Novel cellobiose 2-epimerases for the production of epilactose from milk ultrafiltrate containing lactose

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ABSTRACT

A selected number of enzymes have recently been assigned to the emerging class of cellobiose 2-epimerases (CE). All CE convert lactose to the rare sugar epilactose, which is regarded as a new prebiotic. Within this study, the gene products of 2 potential CE genes originating from the mesophilic bacteria *Cellulosilyticum lentocellum* and *Dysgonomonas gadei* were recombinantly produced in *Escherichia coli* and purified by chromatography. The enzymes have been identified as novel CE by sequence analysis and biochemical characterizations. The biochemical characterizations included the determination of the molecular weight, the substrate spectrum, and the kinetic parameters, as well as the pH and temperature profiles in buffer and food matrices. Both identified CE epimerize cellobiose and lactose into the C2 epimerization products glucosylmannose and epilactose, respectively. The epimerization activity for lactose was maximal at pH 8.0 or 7.5 and 40°C in defined buffer systems for the CE from *C. lentocellum* and the CE from *D. gadei*, respectively. In addition, biotransformations of the foodstuff milk ultrafiltrate containing lactose were demonstrated. The CE from *D. gadei* was produced in a stirred-tank reactor (12 L) and purified using an automatic system. Enzyme production and purification in this scale indicates that a future upscaling of CE production is possible. The bioconversions of lactose in milk ultrafiltrate were carried out either in a batch process or in a continuously operated enzyme membrane reactor (EMR) process. Both processes ran at an industrially relevant low temperature of 8°C to reduce undesirable microbial growth. The enzyme was reasonably active at the low process temperature because the CE originated from a mesophilic organism. An epilactose yield of 29.9% was achieved in the batch process within 28 h of operation

time. In the continuous EMR process, the epilactose yield in the product stream was lower, at 18.5%. However, the enzyme productivity was approximately 6 times higher because the continuous epilactose formation was carried out for about 6 d without further addition of biocatalyst. Within this time, 24 g of epilactose in 2.8 L of permeate were produced. The batch and the EMR process showed that the milk ultrafiltrate, which is a sidestream of the milk protein production, might be upgraded to a dairy product of higher value by the enzymatic in situ production of epilactose.

Key words: cellobiose 2-epimerase, epilactose, prebiotic, lactose, milk

INTRODUCTION

Lactose (4-O- β -D-galactopyranosyl-D-glucopyranose) is a disaccharide that occurs naturally in milk. During the heat treatment of milk, some lactose derivatives, namely lactulose (4-O- β -D-galactopyranosyl-D-fructose) and epilactose (4-O- β -D-galactopyranosyl-D-mannose; Olano and Calvo, 1989; Olano et al., 1989, 1992), are generated through the Lobry de Bruyn-Alberda van Ekenstein transformation (Moreno, 2003; Schuster-Wolff-Bühning et al., 2010). Recent literature suggests that epilactose has prebiotic properties (Nishimukai et al., 2008; Watanabe et al., 2008; Suzuki et al., 2010a,b); therefore, it is appropriate to produce epilactose directly in dairy products for potential future application in the food industry.

Cellobiose 2-epimerases (CE; EC 5.1.3.11) catalyze the formation of epilactose (Ito et al., 2007; Ojima et al., 2013); CE activity was first described by Tyler and Leatherwood (1967). Epimerization activity toward cellobiose was detected in the culture broth of the ruminal bacterium *Ruminococcus albus* 7 ATCC 27210 and CE was later identified as a biocatalyst catalyzing the reaction. Currently, 14 CE have been described. These enzymes catalyze the epimerization of the C2 hydroxyl group of the reducing end of 1,4-linked oligosaccharides (Ojima et al., 2013). Three CE have been found to

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catalyze not only the epimerization (e.g., lactose to epilactose), but also the isomerization to lactulose (Park et al., 2011, 2013; Kim et al., 2012). The CE described have a molecular weight of about 45 kDa and the temperatures of optimal activity are in a broad range from 30 to 80°C. All CE show very low AA sequence homologies, but all feature common structural elements. They share structural similarities with *N*-acyl-D-glucosamine 2-epimerases (**AGE**; Ito, 2009), including the (α/α)₆ fold. Therefore, the AA sequences of AGE have been used as a search template for the identification of new CE from databases.

The crystal structure of the CE from *R. albus* (**RaCE**; Fujiwara et al., 2013a) and *Rhodothermus marinus* (**RmCE**; Fujiwara et al., 2014) were released recently. The crystal structures confirmed the structure proposed previously by Ito et al. (2007). The basic conformation of CE consists of 12 α -helices arranged in an (α/α)₆ barrel structure. The active site, with its triple histidine center (Fujiwara et al., 2013a), is located inside the barrel.

In the current study, we identified 2 novel CE, 1 each from the obligate anaerobic, mesophilic bacterium *Cellulosilyticum lentocellum* and from the facultative anaerobic, mesophilic bacterium *Dysgonomonas gadei*. The focus of our study was the characterization of the undescribed CE with regard to potential industrial application in milk products. Therefore, biochemical characterizations of the 2 enzymes were carried out in buffer matrix and in milk and milk ultrafiltrate (**MUF**). An industrial application potential of the novel CE was demonstrated. Lactose in the food matrix MUF has been enzymatically converted to epilactose in both a batch and a continuous process.

MATERIALS AND METHODS

Chemicals, Enzymes, Milk, and MUF

All chemicals were of analytical grade or higher and supplied from Sigma-Aldrich (St. Louis, MO), Carl Roth (Karlsruhe, Germany), or Merck (Darmstadt, Germany). The T4 DNA ligase was purchased from Fisher Scientific (Schwerte, Germany) and restriction enzymes and DNA polymerase were purchased from New England Biolabs (Ipswich, MA). Milk for biochemical characterizations was obtained from a local supermarket (Weidemilch, Ultrahocherhitzt, 1.5% Fett, Schwarzwaldmilch GmbH, Freiburg, Germany).

Biotransformations were carried out in UHT MUF (obtained from the University of Hohenheim-owned Dairy for Training and Research). This ultrafiltrate was produced from UHT milk by membrane fractionation via a 10 kDa molecular cut-off membrane

(GR73PP-6338/48; Alfa Laval, Lund, Sweden). The lactose content was 48.3 ± 1.3 g/L and the protein content was 581 mg/L (using the Bradford method).

Microorganisms, Plasmids, and Cloning

The AA sequences representing putative CE from *C. lentocellum* and *D. gadei* were identified in UniProtKB/TrEMBL (association no. F2JR71 and F5ITJ6; The UniProt Consortium, 2014). The DNA preparations from *C. lentocellum* DSM 5427 and *D. gadei* DSM 22811 were obtained from DSMZ (Leibniz Institut DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany) for later expression of the wild-type genes heterologously in *Escherichia coli*. The specific primer pair *clce_fw* (5' CGCGGGATC-CGTGTTTGTTCAGAAAGTAAAAG 3') and *clce_rev* (5' GCGCGGATCCTAACTCGAGAACAT-TAATATCCCTCCTGATG 3') were used for PCR amplification of the coding sequence for F2JR71. The resulting PCR fragment was called *CLCE*. The coding sequence for F5ITJ6 (named *DGCE*) was amplified by PCR with the primer pair *dgce_fw* (5' CGCGGGATC-CATGAACGGATTTCAGGAAAGAATTAATCG-GAAAC 3') and *dgce_rev* (5' CGCGGATCCTTACTC-GAGATCAATTCCAAAATGTTCTAACATTTC 3'). The *Bam*HI and *Xho*I restriction sites (underlined) for cloning were added to the wild-type sequences via both primer pairs. A hexahistidine-tag (**His₆-tag**) was added by cloning into pET-20b (+) vectors. The resulting plasmids, pET-20b*CLCEHIS* and pET-20b*DGCEHIS*, were introduced into *E. coli* XL-1 Blue (New England Biolabs, Ipswich, MA). The plasmids were transformed in *E. coli* JM109(DE3) and *E. coli* Rosetta-gami(DE3) for protein production.

Culture Conditions

The CE were produced in baffled shaking flasks for analytical purposes. Thus, 1-L shaking flasks were inoculated with 4 mL of an overnight preculture of *E. coli* JM109 pET-20b*CLCEHIS* or *E. coli* JM109 pET-20b*DGCEHIS*. The main culture was propagated in 200 mL of Terrific broth medium (24 g/L of yeast extract, 12 g/L of tryptone, 4 mL/L of glycerol, 17 mM KH₂PO₄, 72 mM K₂HPO₄, pH 7.0, 100 μ g/mL of ampicillin; Carl Roth, Karlsruhe, Germany). The cultures were cultivated at 37°C until the transcription of the CE was induced at an optical density of 600 nm of 0.6 with 0.5 mM isopropyl β -D-1-thiogalactopyranoside. Thereafter, the cultivation was continued at 20°C for an additional 24 h.

Additionally, *E. coli* Rosetta-gami pET-20b*DGCEHIS* was cultivated in 12 L of Terrific broth medium

supplemented with 100 µg/mL of ampicillin and 25 µg/mL of chloramphenicol in a 23-L stirred-tank reactor (Biostat E, B. Braun, Melsungen, Germany) at 37°C. The pH was maintained at 7.0 by 0.6 M H₃PO₄. Expression was induced by adding 0.5 mM isopropyl β-D-1-thiogalactopyranoside at an optical density of 600 nm of 3.2. After an overall cultivation time of 9 h, cells were harvested by centrifugation (8,000 × g, 4°C, 15 min).

Protein Quantification

The determination of the concentration of protein was carried out via the Bradford method (Bradford, 1976). Bovine serum albumin was used as a standard; SDS-PAGE was used for the evaluation of protein expression and purity after purification. The gels consisted of a 12% (wt/vol) acrylamide separation gel and a 6% (wt/vol) acrylamide stacking gel. The visualization of protein bands was carried out using Coomassie Brilliant Blue staining, according to the Fairbanks method (Wong et al., 2000). Broad Range Protein Marker (2–212 kDa) from New England Biolabs was used as a protein standard.

Purification of CE

Harvested cells were resuspended in 20 mM Tris-HCl buffer pH 8 containing 150 mM NaCl and 20 mM imidazole resulting in a 30% (wt/vol) cell suspension. This suspension was subjected to a sonication on ice for 10 cycles each of 1 min of sonication (95% amplitude, 0.5 cycles) and a 1-min pause (UP200S/S3; Hielscher Ultrasonics, Teltow, Germany) for cell lysis. Cell debris was removed by centrifugation (8,000 × g, 4°C, 15 min) and subsequent filtration (0.45 µm).

Batch purifications were carried out for analytical preparations of CE from *Cellulosilyticum lentocellum* and *Dysgonomonas gadei* (**CICE** and **DgCE**, respectively). The crude extract was injected into an ÄKTA System (GE Healthcare, Little Chalfont, UK). The purification was carried out using affinity chromatography (**IMAC**) with a 12-mL Ni-IDA column (Ni-Profinity IMAC, Bio-Rad, Hercules, CA) equilibrated with loading buffer (20 mM Tris-HCl containing 150 mM NaCl and 20 mM imidazole, pH 8.0). The protein of interest was eluted using a linear gradient of imidazole (20–500 mM) for 4 column volumes with a flow rate of 1 mL/min at room temperature. Active fractions were pooled and the buffer was changed by size-exclusion chromatography (**SEC**; PD-10 column, GE Healthcare) to 20 mM Tris-HCl buffer, pH 8.0 for **CICE** and pH 7.5 for **DgCE** for the determinations of the temperature profiles in buffer and the kinetic parameters. The

buffer was exchanged to 2 mM 3-morpholinopropane-1-sulfonic acid/NaOH (**MOPS**) buffer, pH 7.5 or 8.0, for the pH profile and the temperature profile in milk and MUF. These buffer exchanges were carried out via SEC (PD-10 column, GE Healthcare).

The **DgCE** was purified by an automated enzyme purification system (Bioline chromatography system, Knauer Wissenschaftliche Geräte GmbH, Berlin, Germany) for the biotransformations. The instrumental setup was the same as described previously (Eisele et al., 2012; Stressler et al., 2012, 2013) apart from the columns used. For the purification of **DgCE**, about 150 mL of *E. coli* crude extract obtained by sonication was loaded via a superloop (150 mL; GE Healthcare) onto a 12-mL Ni²⁺ charged Profinity IDA column (Ni-Profinity IMAC, Bio-Rad) equilibrated with loading buffer (20 mM Tris-HCl containing 150 mM NaCl and 20 mM imidazole, pH 7.5). The whole purification procedure was carried out at a flow rate of 2 mL/min at room temperature. Elution from the first column was achieved by a buffer shift to 20 mM Tris-HCl containing 150 mM NaCl and 200 mM imidazole, pH 7.5. The eluting protein fraction was parked in a second superloop (50 mL; GE Healthcare) and loaded onto a desalting column afterward (HiPrep 26/10 Desalting 53 mL; GE Healthcare). This SEC step was carried out to exchange the buffer containing imidazole with a 2 mM MOPS buffer pH 7.5. The final fraction (20 mL) was freeze-dried (Alpha 1–2; Martin Christ Gefriertrocknungsanlagen, Osterode am Harz, Germany) to obtain a lyophilized **DgCE** preparation for biotransformation applications and later use.

Enzyme Assays

Thin layer chromatography was carried out for the qualitative activity evaluation of the CE. Thus, about 50 µL of glucose, fructose, lactose, lactulose, cellobiose, or maltose at concentrations of 300 mM in 20 mM Tris-HCl buffer were mixed with 50 µL of purified CE solution. After 16 h of incubation at 40°C, the samples were diluted 1:5 with water and 1 µL was loaded onto a thin layer chromatography plate (Silica 60 F245; Merck). Visualization of the reaction products was carried out with diphenylamine aniline, as described by Stahl (1967). *N*-Acetylglucosamine was also tested qualitatively for conversion. The assay was the same as for the other sugars, but 1 nmol of ATP was added to the reaction tube. Investigation of the reaction products was carried out by HPLC.

The standard assay for the determination of enzyme activity was carried out as follows. An amount of 100 µL of substrate solution (50 g/L of lactose in 20 mM Tris-HCl, pH 8.0 for **CICE** or pH 7.5 for **DgCE**)

was added to 100 μ L of sample containing CE. The reaction at 40°C was stopped after 10 min for *CICE* and after 2 min for *DgCE* with 400 μ L of 1 M HClO₄.

Samples of 1 mL were taken and a SEC (PD-10 column, GE Healthcare) for matrix exchange and sugar separation was carried out for the evaluation of the enzyme activity during biotransformations. The assay was performed as stated previously with an adapted incubation time, with the resulting CE fraction in 20 mM Tris-HCl, pH 8.0 for *CICE* or pH 7.5 for *DgCE*. These assays were followed by HPLC analysis to determine the concentration of the reaction products.

HPLC Method

An HPLC system (Spectra System, Thermo Fisher Scientific, Waltham, MA) was used for the determination of reaction products from conversions catalyzed by CE. Therefore, a sample preparation adapted from Erich et al. (2012) was carried out as follows. After stopping the reaction, 200 μ L of 2 M KOH was added to the reaction mixture to reach a neutralized solution. Subsequently, 200 μ L of a 50 mM inositol solution (serving as an internal standard) was added, and double-distilled water was also added to achieve a final volume of 2 mL. After incubation on ice for 15 min, the sample was centrifuged (20,000 $\times g$, 4°C, 3 min) and 50 μ L of the sample was injected into the HPLC system. The HPLC was equipped with a Eurokat Pb²⁺ column (Vertex Plus 300 $\times$ 8 mm 10 μ m; Knauer Wissenschaftliche Geräte GmbH) at 85°C and a refractive index detector (Spectra System RI-150, Thermo Fisher Scientific) at 50°C. The mobile phase was double-distilled water with a flow rate of 0.60 mL/min. Retention times were 13.1 min for lactose, 16.1 min for epilactose, and 27.0 min for inositol.

Determination of Biochemical Parameters

The native size of the CE was evaluated by SEC. Therefore, 30 μ L of the purified enzyme solution was loaded onto a 24-mL Sephadex 200 10/300 GL column (GE Healthcare). The flow rate was 0.5 mL/min and the mobile phase was 50 mM sodium phosphate buffer at pH 7.0 supplemented with 150 mM NaCl. The sizes were calculated according to a calibration curve (Gel filtration calibration Kit LMW; GE Healthcare) based on the retention time obtained. Furthermore, the activity of the peak fraction was tested by the standard assay with overnight incubation.

Purified CE in 2 mM MOPS buffer was used for the determination of the pH profile. A 50-g/L lactose solution in 20 mM modified Britton-Robinson buffer (20 mM acetic acid, 20 mM phosphoric acid, and

20 mM glycine) was used as a substrate (Britton and Robinson, 1931). The pH value of the substrate solution varied between pH 4 and 11 by the addition of NaOH. Conversions were carried out with 100 μ L of enzyme solution and 100 μ L of buffered substrate solution. After an appropriate time, the reaction was stopped by the addition of 400 μ L of 1 M HClO₄. Sugar contents were determined by HPLC analysis.

A 20 mM Tris-HCl buffer was used at pH 8.0 for *CICE* and pH 7.5 for *DgCE*, respectively, to determine the temperature profile in buffer. An amount of 100 μ L of enzyme solution and 100 μ L of 50 g/L of lactose in 20 mM Tris-HCl at the accordant pH was added. Assay temperature varied between 8 and 90°C. After an appropriate time, the reaction was stopped by the addition of 400 μ L of 1 M HClO₄ and the reaction mixtures were analyzed by HPLC.

The determination of the temperature profile in 50% (vol/vol) milk and 100% MUF was carried out using a purified enzyme preparation in 2 mM MOPS and 100 μ L of substrate. The temperature varied between 8 and 90°C. The determination of the temperature-dependent enzyme activity was carried out using the standard enzyme assay with an adapted conversion time and HPLC analysis.

Purified enzyme in 20 mM Tris-HCl buffer was used for the determination of the kinetic parameters. Lactose in 20 mM Tris-HCl buffer at pH 8.0 for *CICE* and pH 7.5 for *DgCE* served as the substrate, and the lactose concentration varied between 20 and 437.5 mM. The assay was carried out by adding 175 μ L of substrate solution to 25 μ L of enzyme solution. The reaction was stopped after 10 min for *CICE* and 5 min *DgCE* by adding 400 μ L of 1 M HClO₄. Analysis of the sugars was performed by HPLC.

Biotransformations

Batch biotransformations were carried out in 50-mL stirred vessels with a 20 mL working volume at 8 $\pm$ 2°C for 28 h. First, 433.2 $\pm$ 18.8 nkat of purified *DgCE* in 2 mM MOPS (equivalent to 21.7 nkat/mL of MUF) was freeze-dried directly in the reaction vessel. Then the bioconversion was started by the addition of MUF to the experimental setup. All experiments were carried out in duplicate in 2 separate vessels. A third vessel without enzyme served as a negative control. Samples, each of 1.5 mL, were taken for the quantification of sugars and measurement of residual enzyme activity. The sugar content was determined by HPLC. To determine the residual CE activity, 1 mL of the sample was subjected to SEC (PD-10 column, GE Healthcare) to exchange the matrix and to remove sugars. Subsequently, the standard assay was carried out. The mi-

Figure 1. Schematic flow diagram of the enzyme membrane reactor (EMR) setting. P1 = hose pump; PI1, PI2 = pressure gauge; F1 = tangential flow membrane module; B1 = feed tank (2.0 L); B2 = stirred reaction vessel (0.5 L); V1 = transmembrane pressure regulation valve.

crobial stability of the systems was checked by plating aliquots of 100 μ L on plate count agar plates (2.5 g/L of yeast extract, 5 g/L of peptone, 1 g/L of glucose, and 15 g/L of agar). The number of colony-forming units was counted after 48 h of incubation at 30°C.

A continuous biotransformation was carried out in an enzyme membrane reactor (EMR). The setup consisted of a 500-mL stirred vessel, a 10-kDa molecular weight cut-off (MWCO) polyethersulfone tangential flow membrane (Minimate TFF Capsule; Pall Corporation, Port Washington, NY), and a feed tank (Figure 1). The whole setup was operated at $8 \pm 2^\circ\text{C}$; MUF from the same batch as in the batch biotransformation served as substrate and feed solution. During the operation time of 6 d, 2.8 L of MUF was processed. About $5.20 \pm 0.23 \mu\text{kat}$ *DgCE* in 2 mM MOPS (equivalent to 21.7 nkat/mL of MUF), obtained from the automated purification, was freeze-dried in the reaction vessel (as described previously) and the EMR was started by the addition of 240 mL of MUF. Transmembrane pressure was continuously monitored by a pressure gauge and initially set to 50 kPa. The resulting eluate flux was aimed to be 20 mL/h. This flux was kept constant by adjusting the transmembrane pressure. This resulted in a residence time of 12 h. Eluate samples were taken on a regular basis by collecting the efflux for 3 min. From these samples, the flux (by weighing) and sugar content (by HPLC) were determined. Moreover, samples from the reaction vessel were taken to monitor microbial stability and the CE activity. For the microbial stability, 100 μ L of sample was spread on plate count agar plates and microbial counts were evaluated. The enzyme activity was measured after SEC (PD-10 column, GE Healthcare) by the standard assay (see above). The eluate was collected in separate batches (12 h each). Sugar content (by HPLC) and enzyme activity was also measured in these batches to check for possible enzyme leakage (by standard assay after SEC with 24 h of incubation time).

Statistics and Replication of Experiments

All enzymatic assays and analytic measurements were performed at least in duplicate. Statistical analyses, calculation, and visualization were carried out using SigmaPlot 12 (Systat Software, San Jose, CA) or Excel 2007 (Microsoft Corporation, Redmond, WA). The kinetic parameters were calculated by nonlinear regression fitting with the Enzyme Kinetics Module of SigmaPlot 12 (Systat Software).

RESULTS

Selection of Putative CE and Confirmation of Epimerization Activity

We carried out a sequence similarity search with the Basic Local Alignment Search Tool (The UniProt Consortium, 2014) for the in silico identification of novel CE. Whole AA sequences of the known CE from *R. albus* (UniProtKB E6UB41; Ito et al., 2007), as well as the whole AA sequence of *Caldicellulosiruptor saccharolyticus* CE (UniProtKB A4XGA6; Kim and Oh, 2012) were used as search templates. The search was refined by checking the results for the known (α/α)₆ barrel structure of AGE (Lee et al., 2007; Ito, 2010) by PSIPRED (Buchan et al., 2013) and the presence of reported residues responsible for the CE activity (Ito et al., 2009). Among others, the CE-like proteins UniProtKB F2JR71 and UniProtKB F5ITJ6 were identified with identities of 54 and 40% compared with *RaCE* (Figure 2). The sequence identity between both F2JR71 and F5ITJ6 was also low, at 40%. The catalytic residues of *RaCE* (His184, His243, and His374) have been identified in both sequences (Mu et al., 2013) and been predicted to be His190, His249, and His380 in F2JR71, and His184, His243, and His373 in F5ITJ6. The 395-AA-long sequence F2JR71 was automatically translated from the gene locus European Nucleotide Archive, association number ADZ85052.1, of *Cellulosilyticum lentocellum* DSM 5427. This microorganism was isolated by Murray et al. (1986) from river sediment with paper mill waste. *Cellulosilyticum lentocellum* is an anaerobic bacterium belonging to the class Clostridia and the phylum of *Firmicutes*. The second extracted AA sequence (F5ITJ6) is 388 AA long. The coding nucleotide sequence is EGJ99380.1, which was obtained from *Dysgonomonas gadei* DSM 22811. *Dysgonomonas gadei* is a facultative anaerobically growing bacterium belonging to the class and phylum of *Bacteroidetes* and was isolated from a human gall bladder (Hofstad et al., 2000).

After cloning the wild-type genes (Supplementary Figures S1 and S2; [Journal of Dairy Science Vol. 98 No. 6, 2015](http://dx.doi.org/10.3168/jds.2015-</p>
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Figure 2. Alignment of *Ruminococcus albus* cellobiose 2-epimerase (RaCE; E6UB41), *Cellulosilyticum lentocellum* cellobiose 2-epimerase (CICE; F2JR71), and *Dysgonomonas gadei* cellobiose 2-epimerase (DgCE; F5ITJ6). Similar AA are marked with periods (.) and colons (:); identical amino acids are highlighted by an asterisk (*). Catalytic active residues in RaCE are marked by ▼. Alignment was constructed with UniProt/Clustal (The UniProt Consortium, 2014).

did not serve as substrates for either enzymes (Table 1). Therefore, F2JR71 was identified as a novel CE, which we named *C*CE; the second novel CE (F5ITJ6) identified was named *Dg*CE. Quantitative HPLC analyses of the conversion products of lactose showed that lactose was epimerized to epilactose for both CE. No formation of lactulose was detected, even in elongated

Table 1. Substrate spectrum of novel cellobiose 2-epimerases (CE) from *Cellulosilyticum lentocellum* and *Dysgonomonas gadei*¹

Item ²	Glc	Fru	Lac	Lactu	Cello	Malto	GlcNAc ³
<i>Cellulosilyticum lentocellum</i> CE	–	–	+	–	+	–	–
<i>Dysgonomonas gadei</i> CE	–	–	+	–	+	–	–

¹Glc = glucose; Fru = fructose; Lac = lactose; Lactu = lactulose; Cello = cellobiose; Malto = maltose; GlcNAc = *N*-acetyl glucosamine.

²+ = reaction products detected by thin layer chromatography; – = reaction products not detected by thin layer chromatography.

³Analysis of the reaction mixture containing GlcNAc was not carried out by thin layer chromatography, but by HPLC.

incubation for 24 h, by either enzyme. Moreover, the cellobiose was converted to a single reaction product, which was assumed to be glucosylmannose (Ojima et al., 2011). The measured activity was higher in both CE toward cellobiose (*CICE*: 24.3 nkat/mL; *DgCE*: 118.0 nkat/mL) than toward lactose (*CICE*: 12.6 nkat/mL; *DgCE*: 108.0 nkat/mL). Also, the molecular size of the purified enzymes was determined (Table 2). The theoretically calculated size for *CICE* was 46 kDa. Similar sizes were observed under denaturing conditions by SDS-PAGE (Figure 3) and SEC under native conditions with sizes of 43 and 52 kDa, respectively. A theoretical size of 45 kDa was calculated for *DgCE*, which was confirmed by SDS-PAGE with a size of 42 kDa (Figure 3). A size of 33 kDa was evaluated via SEC under native conditions.

Effects of pH and Temperature on Enzyme Activity

We investigated the pH and the temperature profiles of both CE (Table 2). The pH profile was tested in universal Britton-Robinson buffer (Britton and Robinson, 1931), whereas the temperature profile was tested in buffer system, as well as in milk and MUF.

The highest activity was exhibited by *CICE* at pH 8.0 (100% = 83.0 nkat/mL). Besides the pH optimum, the activity dropped quickly. No activity was measured below pH 4.0 or above 11.0. At pH 7.0, we detected about 85% residual activity. Concerning the optimal assay temperature, the enzyme showed maximal activity at 40°C in Tris-HCl buffer pH 8.0, in 50% milk and in MUF (applied enzyme activities: 40.1 nkat/mL in buffer, 35.4 nkat/mL in milk, and 18.4 nkat/mL in MUF). The temperature optimum is the same in defined buffer solution at an optimal pH, in milk and in MUF. In comparison to the temperature optimum, an activity of 16% in buffer and milk and 20% in MUF was measured at 8°C. No activity was measured at temperatures higher than 80°C in milk and MUF matrix and 90°C in buffer matrix. The optima of *DgCE* differed moderately by means of the pH optimum and the temperature optima. The pH profile of *DgCE* showed an optimum at pH 7.5 (100% = 192.3 nkat/mL). No activity was measured below pH 4.0 or above 11.0, which is identical for

DgCE and *CICE*, and we observed about 97% activity at a pH of 7.0.

Differences in the temperature profiles of *DgCE* in buffer and 50% milk, as well as in MUF matrix were observed. The optimal activity could be detected at 40°C in buffer matrix (100% = 467.5 nkat/mL), whereas it was 50°C in milk and MUF matrix (100% = 112.0 nkat/mL for milk and 277.3 for MUF). Above the optima at 60°C, the activity dropped fast to 3% in buffer, and 17% in milk and MUF. At an assay temperature of 8°C activities of 2, 9, and 7% were measured in buffer, milk, and MUF, respectively.

Kinetic Parameters of Lactose Epimerization

For comparison of the kinetic performance of *CICE* and *DgCE* in lactose epimerization, the kinetic parameters were investigated. For the epimerization reaction, a Michaelis-Menten-like kinetic was observed for both CE. The *CICE* showed an apparent K_m (Michaelis

Table 2. Biochemical characterization of novel cellobiose 2-epimerases (CE) from *Cellulosilyticum lentocellum* and *Dysgonomonas gadei*

Item	<i>Cellulosilyticum lentocellum</i> CE	<i>Dysgonomonas gadei</i> CE
Molecular mass (kDa)		
Calculated ¹	46	45
SDS-PAGE	43	42
Size exclusion chromatography	52	33
pH optimum ²	8.0	7.5
Temperature optimum ³ (°C)		
Buffer	40	40
Milk	40	50
MUF	40	50
K_m ⁴ (mM)	129	108
v_{max} ⁴ (nkat/mL)	92	173

¹Sizes were calculated by ExPASy (<http://www.expasy.org/>).

²The pH profiles were evaluated in 20 mM modified Britton-Robinson buffer of the pH range from 4 to 11.

³Temperature profiles were measured in Tris-HCl buffer at the pH optimum, in 50% (vol/vol) milk or milk-ultrafiltrate (MUF), respectively.

⁴Kinetic parameters (K_m = Michaelis constant; v_{max} = maximum enzyme rate) were determined at the pH and temperature optimum with lactose concentrations from 20 to 437.5 mM. Parameters were calculated by nonlinear regression fitting with the Enzyme Kinetics Module of SigmaPlot 12 (Systat Software Inc., San Jose, CA).

Figure 3. Sodium dodecyl sulfate-PAGE of novel cellobiose 2-epimerases (CE) fractions: *Cellulosilyticum lentocellum* CE (A) and *Dysgonomonas gadei* CE (B). Lane M = molecular mass marker; lane I = crude protein solution at time of induction; lane H = CP solution at time of harvest; lane P = CE-active fraction after purification (affinity chromatography and desalting by size exclusion chromatography).

constant) value of 129 ± 14 mM and $v_{\max}$ (maximum enzyme rate) of 92 ± 4 nkat/mL toward lactose. The *DgCE* showed a slightly higher affinity for lactose and a higher maximal conversion speed: the K_m value was 108 ± 5 mM and the $v_{\max}$ was 173 ± 3 nkat/mL (Table 2).

Production and Purification of *DgCE* for Biotransformations

With the aim of preparing 1 standardized batch of a *DgCE* formulation, the enzyme was produced in a 12-L stirred-tank reactor. *Escherichia coli* Rosetta-gami contained the plasmid pET-20b*DGCEHIS*, which was the expression host. During the 9 h of cultivation (Figure 4), bacterial growth was observed for 5.5 h after inoculation. Thereafter, the cell mass was constant at 9.5 g/L (bio DM) until the cells were harvested at the end of cultivation. The CE formation was growth-related, reaching a total activity of 49.0 μ kat at the end of cultivation.

For purification of *DgCE* from 114 g (bio DM) of *E. coli* cells, an automated 2-step purification was carried out. After cell disruption, 150 mL of cell-free extract was handled automatically and, after a total runtime of 290 min, a 20-mL fraction containing *DgCE* was obtained in a single run (Figure 5). In total, 20.3 μ kat (specific activity: 572.6 nkat/mg) of *DgCE* were obtained after 7 single runs in buffer for lyophilization

(content of the whole bioreactor). This *DgCE* activity is according to a yield of 41%.

Production of Epilactose in MUF by *DgCE*

For biotransformations, a lyophilized, standardized *DgCE* preparation from the automated purification was used (CE activity: 572.6 nkat/mg). The epilactose formation was investigated with *DgCE* as a biocatalyst and MUF as matrix and substrate in batch biotransformations and in a continuously operated EMR. A process temperature of 8°C, a temperature suitable for milk processing in the dairy industry, was chosen.

The mean values of the batch biotransformations are shown in Figure 6. The biotransformation with 21.7 nkat/mL of *DgCE* resulted in 14.44 ± 0.53 g/L of epilactose. Therefore, the epilactose yield compared with the initial lactose content of MUF was 29.9%. The apparent reaction equilibrium was established after 10 h and remained constant until the biotransformation was stopped after 28 h. After this time, *DgCE* showed 90% of the initial activity. For verification of the assumed reaction equilibrium, fresh *DgCE* was added to the reaction mixture again. This did not change the sugar concentrations, indicating the true equilibrium. Calculating the enzyme productivity (expressed as the amount of epilactose formed per *DgCE* activity applied) at the time of the establishment of equilibrium (10 h), a value of 0.68 mg/nkat was obtained. The whole system

Figure 4. Bioreactor cultivation of *Escherichia coli* Rosetta-gami pET-20b*DGCEHIS* on a 12-L scale [medium: Terrific broth (Carl Roth, Karlsruhe, Germany), 37°C, pH 7.0]. Enzyme activity was measured after cell disruption and purification. The culture was induced with 0.5 mM isopropyl β -D-1-thiogalactopyranoside at 2 h after inoculation. BDM = bio dry matter; *DgCE* = *Dysgonomonas gadei* cellobiose 2-epimerase. Error bars represent SD.

Figure 5. Automated 2-step purification of *Dysgonomonas gadei* cellobiose 2-epimerase (*DgCE*). About 150 mL of cell-free extract was loaded into a Ni-IDA column (1 column volume = 12 mL; Ni-Profinity IMAC, Bio-Rad, Hercules, CA). The first peak corresponds to the cellobiose 2-epimerase-containing elution peak from the Ni-IDA. In a subsequent step, the buffer of this fraction was exchanged with 2 mM 3-morpholinopropane-1-sulfonic acid at pH 7.5 by size exclusion chromatography (SEC) utilizing a Hi Prep 26/10 desalting column (1CV = 15 mL; HiPrep 26/10 Desalting 53 mL, GE Healthcare, Little Chalfont, UK).

was microbiologically stable (cell counts after 28 h = $2 \cdot 10^2$ cfu/mL).

We carried out a continuous biotransformation under identical conditions as in the batch transformation by using EMR technology. A 10,000-Da MWCO membrane retained the *DgCE* within the reaction vessel. Enzyme leakage of *DgCE*, which is 3 times larger than the pore size, was not observed by checking CE activity in the permeate. Moreover, the system could be operated under stable flux for 6 d, leading to the assumption that no or very little membrane fouling, caused by the low residual protein content (581 mg/L) of MUF, was occurring. The *DgCE* showed an operational stability for the 6 d of operation with a residual activity of 31% at the end. After 2 d of operation, a plate count of 3.6×10^5 cfu/mL was determined. To prevent further growth, NaN_3 was added at this point.

The enzyme activity was the same in both processes, batch and EMR, with 21.6 nkat/mL. We chose a residence time of 12 h for the EMR process because the time to reach equilibrium in the batch process was approximately 10 h. According to the reactor volume

of 240 mL, a theoretical permeate flux of 20 mL/h was targeted. The actual average flux was 19.8 ± 1.2 mL/h. This resulted in a total permeate volume of 2.8 L within the operation time of 144 h [equal to 11.6 volume replacements (VR)]. Stable epilactose concentration in the permeate at a mean concentration of 8.93 ± 0.67 g/L was measured 8 h (0.7 VR) after starting the EMR. This corresponded to a yield of 18.5% epilactose. In total, the EMR process produced about 24 g of epilactose. During the operation time, the epilactose concentration decreased slightly from 9.93 ± 0.43 (after 14 h; 1.1 VR) to 7.27 ± 0.16 g/L (after 132 h; 10.9 VR). The respective space-time yield decreased from the maximum of 0.80 to 0.57 g/L per hour. An average enzyme productivity of 4.56 mg/nkat was achieved after 12 VR (Figure 7). In the batch process, an enzyme productivity of only 0.68 mg/nkat was achieved because the biocatalyst has to be replaced with every new batch (1 VR). This means that about 571% more epilactose could be produced in the EMR process with the same *DgCE* activity as used in the batch process.

Figure 6. Time course of epilactose production in milk ultrafiltrate by *Dysgonomonas gadei* cellobiose 2-epimerase (CE) in batch mode (mean values of 2 independent biotransformations). The working volume was 20 mL. Error bars represent SD.

DISCUSSION

Assignment of the Gene Products Obtained to the Enzyme Class of CE

We provide evidence that 2 DNA sequences from *C. lentocellum* and *D. gadei* are coding for CE activity. We proved that the recombinant enzymes catalyze a C2-epimerization reaction of the glucose or mannose motive of β -1,4-linked disaccharides. An activity against monosaccharides was not detected. This substrate spectrum is similar to the CE, which form epilactose as previously described (Ito et al., 2008; Taguchi et al., 2008; Senoura et al., 2009; Ojima et al., 2011, 2013). Lactulose also does not serving as a substrate for *CICE* and *DgCE*. This distinguishes the novel CE identified from the lactulose-forming CE, *Caldicellulosiruptor saccharolyticus* CE (Park et al., 2011), *Dictyoglomus turgidum* CE (Kim et al., 2012), and *Spirochaeta thermophila* CE (Park et al., 2013). The α -1,4-linked disaccharide maltose is not accepted as a substrate for *CICE* or *DgCE*, which was also reported for *RaCE* (Ito et al., 2007) and *RmCE* (Ojima et al., 2011).

The *CICE* and *DgCE* both originate from mesophilic, anaerobic bacteria, similar to most known CE that produce epilactose. *Cellulosilyticum lentocellum* is taxonomically grouped to the Clostridia, whereas *D. gadei* is grouped to the phylogenetically very distant Bacteroides. Involvement of *CICE* in a cellulose pathway is imaginable because *C. lentocellum* is a bacterium

which degrades cellulose slowly. Cellulose degradation was not reported for *D. gadei*. However, an involvement of both enzymes in a mannan-degrading pathway, as assumed by Senoura et al. (2009) for other CE, might be possible. This mannan-degrading pathway has been suggested for *Bacteroides fragilis* CE (*BfCE*; Senoura et al., 2011), *RaCE* (Kawahara et al., 2012) and *RmCE* (Kawahara et al., 2012). The physiological function of *CICE* and *DgCE* is not clear and has to be proven by further sequence analyses and experimental data.

Similar to the taxonomical distance between the bacteria from which *CICE* and *DgCE* are derived, the sequences of the CE share low overall sequence homologies of only 40%. The low sequence homology observed between the 2 CE is also noticeable between all CE reported (Mu et al., 2013). However, the secondary structure prediction suggests that the protein structure of the novel CE is similar to the enzymes reported. Moreover, AA residues reported responsible for disaccharide binding and catalysis are present in the structures (Figure 2). Three histidine residues, which have been reported to be essential for catalysis in *RaCE* (Fujiwara et al., 2013a) and *RmCE* (Fujiwara et al., 2014), have been identified in the primary sequences of *CICE* and *DgCE*. These findings on the AA sequence level indicate that the newly identified enzymes follow the same catalytic mechanism as that suggested for *RaCE* (Amein and Leatherwood, 1969; Ito et al., 2009; Fujiwara et al., 2013a) and confirmed for *RmCE* (Fujiwara et al., 2014).

Figure 7. Dependency of the *Dysgonomonas gadei* cellobiose 2-epimerase (*DgCE*) efficiency on the number of volume replacements in the batch process and in enzyme membrane reactor (EMR) process.

Biochemical Properties of *CICE* and *DgCE*

The biochemical characterizations of the enzymes identified are in accordance with literature about CE reported previously. As is often observed for enzymes from mesophilic organisms, both enzymes exhibit the temperature optimum at a mesophilic temperature of 40°C in buffer. For CE that form epilactose, temperature optima in different buffer systems ranging from 35 to 50°C have been reported. Currently, only 1 enzyme that forms epilactose, *RmCE*, with a higher temperature optimum of 80°C has been reported (Ojima et al., 2011). For *DgCE*, a stabilizing effect shifting the temperature optimum to 50°C in milk and MUF was observed. This is probably caused by the ionic composition of these matrices. We did not observe this effect for *CICE*. About 7% activity of the maximal *DgCE* activity was preserved at a process temperature for the biotransformations of 8°C. This activity was suitable to carry out batch and continuous biotransformations of MUF. Similar residual activities of 5.5% (*Flavobacterium johnsoniae* CE; *FjCE*) and 15.9% (*Pedobacter heparinus* CE; *PhCE*) were shown to be suitable in biotransformations of milk published previously (Krewinkel et al., 2014). The pH profiles of *CICE* and *DgCE* determined showed the highest activities at slightly alkaline pH values. This is also true for all CE reported. The optimum of *CICE* is pH 8.0 and of *DgCE* is pH 7.5. Activities at neutral pH values found in milk and MUF are appropriate with 85 (*CICE*) and 97% (*DgCE*) for

biotransformations in these matrices. The pH profiles are consistent with the assumption that histidine residues with a pK_a (acid dissociation constant) of 6.0 are involved in the catalytic mechanism.

The molecular sizes of *CICE* and *DgCE* actually determined under denaturing conditions on SDS-PAGE are in accordance with the theoretically calculated molecular mass. With a molecular mass of about 43 (*CICE*) and 42 kDa (*DgCE*), the sizes of the enzymes are similar to the sizes for CE reported previously, ranging from 41 to 47 kDa. Under native conditions, the size for *CICE* was slightly larger (52 kDa), indicating that the enzyme is active in a monomeric state. We also confirmed a monomeric state for *DgCE*. However, the size measured by SEC under native conditions was 33 kDa. Activity was confirmed in the elution fractions of SEC, verifying the identity of the enzyme and the active monomeric state. Based on the size of 33 kDa for *DgCE*, a membrane with an MWCO of 10 kDa was selected for the EMR setup and later proven to be suitable by measuring no enzyme leakage.

The kinetic parameters for both CE were determined under similar conditions as those described in literature (Taguchi et al., 2008; Ojima et al., 2011, 2013). Nevertheless, comparing the values was difficult. Reported K_m values for lactose among recombinant CE are diverse, ranging from 6.6 mM (*BfCE*; Senoura et al., 2009) to 238 mM (*Teredinibacter turnerae* CE; Ojima et al., 2013). The novel CE identified exhibit high K_m values for lactose. The K_m of *CICE* is 129 mM and for

DgCE is 108 mM. These values support the assumption that lactose might not be the prior substrate. Nevertheless, the affinity of *DgCE* to lactose was suitable to carry out the biotransformations of lactose in MUF. However, utilization of CE with a higher affinity to lactose, such as the mesophilic *BfCE* (Senoura et al., 2009), might lead to even more effective processes in diluted lactose solution. Alternatively, utilization of lactose concentrates with higher lactose concentrations compared with the K_m values of CE would be advantageous for achieving more efficient processes.

Production of *DgCE*

For the biotransformations, *DgCE* was produced in a 12-L stirred-tank reactor indicating the possibility of a later upscaling to larger fermentation processes. This resulted in a total *DgCE* activity of 49.0 μ kat. After automated purification, 20.3 μ kat of *DgCE* were obtained, which accounts for a yield of 41% and a specific activity of 572.6 nkat/mg. The purification yield and specific activity are comparable to yields for *PhCE* reported previously (Krewinkel et al., 2014). The utilized expression system, *E. coli* Rosetta-gami, in combination with the pET system providing the His₆-tag, was suitable for CE production. The His₆-tag allowed a reproducible and fast automated purification of *DgCE* with short hands-on time (*DgCE* activity in the final fractions from 7 consecutive runs varied between 125.3 and 153.0 nkat/mL). With respect to a later application of these enzymes in food technology, an expression in antibiotic-free food-grade expression systems seems indispensable.

Biotransformation of Lactose-Containing Foodstuff

The biotransformations with *DgCE* as biocatalyst were carried out at 8°C, which is a suitable storage and process temperature for milk. The substrate and matrix of both biotransformations was MUF, a highly available sidestream product of milk protein production. The lactose content of MUF is comparable with the content of milk, but only marginal protein is present. The lactose content of about 48.3 g/L makes this foodstuff attractive as a valuable source for epilactose production. Due to the low protein content, a long operation time of more than 6 d with stable fluxes were achieved. Membrane fouling, which is caused by macromolecule deposition at the EMR membrane, was not observed. The lactose of MUF could be converted to a product of higher value with both processes, batch and continuous, because the in situ production of the putative prebiotic disaccharide epilactose (Lange and Vogel, 2009) was shown.

Within 10 h of operation of the batch process, 14.4 g/L of epilactose was produced from the initial lactose content (48.3 g/L) of MUF. This led to a mean conversion yield of 29.9%. This equilibrium was confirmed by further operation of the process up to 28 h and the addition of fresh enzyme.

A similar biotransformation in milk was reported in earlier works by Krewinkel et al. (2014) and led to comparable results. In our study, the mesophilic CE *PhCE* and *FjCE* were used under identical reaction conditions (8°C, 20-mL reaction volume) for a biotransformation of milk lactose. With enzyme activities of 352 and 199 nkat, about 30.5 and 33.6% conversion yield were achieved within 9 h of biotransformation for *PhCE* and *FjCE*, respectively. Saburi et al. (2010) showed a conversion yield of approximately 27% in a bioconversion carried out by *RaCE*. This biotransformation was performed at room temperature in 15 L of sodium phosphate buffer containing 3 kg of lactose monohydrate for 48 h (Saburi et al., 2010). The *RmCE* reached a conversion yield of about 30% epilactose in a biochemical sodium phosphate buffer system in another bioconversion (Sato et al., 2012). This reaction was carried out with an immobilized CE at 50°C for 10 d.

To enhance the enzyme productivity and lower the effort for enzyme production for epilactose production a continuous biotransformation with an EMR was carried out. It was remarkable that the EMR process ran with a stable product flux for 6 d. In total, 24 g of epilactose were produced. This yield was achieved with the same *DgCE* activity (21.7 nkat/mL) as in the batch process, where 288 mg of epilactose were produced. Considering the enzyme productivity of both processes, the EMR, with 144 h of operation time (11.6 VR) was about 6 times more productive than the batch process. Considering that enzyme inactivation and leakage were negligibly low and *DgCE* was acceptably active when stopping the EMR after 6 d of operation, a longer EMR runtime, leading to even higher enzyme productivity, would have been possible. Microbiological stability was acceptable until d 3. Thereafter, addition of a bacteriostatic agent was necessary to keep this laboratory process stable. Bacterial growth was caused by a nonsterile handling of the feed solution and subsequent concentration of the MUF-inherent microorganisms during the EMR operation. These difficulties are to be avoided by sterile handling of the feed MUF or a direct implementation of the EMR module after the UHT process in a sterile fashion.

Nevertheless, with this proof of principle study, we showed for the first time that epilactose could be produced continuously from a sidestream food matrix. A direct application of the MUF that contains epilactose

for food applications or a further purification of the epilactose is conceivable.

In general, the industrial application of enzymatic epilactose production seems interesting due to the higher value of epilactose compared with lactose. Watanabe et al. (2008) recently reported on a bifidogenic effect of epilactose similar to lactulose in animal models. Currently, there is no industrial production process for epilactose; but the enzymatic process reported demonstrates the feasibility of epilactose formation from a cheap food sidestream product without formation of detectable by-products.

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