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Enzymatic production of lactulose and epilactose in milk

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ABSTRACT

The enzymatic production of lactulose was described recently through conversion of lactose by a thermophilic cellobiose 2-epimerase from *Caldicellulosiruptor saccharolyticus* (*Cs*CE). In the current study, we examined the application of *Cs*CE for lactulose and epilactose production in milk (1.5% fat). The bioconversions were carried out in stirred reaction vessels at 2 different temperatures (50 and 8°C) at a scale of 25 mL volume. At 50°C, 2 highly different *Cs*CE amounts were investigated for the time course of formation of lactulose and epilactose. The conversion of milk lactose (initial lactose content of 48.5 ± 2.1 g/L) resulted in a final yield of 57.7% (28.0 g/L) lactulose and 15.5% (7.49 g/L) epilactose in the case of the approximately 9.5-fold higher *Cs*CE amount (39.5 $\mu kat_{epilactose,\ 50°C}$) after 24 h. Another enzymatic lactose conversion was carried out at low 8°C, an industrially relevant temperature for milk processing. Although the *Cs*CE originated from a thermophilic microorganism, it was still applicable at 8°C. This enzymatic lactose conversion resulted in 56.7% (27.5 g/L) lactulose and 13.6% (6.57 g/L) epilactose from initial milk lactose after 72 h. The time courses of lactose conversion by *Cs*CE suggested that first epilactose formed and afterward lactulose via epilactose. To the best of our knowledge, this is the first time that an enzyme has produced lactulose directly in milk in situ at industrially relevant temperatures.
Key words: cellobiose 2-epimerase, lactulose, epilactose, milk, prebiotic

INTRODUCTION

The disaccharide lactulose (4-O-β-D-galactopyranosyl-D-fructofuranose) is one of the most valuable lactose derivatives and possesses prebiotic properties (Petuely, 1957; Olano and Corzo, 2009). The application of lactulose as a functional food ingredient has been researched since 1957 (Petuely, 1957; Panesar and Kumari, 2011; Aït-Aissa and Aïder, 2014). An intake from 2 to 10 g of lactulose/d has been assigned to present prebiotic action (Terada et al., 1992; Mizota et al., 2002; Tuohy et al., 2002; Seki et al., 2007). This positive influence on the intestinal function has the potential to provide health benefits by alteration of colon microbiota as well as enhancing mineral adsorption as determined in several clinical trials (Oku and Okazaki, 1998; Seki et al., 2007; Sharma et al., 2008; Schuster-Wolff-Bühring et al., 2010). In Japan, the nutritional benefits of lactulose were acknowledged by the Japanese Ministry of Health and Welfare who claimed it as a "food of specific health use." Seki and Saito (2012) mentioned some functional food products containing lactulose (e.g., infant formula, soft drinks, and yogurt).

Lactose (4-O-β-D-galactopyranosyl-D-glucose) is isomerized to lactulose in milk during milk processing by a chemical reaction via the Lobry de Bruyn-Alberda van Ekenstein rearrangement, which is catalyzed by heat treatment and alkaline conditions (Schuster-Wolff-Bühring et al., 2010). According to this reaction, 0.1 to 0.9 g/L of lactulose can be found in UHT milk (Cattaneo et al., 2008; Manzi and Pizzoferrato, 2013). Industrial lactulose production has been carried out by chemical isomerization up to now. This process includes the use of chemical catalysts and various purification steps (Aïder and de Halleux, 2007; Schuster-Wolff-Bühring et al., 2010). Research has focused on finding alternative enzymatic processes to overcome these drawbacks during recent years. One approach is the use of β-galactosidases via the transgalactosylation reaction with lactose and fructose as substrates (Kim et al., 2006; Mayer et al., 2010; Guerrero et al., 2011). The disadvantage of this process is the need of the sweet co-substrate fructose (galactose acceptor), which is a cost and taste factor, respectively.

Regarding dairy products as yogurt or formula milk, the addition of commercially available lactulose can be redundant by generating lactulose in situ from initial

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milk lactose. This new approach of integrating the lactulose formation in the processing of a dairy product was recently described by Förster-Fromme et al. (2011). The research group produced a lactulose-containing milk serum drink by application of a β-galactosidase from *Aspergillus oryzae* to whey powder (6.9 g/L of lactose) along with 90 g/L of fructose.

Ito et al. (2008) reported that a cellobiose 2-epimerase (**CE**; EC 5.1.3.11) from *Ruminococcus albus* catalyzed the epimerization reaction of lactose into epilactose (4-*O*-β-D-galactosyl-D-mannose), another valuable lactose derivate (Watanabe et al., 2008). Further research regarding this enzyme class led to the identification of 2 thermophilic CE from *Caldicellulosiruptor saccharolyticus* (***Cs*CE**) and *Dictyoglomus turgidum*. Both enzymes catalyzed not only the epimerization reaction, but also a further isomerization reaction (Park et al., 2011; Kim et al., 2012). This novel isomerization reaction led to the enzymatic formation of lactulose from lactose. Thus, the application of *Cs*CE to milk may lead to a novel way of generating lactulose-enriched dairy products with prebiotic properties in situ. The enzymatic synthesis could be integrated in the processing of dairy products, which offers the opportunity to turn a by-product like whey or whey permeate into a valuable prebiotic product.

The enzymatic lactulose production from lactose by CE has only been investigated in synthetic buffer systems at high temperatures up to now (Kim and Oh, 2012; Kim et al., 2012). In the current study, the enzymatic lactulose production in milk was investigated for the first time. The application of the CE from *C. saccharolyticus* was performed under varied reaction conditions, such as altered initial enzyme activity and temperature. Experiments were carried out at 8°C, a typical temperature used in the milk industry, to illustrate the possible industrial applications of this enzymatic process.

MATERIALS AND METHODS

Chemicals, Enzymes, and Milk

All chemicals were of analytical grade or higher and supplied by Alfa Aesar (Heysham, UK), Merck (Darmstadt, Germany), Carl Roth (Karlsruhe, Germany), or Sigma-Aldrich (St. Louis, MO). 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). The UHT milk containing 1.5% fat (fettarme, haltbare Weidemilch 1.5%, Schwarzwaldmilch GmbH, Freiburg, Germany) was obtained from a local supermarket.

Microorganisms, Plasmids, and Gene Cloning

The strains *Escherichia coli* XL-1 Blue (New England Biolabs, Ipswich, MA) and *E. coli* BL21 (DE3) were used for cloning and expression. The genomic DNA sequence of CE from *C. saccharolyticus* DSM 8903 was obtained from the European Molecular Biology Laboratory, European Bioinformatics Institute (ENA Association No. ABP65941.1). The *Cs*CE gene was purchased as a synthetic gene with added *Nde*I and *Bam*HI restriction sites in a pMA vector from Life Technologies (Carlsbad, CA). Prior to the synthesis, optimized codon usage for expression in *E. coli* was achieved using Gene Designer (DNA2.0 Inc., Menlo Park, CA). In general, standard molecular biology methods according to Sambrook and Russell (2001) were used. After subcloning in pET-16b by *Nde*I and *Bam*HI, a PCR was performed using the primer pair csce_fw (5′ GACCACAACG-GTTTCCCTCTAG 3′) and csce_rev (5′ GATCTCGA-GATCCACGCG-TTTGATAATTTCC 3′). Subcloning in pET-20b (+) by *Nde*I and *Xho*I was carried out to introduce a C-terminal hexahistidine tag (**His_6-Tag**), followed by transformation in *E. coli* XL-1 Blue. The resulting construct pET-20b*CSCE* was transformed in *E. coli* BL21 (DE3) for the expression of the *Cs*CE.

Production, Purification, and Molecular Mass Determination of CsCE

Escherichia coli BL21 (DE3) pET-20b*CSCE* was cultivated in 15 L of 2-yeast-tryptone medium containing 16 g/L of tryptone, 10 g/L of yeast extract, 20 g/L of glucose, and 100 μg/mL of ampicillin in a 23-L stirred tank reactor (Biostat E. B. Braun, Melsungen, Germany) for 11 h. The expression of the *Cs*CE was induced at an optical density (600 nm) of 6.5 with 0.5 m*M* isopropyl-β-D-thiogalactopyranoside after 2.25 h of cultivation at 37°C. The cultivation was continued for 8.75 h at 20°C. The pH was regulated to 7.0 by adding 2 *M* NaOH and 0.6 *M* H_3PO_4.

The recombinant *E. coli* cells were harvested by centrifugation (8,000 *g*, 15 min, 4°C) and washed twice with 0.9% (wt/vol) NaCl solution. Subsequently, the cells were resuspended in 50 m*M* sodium phosphate buffer, pH 7.5, containing 300 m*M* NaCl, to obtain a 30% (wt/vol) cell suspension. The cell disruption was carried out by a 10 cycle sonication program on ice. Each cycle consisted of 1 min of sonication (95% amplitude, 0.5 cycles; UP200S/S3, Hielscher Ultrasonics, Teltow, Germany) and a break of 1 min. Cell debris was removed by centrifugation (8,000 × *g*, 15 min, 4°C). Afterward, the supernatant was heated for 15 min at 60°C and, subsequently, centrifuged (8,000 × *g*, 15 min, 4°C)