzymatic properties of the selected mutants and wild-type CsCE (WT-CsCE) were analyzed and compared after purification.

2. Materials and methods

2.1 Materials

Unless otherwise stated, all materials and reagents were of the highest grade and purchased from Sigma (St. Louis, MO). Isopropyl β -D-1-thiogalactopyranoside (IPTG) was obtained from Calbiochem (Gibbstown, NJ). The bacterial strain *Escherichia coli* BL21 (DE3) pLysS competent cells were obtained from Promega (Madison, WI). The primers were ordered from ElimBio (Hayward, CA). *Caldicellulosiruptor saccharolyticus* DSM 8903 genomic DNA was kindly provided by Alejandro Muñoz of the Department of Applied Microbiology, University of Lund, Sweden.

2.2 Random mutagenesis

The random mutant library was prepared via error-prone PCR (ep-PCR) using the Stratagene GeneMorph II Random Mutagenesis Kit (Agilent, USA) according to its protocol. The reaction mixture contained plasmid DNA (500 ng of target DNA), 125 ng of each primer, 0.8 mM dNTP mix (0.2 mM each) and 2.5 U Mutazyme II DNA polymerase in a 50 μ l reaction volume. The cycling parameters were 30 cycles of 95 °C for 30 s, 58 °C for 30 s, and 72 °C for 1.5 min. The primers used were Forward (5'-CGCGGATCCATGGATATTACAAGGTTTAAG-3') and Reverse (5'-

CCGGAATTCTTAGTCAACCCTTTTATTATC-3'). Amplification of the 1.2 kb product was verified through nucleic acid electrophoresis on 0.7% agarose gel. The amplified product was then extracted and purified using Wizard SV Gel and PCR Clean System (Promega, USA). The purified PCR product and the pET28a(+) vector were digested with *Bam*HI and *Eco*RI enzymes and ligated together using T4 DNA ligase (Biolabs, New England). The recombinant plasmids were transformed into competent *E.coli* BL21 (DE3) cells (Novagen, Darmstadt, Germany). The transformants were plated on Luria–Bertani (LB) agar containing kanamycin (30 µg/mL) and incubated at 37 °C overnight.

2.3 Screening procedure

Individual colonies from the mutant library were transferred with sterile toothpicks or by a QPix2 colony picker (Genetix Ltd., New Milton, UK) and directly inoculated into wells of a round-bottomed 96-deep-well microplate containing 900 µL of LB medium supplemented with 10 µg/mL kanamycin and 0.4 mM IPTG (Figure 1). The culture plates were tightly sealed with Breathe-Easy® sealing membrane (Sigma, St. Louis, MO) and incubated at 30 °C and 800 rpm for 24 h. One hundred microliters of each of the culture was transferred to the corresponding well of a new autoclaved microplate for preservation. Meanwhile, the remaining samples were centrifuged at 3500 rpm for 15 min for cell harvest. The cells were then resuspended in 50 µL of 50 mM piperazine-1,4-bisethanesulfonic acid (PIPES) buffer containing 10 mg/mL lysozyme, incubated at 37 °C for 1 h, and subsequently subjected to three freeze/thaw cycles at –80 °C. Each well was added with 50 µL of lactose solution (10 mg/mL) after cell disruption. The enzymatic reactions were then performed at 80 °C for 5 min. Lactulose assay reagent (300 µL) was added into each well, and the solution was mixed by shaking at 500 rpm for 10 min using a microplate shaker (Southwest

Science, Hamilton, NJ). The microplates were then kept in a 46 °C water bath for 70 min. The assay reagent consisted of 2.5% cysteine hydrochloride and 0.08% tryptophan dissolved in 75% sulfuric acid. Absorbance was measured at 518 nm by using a microplate reader (SpectraMax M2, Molecular Devices Corporation, CA). Mutants with higher absorbance values at 518 nm were selected for further identification by flask culture and activity analysis by HPLC (see below). The Variant that demonstrated the highest activity was selected as template for the next round of random mutagenesis and screening.

2.4 Enzyme production and purification

The WT and mutant enzymes without His-tag were produced in *E.coli* BL21 (DE3). The bacteria were grown in 1 L batches of LB medium containing kanamycin (kana, 50 mg L⁻¹) at 37 °C until the OD₆₀₀ of the culture reached 0.6~0.8. Isopropyl-beta-D-thiogalactopyranoside (IPTG) was added to the culture at a final concentration of 0.4 mM. The cells were collected by centrifugation after 16 h of induction at 30 °C and then disrupted by sonication in 10 mM Tris-HCl buffer (pH 7.5). The cell lysate was centrifuged at 6,000 rpm and 4 °C for 30 min. The obtained crude enzyme was purified by three steps using Mono Q column, Hitrap phenyl HP column and Superdex-75 column (Qiuyun Shen, Zhang, Yang, Hua, Zhang, & Zhao, 2015). Protein purity was verified through sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using NuPAGE[®] Novex[®] Bis-Tris Gel (Life TechnologiesTM, Carlsbad, CA). The concentration of the purified enzymes was determined using Nanodrop 2000C spectrophotometer (Thermo, Waltham, USA) based on the theoretical molar extinction coefficient (105310) of the protein at 280 nm (Gasteiger E., 2005) .

2.5 Enzyme assay

Enzymes thermally inactivated by boiling were used as control. Unless otherwise stated, the reaction in the activity assay was performed at 80 °C for 5 min in 50 mM PIPES buffer (pH 7.5) containing 150 mM substrate and 50 µg enzyme in a total volume of 100 µL. The reaction was terminated by adding HCl into the solution at a final concentration of 200 mM. After being diluted to adjust the concentration of sugars to 0.1%–1.0% (w/v), the reaction solution was analyzed by Dionex ion chromatography ICS-5000 (Sunnyvale, CA, USA) with a pulsed amperometric detector (HPAEC-PAD) equipped with an Au electrode and a Dionex Carbopac PA20 column (3 mm id × 150 mm, Sunnyvale, CA, USA). The column was eluted with 1.5 mM NaOH at 30 °C and 0.5 mL min⁻¹. One Unit (U) of activity was defined as the amount of enzyme required to produce 1 µmol lactulose per minute.

The kinetic parameters of WT enzyme and mutant G4-C5 were determined using 60–900 mM lactose by measuring the lactulose increase. Parameters, including Michaelis–Menten constant (K_m) and turnover number (k_{cat}) values, were determined by non-linear regression to the Michaelis-Menten equation analyzed using GraphPad Prism v6.0 (GraphPad Software, San Diego, CA, USA). The reaction conditions, except for lactose concentration, were the same as the standard conditions described above. All measurements in this section were conducted in triplicate, and the mean value was presented with the standard deviation.

2.6 Thermostability measurement

The irreversible thermal inactivation of the purified enzyme was determined through incubation in 50 mM PIPES buffer (pH 7.5) at 80 °C for indicated periods (30, 60, 90, 120, 180, 240, and 300 min). The first-order rate constant, k , of irreversible thermal denaturation was obtained from the slope of the semi-logarithmic plot of incubation time versus residual activity. The half-life of thermal inactivation was

calculated as $\ln 2/k$ (O'Fagain, 2003). Circular dichroism (CD) spectrum signal at 220 nm was used to study the unfolding of WT-CsCE and mutant G4-C5 induced by denaturing reagent guanidine hydrochloride (GuHCl) as described previously (Qiuyun Shen, Zhang, Yang, Hua, Zhang, & Zhao, 2015). The free energy of denaturation (ΔG_0) was calculated by fitting the data to a two-state transition using Origin 9.0 software.

2.7 Differential scanning calorimetry (DSC)

Thermal measurements were performed using a Q2000 DSC (TA Instruments, DE). Purified protein was concentrated in a 15 mL ultra centrifugation device (Millipore, Billerica, MA) and the concentration was measured using a Nanodrop 2000C spectrophotometer (see Section 2.4). All measurements were performed using 30 μ L high pressure stainless steel crucibles and crucible filled with distilled water was used as a reference. To eliminate signal baseline drift of the equipment, we allowed the DSC to warm up for 10 min. The samples were heated from 40 to 100 °C at a scan rate of 5 °C/min. The onset denaturation temperature T_0 was determined by taking the intercept of the baseline and the extrapolated from edge of the peak. The temperature at maximum of the peak was taken as the denaturation temperature T_p .

2.8 Effects of pH and temperature on activity

The effect of temperature on enzyme activity was investigated at 50 to 90 °C in 50 mM PIPES buffer (pH 7.5). Moreover, the effect of pH was determined at pH 6.5 to 8.5 in 50 mM PIPES buffer (pH 6.5–7.5) and 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (pH 7.5-8.5) at 80 °C.

2.9 Lactulose production by CsCE and its mutant G4-C5

The formation of lactulose was performed in **nearly** saturated lactose solution (500 g/L lactose) at 80 °C, pH 7.5 in 1 mL of reaction volume. **The samples were**

obtained at regular time intervals, and the reaction was terminated by adding HCl. The concentrations of lactulose, epilactose, and lactose in the reaction mixture were determined by HPLC, as described in section 2.5. The yield of lactulose was calculated by dividing the lactulose concentration by the initial concentration of lactose.

3.0 Amino acid sequence alignment and structure prediction

Combined sequence and secondary structure alignments were performed using ESPript program and manually refined. Three-dimensional structures of WT-C5CE and mutant G4-C5 were predicted by the online system Phyre2 (Kelley & Sternberg, 2009).

3. Results and discussion

3.1 Activity assay in microplate

The screening procedure mainly involved culture in 96-well microplate and lactulose quantification by colorimetric method (Figure 1). The colorimetric assay was based on hydrolysis of lactulose into galactose and fructose, by sulfuric acid. Fructose was much more rapidly transformed into hydroxymethylfurfural than aldoses and reacted with cysteine hydrochloride–tryptophan reagent, to produce colored compounds. It was demonstrated that the maximum difference of this colorimetric method and HPLC method was just 2% (Z. Zhang, Wang, Yang, & Jiang, 2010). The calculated linear equation of 5–250 μ M lactulose in microplate wells was $y = 0.0055x + 0.0348$, with $R^2 = 0.999$, whereas that of fructose was $y = 0.0055x + 0.0252$, with $R^2 = 0.999$. This finding indicated lactulose can be quantified from the standard curve of equimolar fructose. In addition, lactulose concentration higher than 5 μ M can be detected during microplate screening.

The influence of other components in the enzymatic reaction solution, such as lactose, epilactose, and bacterial debris, on lactulose quantification by this colorimetric method was also investigated, because this method was developed using standard lactulose solution and validated using lactulose syrups produced through chemical isomerization. The absorbance value of the equivalent lactose and epilactose was only approximately 1.0% of those of fructose and lactulose (data not shown) because cysteine hydrochloride–tryptophan reaction was specific for ketohexoses (Messineo & Musarra, 1972). Addition of excess bacterial debris reduced the absorbance by almost 5% (data not shown), which could be due to the reaction between sulfuric acid and bacterial protein. However, turbidity produced by insoluble protein was eliminated by sulfuric acid, and the possible interference could be eliminated by adding bacterial debris to standard and blank solutions. Thus, the colorimetric assay can be used for the sensitive, simple, and rapid measurement of the isomerization activity toward lactose of CsCE and its mutants from the standard curve of equimolar fructose. Moreover, crude enzyme with bacterial debris can be directly applied to the enzyme reaction.

Microplate screening was determined based on the colorimetric activity measurement, including colony transfer, cultivation, dilution, activity assay. All operations were conducted at the microscale level and could be automated using microplates and automatic pipetting systems, which could significantly increase the possible number of mutant colonies that can be screened (Figure 1).

3.2 Construction and screening of random mutagenesis libraries

The established microplate screening procedure was successfully implemented in directed evolution of CsCE. The CsCE gene was subjected to sequential random mutagenesis. Random mutations were introduced into the gene via error-prone PCR,

and the mutagenesis conditions were adjusted to yield an average of one to two mutations per gene which was deemed optimal for the improvement of specific properties by directed evolution (Shafikhani, Siegel, Ferrari, & Schellenberger, 1997; You & Arnold, 1996) . Wild type CsCE gene (GenBank accession no. FW496353.1) was used as template in the first round of random mutagenesis, and more than 1,000 colonies were screened for their isomerization activity. Mutants showed at least 20% increase in absorbance at 518 nm were selected from the initial screening, and their activities were reconfirmed by flask cultivation and activity assay through HPLC. The mutant displaying the highest activity from each round of ep-PCR was used as template for the next generation of random mutagenesis. After four rounds of mutagenesis and screening, 28 mutants were selected and 19 of them were identified as positive variants compared with their templates. The existence of false positives is most likely caused by variation in enzyme expression among cultures in each well.

The WT and mutant enzymes demonstrating the highest activity from each generation (G1-E5, G2-C11, G3-D7 and G4-C5) were produced in *E. coli* BL21(DE3) and then purified (Supplementary Figure 1, Supplementary Tables 1-5). The activity of the mutant enzymes was 1.27–2.79 fold higher than that of the WT enzyme (Table 1). Hence, the screening procedure was considered efficient for directed evolution of CsCE.

Changes in the amino acid sequence of mutant enzymes (G1-E5, G2-C11, G3-D7 and G4-C5) were identified by analyzing the nucleotide sequence of the plasmids (Table 1). Mutants G1-E5 and G2-C11 harbored one (A12S) and two (A12S/K328I) amino acid mutations, respectively. In addition, four substitutes (R5M/A12S/F231L/K328I) were identified in mutant G3-D7, and one additional

amino acid replacement (I52V) was detected in mutant G4-C5 compared with the template G3-D7.

Iterative cycles of diversity generation and screening were widely used to improve enzyme properties, including activity, stability, substrate specificity, and enantioselectivity. The specific activity of endoglucanase was increased to 193% of that of the wild type after three rounds of error-prone PCR and screening (Liang, Fioroni, Rodriguez-Ropero, Xue, Schwaneberg, & Ma, 2011). The product yield of toluene dioxygenase was increased by 40% after three rounds of mutagenesis and screening (N. Zhang, Stewart, Moore, Greasham, Robinson, Buckland, et al., 2000). This is the first case on activity improvement of cellobiose 2-epimerase by this strategy.

3.3 Thermostability

The half-lives of inactivation ($t_{1/2}$) at 80 °C of WT-CsCE and its mutants was evaluated by measuring enzyme activity over time in 50 mM PIPES buffer (pH 7.5) at 80 °C (Table 1). The $t_{1/2}$ of mutant G1-E5 increased by approximately 20 min compared with that of the WT enzyme, indicating that directed evolution can simultaneously improve enzyme activity and stability. Conversely, $t_{1/2}$ of mutants G2-C11 and G3-D7 successively decreased, indicating that sometimes improved catalytic activity might negatively affect the thermal tolerance. This phenomenon could be due to the fact that enzyme activity requires flexible spatial conformation, whereas enzyme thermostability requires a rigid conformation to resist high-temperature passivation damage (Nakazawa, Okada, Onodera, Ogasawara, Okada, & Morikawa, 2009). Additional amino acid substitution (I52V) in G3-D7 increased the $t_{1/2}$ of mutant G4-C5 at 80 °C from 116.9 min to 159.4 min, which was slightly higher than that of the WT enzyme.

The ΔG_0 values obtained through folding/unfolding equilibrium in the presence of GuHCl were 16.24 ± 1.64 kcal/mol and 16.32 ± 1.10 kcal/mol for WT enzyme and mutant G4-C5, respectively (data not shown). Moreover, DSC analysis showed an endotherm peak for the WT enzyme with an onset temperature $T_0 = 82.81$ °C and a denaturation temperature $T_p = 86.84$ °C. The onset temperature and denaturation temperature for mutant G4-C5 were 83.07 °C and 86.88 °C, respectively, which were both a little higher than the corresponding values for WT enzyme (Supplementary Figure 2). These results implied that acquisition of catalytic activity by evolutionary engineering was possible without compromising thermostability.

3.4 Kinetic parameters

The kinetic parameters of WT enzyme and the selected mutants were determined by non-linear regression to the Michaelis–Menten equation (Table 1). All mutants from different generations showed gradual reduction in K_m value and increase in k_{cat} value. The K_m value of mutant G4-C5 for lactulose production (358.24 mM) was 58% of that of WT-CsCE (613.54 mM), and k_{cat} of the former (75.27 s⁻¹) was 1.74-fold higher than that of the latter (43.26 s⁻¹), resulted in an approximately threefold increase in k_{cat}/K_m value for the mutant. These results indicated that the catalytic efficiency for lactulose production of engineered CsCE was increased.

3.5 Effects of pH and temperature on activity

The effects of temperature and pH on the activity of WT-CsCE and the optimal mutant G4-C5 were investigated (Figure 2). The activity of both enzymes significantly increased as the temperature increased to 80 °C. At temperatures higher than 80 °C, the isomerization activity of both enzymes rapidly decreased with increasing temperature. Moreover, mutant G4-C5 retained higher activity than the WT enzyme at low temperatures. The effect of pH on enzyme activities was tested in 50

mM PIPES buffer (pH 6.0, 6.5, 7.0, and 7.5) and 50 mM HEPES buffer (pH 7.5, 8.0, and 8.5). The activity of both enzymes peaked at pH 7.5. Mutant G4-C5 showed a broader pH-activity profile than the WT enzyme and demonstrated approximately 10% increase in activity in HEPES buffer than that in PIPES buffer at pH 7.5.

3.6 Lactulose production by mutant G4-C5

Lactulose production by WT enzyme and mutant G4-C5 was performed in 50 mM HEPES buffer (pH 7.5) containing 500 g/L lactose (~saturation) and 3 mg/mL of each enzyme at 80 °C for up to 240 min. The reaction solution was analyzed by HPLC at different time intervals (Figure 3). The yields of lactulose and epilactose catalyzed by the WT enzyme reached around 57% and 16%, respectively, after 4 h. The concentration of lactulose catalyzed by mutant G4-C5 rapidly increased from 0 min to 90 min and then increased gradually to the steady-state level with a yield of lactulose up to approximately 76%. No obvious epilactose was detected at any time intervals for mutant G4-C5. CsCE was demonstrated to interconvert lactose, lactulose and epilactose (J. E. Kim, Kim, Kang, & Oh, 2012; Y. S. Kim & Oh, 2012; Park, Kim, Choi, & Oh, 2011). The formed epilactose might be catalyzed to lactulose very quickly, thus no epilactose was detected. Although epilactose is also a bifidus factor (Watanabe, Nishimukai, Taguchi, Senoura, Hamada, Matsui, et al., 2008), formation of epilactose could complicate lactulose purification. Therefore, mutant G4-C5 is more suitable for lactulose synthesis than the WT enzyme.

3.7 Identification of putative active site

Three-dimensional structure of CsCE was predicted by the online system Phyre2 (Kelley & Sternberg, 2009). The top template was identified as the crystal structure of *Rhodothermus marinus* cellobiose 2-epimerase (RmCE, pdb3wkf) (Fujiwara, Saburi, Matsui, Mori, & Yao, 2014) which showed 39% sequence identity to CsCE. All of the

390 residues were modeled with more than 90% confidence. The predicted structure consisted of 12 α -helices forming a typical (α/α)₆-barrel structure with a deep cleft (Figure 5). The deep cleft was considered to be an active site. Although sequence identity between CsCE and other sugar epimerases, namely, AGEs and yihS-encoded proteins (YihSs), is low (approximately 15%), the predicted CsCE structure was similar to that of *Ruminococcus albus* CE (RaCE), YihSs from *Salmonella enterica* (SeYihS) and *E. coli* (EcYihS), *Anabaena* sp. CH1 AGE (AsAGE) and AGE from porcine kidney (pAGE). Sequence alignment of CsCE with CEs, AGEs, and YihSs showed that 13 residues were strictly conserved (Figure 4) and seven of these residues, Arg56, Phe118, His247, Glu250, Trp308, Glu312, and His377 were located in the putative catalytic center and in close proximity to each other. Moreover, His188, Glu191, Tyr114 and Asn184 were only conserved among CEs and YihSs but not in AGEs. Trp372 at the entrance of the cleft, whose ring is parallel to that of Tyr308, was only conserved in CEs. These conserved and partially conserved amino acids protruded into the putative active site pocket of CsCE. Some of these amino acids are potential acid/base catalysts for isomerization and epimerization reactions while the others possibly play roles in substrates binding or orientation.

3.8 Location of the substituted residues

The substituted residues in CsCE mutants isolated in this study were mapped in the tertiary level of the enzyme and no mutation was found in the conserved residues (Figure 5). Three mutations, namely, A12S, R5W, and K328I, were found in different α -helices at the surface region and far from the putative catalytic center. Mutation K328I had a relatively long distance to the putative active site, which excluded any direct effects on residues at the active site. Increased activity could be a result of long-range structural effects. In other enzymes, mutations far from the active site can also

improve catalytic properties. These mutations may influence substrate access, produce exit, or the ability to undergo conformational changes that occur in the catalytic cycle or may also affect the global structure that impacts on residues in the active site (Balci, Ozturk, Pijning, Ozturk, & Gumusel, 2014; Fan, Fang, Xiao, Yang, Zhang, Bidochka, et al., 2007). Moreover, mutant G1-E5 with A12S substitution, showed improved activity and enhanced thermostability at 80 °C compared with WT-CsCE. Serine has a polar hydroxyl group on its side chain and can form more hydrogen bonds. Increase in hydrogen bonding has been suggested as one of the principal determinants of enhanced stability (Kumar, Tsai, & Nussinov, 2000; Vieille & Zeikus, 2001).

The amino acid mutation F231L was also observed at protein surface, but in a β -strand region and close to the entrance of the active cleft. The replacement of phenylalanine at position 231, a bulky residue close the substrate-binding channel, might provide easy substrate access and therefore increased lactose affinity. This sterical effect was also found in pyruvate decarboxylase from *Zymomonas mobilis*, mutation of Trp392 in the substrate-binding channel increased the carbolligase side-reaction of the enzyme by a factor of six (Pohl, 2001). Another mutation, I52V, was observed in an inner α -helix and located close to the conserved residues Arg56, Tyr114 and Trp372. The replacement of hydrophobic isoleucine by smaller valine residue might confer flexibility to the active site pocket and be beneficial for substrates binding or orientation for reaction. The thermostability enhancement might be a result of long-range structural effects because that the side chains of Ile and Val are both alkyls and no additional interaction was observed after the amino acid substitutions.

The roles of mutated residues remain difficult to elucidate because the structural basis and catalytic mechanism of CsCE for lactulose production are unavailable. An

on-going X-ray analysis of WT-CsCE and mutant G4-C5 is expected to provide sufficient information on the structure–function relationship.

4. Conclusion

Mutant G4-C5, which demonstrated a 2.8-fold increase in activity for lactulose production was obtained through four rounds of random mutagenesis. The thermostability of this enzyme was also enhanced. Under the optimal condition, the yield of lactulose catalyzed by mutant G4-C5 increased to 76% without epilactose being detected in the reaction solution. Thus, mutant G4-C5 is more suitable than the WT enzyme for lactulose production. To the best of our knowledge, this work is the first to improve the activity of CsCE through directed evolution for lactulose production.

Notes

Mention of trade names or commercial products in this publication is solely for the purpose for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture.

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Figure legends

Figure 1. Diagram of sequential mutagenesis and microplate screening procedure for CsCE mutants with higher isomerization activity

Figure 2. Temperature and pH dependence of the activity for lactulose production of WT-CsCE (square) and mutant G4-C5 (circle). (A) Temperature profile of WT-CsCE and mutant G4-C5, activities normalized as percentages of the activities at 80 °C; (B) pH profile of WT-CsCE and mutant G4-C5, activity at pH 7.5 in HEPES buffer for each enzyme was defined as 100%.

Figure 3. Bioconversion of lactose catalyzed by WT-CsCE (■, lactulose; ●, epilactose) and mutant G4-C5 (□, lactulose). The reaction was performed in 50 mM HEPES buffer (pH 7.5) containing 500 g/L lactose and 60 U/mL of enzyme at 80 °C up to 240 min. Data represent the means of three separated experiments and error bars represent standard deviation.

Figure 4. Structure-based alignment of CsCE using the program ESPript and identification of critical residues. The sequences are as follows (GenBank accession numbers) 1, CsCE (FW496353.1); 2, DtCE (YP_002352551.1); 3, RmCE (BAK61777.1); 4, RaCE (BAF81108.1); 5, SeYihS (AAL22860.1); 6, EcYihS (AAD13442.2); 7, AsAGE (ABG57043.1); 8, pAGE (BAA12103.1). The strictly and partly conserved residues were indicated as black circle and rhombus respectively, and residue substitutions were marked with asterisk.

Figure 5. Three-dimensional structure of *Caldicellulosiruptor saccharolyticus* cellobiose 2-epimerase predicted by online Phyre2 program and the location of the conserved residues and residue substitutions of mutant G4-C5. Conserved residues located in the deep cleft of the (α/α)₆ structure and were shown by thin sticks. The

545 mutation sites of mutant G4-C5 were indicated by CPK (Corey-Pauling-Koltun)
546 representation scheme and colored by atom type.

1 Tables

2 Table 1. Catalytic properties of WT-CsCE and its mutants for lactulose production

Mutant	Amino acid mutation	Activity (U/mg) ^a	$t_{1/2}$ (min) ^b	kinetic parameters ^c		
				K_m (mM)	k_{cat} (s ⁻¹)	k_{cat}/K_m (s ⁻¹ · mM ⁻¹)
WT-CsCE	None	10.77±0.35 (100% ^d)	141.3±4.8	613.54±15.83	43.26±1.08	7.05×10 ⁻²
G1-E5	A12S	13.68±0.41 (127%)	161.2±2.7	537.12±13.28	47.50±1.04	8.84×10 ⁻²
G2-C11	A12S/K328I	17.45±0.51 (162%)	132.5±5.3	475.43±14.32	51.77±2.20	10.89×10 ⁻²
G3-D7	R5M/A12S/F231L/K328I	19.60±0.45 (182%)	116.9±3.2	402.12±6.37	55.83±1.67	13.89×10 ⁻²
G4-C5	R5M/A12S/I52V/F231L/K328I	30.08±0.86 (279%)	159.4±4.5	358.24±8.14	75.27±2.39	21.01×10 ⁻²

3 ^a Reactions for activity assay were performed at 80 °C for 5 min in 50 mM PIPES buffer (pH 7.5) with 150 mM lactose and 500 µg/mL enzyme.

4 ^b Half-lives of inactivation ($t_{1/2}$) at 80 °C.

5 ^c The kinetic parameters for lactulose production were determined from the initial isomerization reaction rates toward varying concentrations of lactose (60
6 mM to 900 mM) by non-linear regression to the Michaelis-Menten.

7 ^d Numbers in parentheses indicated the relative activity compared with that of the WT-CsCE.

1 **Figures**

Figure 1.

Figure 2.

Figure 3.

Figure 4.

Figure 5.