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Biochemical characterization of two cellobiose 2-epimerases and application for efficient production of lactulose and epilactose

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ABSTRACT

Lactose is the most abundant by-product of the dairy industry and is increasingly recognized as an important feedstock to produce value-added compounds. Lactulose and epilactose are valuable prebiotics that can be generated from lactose with cellobiose 2-epimerases (CEases). Here we describe the characterization of the two CEases *Cb*CEP and *Rf*CEP, originated from the thermophilic microorganism *Caldicellulosiruptor bescii* and the mesophilic microorganism *Roseburia faecis*, respectively. *Cb*CEP showed exceptional pH and temperature stability, with maximal activity at pH 7.5 and 70 °C. *Rf*CEP exhibited maximum activity at 50 °C and pH 8. Under optimal conditions in small-scale experiments with commercial lactose, *Cb*CEP produced both lactulose and epilactose with yields of 29.8% and 21.6%, respectively, while *Rf*CEP produced only epilactose with a yield of 19.3%. Furthermore, we evaluated the application of *Cb*CEP for lactulose and epilactose production in stirred fermenters at two different temperatures (70 and 30 °C) at a scale of 5 L volume and a concentration of cheese-whey permeate of 50 g/L. At 70 °C, lactulose was the predominant product of *Cb*CEP-mediated lactose conversion, with a final yield of 30% (12.8 g/L) lactulose and 24.7% (10.6 g/L) epilactose. At 30 °C, lactose was mainly converted into epilactose, with a final yield of 35% (14.9 g/L), and a minor amount of lactulose (final yield of 4.3%, 1.8 g/L). The findings presented here may guide the design of an industrial strategy, based on the temperature-tunable activity of *Cb*CEP, for production of valuable lactose derivatives at high yields directly from whey permeate.

1. Introduction

Cheese whey is a by-product of the dairy industry that causes significant disposal and environmental pollution problems (Smithers, 2008). Whey corresponds to 80–85% of the total milk volume entering the process and contains about 55% of milk nutrients, of which lactose (4-*O*-β-D-galactopyranosyl-D-glucose) is the main solid constituent. The annual worldwide production of whey reaches nearly 190 million tons, of which 70% is generated in Europe and North America, with an estimated growth of the production rate of 2%/year (Sitanggang et al., 2016). Numerically, just in Norway, about 100,000 tons of milk are processed each year, which generates about 85,000 tons of whey and about 4250 tons of lactose (Marquer, 2013). Although direct utilization of whey is difficult because of the low solubility and sweetening power, one approach for valorization that has attracted interest is the conversion of lactose to various derivatives with applications in the food and pharmaceutical fields (Seki and Saito, 2012).

Prebiotics are non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth of desirable bacteria, mostly Firmicutes, Bacteroidetes and *Bifidobacterium* spp., in the gastrointestinal tract, eventually improving host health (Gibson et al., 2017). Fermentation of the prebiotic carbohydrates by beneficial bacteria results in production of short chain fatty acids (SCFAs), leading to the acidification of the colon, and other metabolites that influence the gut function and beyond (Louis and Flint, 2017). SCFAs are known to serve as energy for the intestinal tissues and play an important role in the regulation of the colonic and intracellular environment, and modulation of cell proliferation and gene expression (Sonnenburg and Backhed, 2016). In addition, the SCFAs contribute to strengthening the immune system, glucose regulation and prevents obesity (Sonnenburg and Backhed, 2016). On the contrary, gut microbial dysbiosis is the leading cause of numerous chronic (Murphy et al., 2015) and metabolic diseases (Cani and Delzenne, 2009).

The disaccharides lactulose (4-*O*-β-D-galactopyranosyl-D-fructose) and epilactose (4-*O*-β-galactopyranosyl-D-mannose) are valuable lac-

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tose derivatives (Seki and Saito, 2012). Lactulose is an approved prebiotic and it is widely used in pharmaceutical, food and feed industries (Xiao et al., 2019). Studies have shown that lactulose intake promotes growth of beneficial bifidobacteria (bifidus factor) and lactobacilli (Panesar and Kumari, 2011). Lactulose is metabolized to acetic and lactic acids, thus lowering the pH of the gastrointestinal tract and eventually preventing the expansion of pathogenic bacteria such as *Salmonella* (Panesar and Kumari, 2011). Applications of lactulose include the treatment of chronic constipation, hepatic encephalopathy and hyperammonemia (Sitanggang et al., 2016). In the food industry, lactulose is used as an ingredient with higher sweetness and solubility than lactose and to enhance food flavor and physicochemical properties (Panesar and Kumari, 2011). Lactulose is routinely prepared by chemical isomerization of lactose using alkali hydroxides or boric acid, according to the Lobry de Bruyn-Alberda van Ekenstein transformation in basic solution (Aider and de Halleuxc, 2007). A second method of production is based on the Amadori rearrangement, in which the reaction of lactose with an amine and subsequent hydrolysis generates lactulose (Ackerman et al., 2017). However, these approaches utilize chemicals that cause environmental pollution and lead to the formation of unwanted by-products, rising the overall cost and energy consumption for product separation and purification. Recently, more sustainable and bio-economic alternatives have been explored including enzymatic transgalactosylation and isomerization methods that exploit β-galactosidases and cellobiose 2-epimerases (CEases) (Xiao et al., 2019; Sitanggang et al., 2016; Guerrero et al., 2015; Mayer et al., 2010). On the one hand, production of lactulose via transgalactosylation requires lactose as a galactose donor and fructose as an acceptor and has a low yield (up to 19%) (Panesar and Kumari, 2011). On the other hand, recent reports have shown that CEases from thermophilic microorganisms, such as *Caldicellulosiruptor saccharolyticus*, *Dictyoglomus turgidum* and *Spirochaeta thermophila* (Kim and Oh, 2012; Kim et al., 2012; Park et al., 2013), and mesophilic bacteria, such as *Bacteroides fragilis* and *Flavobacterium johnsoniae* (Kuschel et al., 2017), are able to catalyze the isomeration of the glucose residue at the reducing end of lactose to a fructose residue with generation of lactulose. These enzymes act without the need of a co-substrate and can generate lactulose with high yields (Kim and Oh, 2012).,

Recent literature has shown that epilactose has prebiotic properties as it resists intestinal enzyme degradation *in vivo* in rats, reaching the lower gastrointestinal tract where it is fermented by bifidobacteria and lactobacilli (Seki and Saito, 2012; Ito et al., 2008). In addition, epilactose promotes calcium absorption in the small intestines (Nishimukai et al., 2008; Suzuki et al., 2010) and reduces the risk of arteriosclerosis development in animal models (Nishimukai et al., 2008). While chemical epilactose production from lactose is challenging, epilactose is generally obtained enzymatically via epimerization of lactose by CEases (Ito et al., 2008; Chen et al., 2015; Taguchi et al., 2008). Thermostable CEases from *Thermoanaerobacterium saccharolyticum* JW/SL-YS485 and *C. saccharolyticus* have been shown to have high epimerization activity toward lactose both when overexpressed in *Escherichia coli* (Kim and Oh, 2012; Chen et al., 2015) or in the food-grade *Bacillus subtilis* expression systems (Chen et al., 2018; Wu et al., 2017). The CEase from *T. saccharolyticum* generates only epilactose from lactose with a yield of 24.0% (Chen et al., 2018). The CEase from *C. saccharolyticus* is the most efficient enzyme for epimerizing lactose to both epilactose and lactulose with yields of 15% and 58%, respectively (Wu et al., 2017). Recently, a cold-active CEase from *Roseburia intestinalis* has been used for the epimerization of lactose at low temperatures (8 °C), giving a yield of 20.9% of epilactose (Chen et al., 2020).

In this paper, we evaluated the use of the CEase from the thermophile *Caldicellulosiruptor bescii* (*Cb*CEP) and the mesophile *Roseburia faecis* (*Rf*CEP) for the efficient conversion of lactose directly from whey permeate to obtain lactulose and epilactose at high yields. The results show that *Cb*CEP produces both lactose derivatives and has higher thermal stability than its homolog in *R. faecis*, which only generates epilactose. Additionally, we demonstrate that *Cb*CEP-mediated conversion of lactose into lactose and epilactose can be controlled and tuned through temperature changes.

2. Materials and methods

2.1. Chemicals and cheese whey permeate

All chemicals were of analytical grade and obtained from Merck (Darmstadt, Germany), Gibco/Thermo Fisher Scientific (MA, USA) or Sigma-Aldrich (St. Louis, MO). Lactose, epilactose and lactulose used for the enzymatic assays were purchased from Sigma-Aldrich (St Louis, MO, USA). Stocks were prepared at 20 g/L in double-distilled water and sterilized by filtration using a 0.22 μm membrane filter (Sarstedt AG & Co, Germany). Liquid chromatography standards such as mannose, glucose, fructose, galactose, lactose, epilactose and lactulose were all purchased from Megazyme International, Wicklow, Ireland. Lactose powder (spray-dried powder containing > 85% w/w lactose) from cheese whey permeate was provided by Tine AS.

2.2. Expression and purification of recombinant proteins

The amino acid sequences encoded by the predicted cellobiose 2-epimerase genes from *Caldicellulosiruptor bescii* DSM 6725 (locus tag Athe_2557) and *Roseburia faecis* M72 (locus tag M72_25631) were codon optimized for *Escherichia coli*, synthesized and cloned into the pET-28a(+) plasmid by GenScript. Plasmids harboring the gene of interest were transformed into competent *E. coli* BL21 STAR cells (Invitrogen) through chemical transformation methods. Recombinant cells were cultivated in 500 mL Tryptone Yeast extract (TYG) supplemented with 50 μg/mL kanamycin for 24 h in a Harbinger system (Harbinger Biotechnology & Engineering, Canada) at 25 °C. Protein overexpression was induced by adding isopropyl β-D-thiogalactopyranoside (IPTG) to a final concentration of 200 μM. After incubation for additional 16 h at 25 °C, cells were collected by centrifugation (14,000*g* for 10 min), resuspended in binding buffer (50 mM Tris buffer pH 8.0, 500 mM sodium chloride, 5 mM imidazole) and sonicated using a Vibracell Ultrasonic Homogenizer (Sonics and Materials, USA). After removal of the cell debris by centrifugation (14,000*g* for 10 min), the cell-free supernatant was loaded onto a 5 mL HisTrap IMAC HP Nickel Sepharose column (GE Healthcare) equilibrated with the same buffer. The target His-tagged protein was eluted using a linear gradient of 0–100% elution buffer (20 mM sodium phosphate pH 7.4, 500 mM sodium chloride, 500 mM imidazole) over 16 column volumes. Fractions containing the purified enzymes were pooled and concentrated using a Vivaspin 20 centrifugal concentrator (10-kDa molecular weight cutoff) with concomitant dialysis against 10 mM TrisHCl buffer (pH 7.0). Protein purity was determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) analysis. The protein concentrations were determined by measuring the absorbance at 280 nm with a Biophotometer UV–VIS spectrophotometer (Eppendorf, Germany), using their theoretical extinction coefficients as determined using web.expasy.org/protparam. The protein stock solutions were stored at 4 °C until use.

2.3. Activity assays

The temperature optima of the enzymes were assessed by incubation of 1 μM *Cb*CEP or 2 μM *Rf*CEP with 0.2 g/L lactose for 60 min at temperatures ranging from 20 °C to 90 °C in 100 mM MOPS (3-[N-morpholino]propanesulfonic acid) buffer at pH 7.0 or 8.0, respectively. Sample blanks, without the addition of enzyme, were used as negative controls to monitor the non-enzymatic formation of lactulose and epilactose as the temperature increased. The pH optima were determined by carrying out the same reactions as above in 100 mM

sodium-phosphate (pH 5.8–8.0) for 1 or 3 h at 70 °C. The enzyme dosage for maximum lactulose and epilactose production was determined using 1, 1.5 or 2 μM of *Cb*CEP or *Rf*CEP. The reactions were set up in 100 mM MOPS pH 7.0 (as a proxy for the pH of milk) containing 30 g/L or 50 g/L lactose and incubated at 37, 40, 50, or 70 °C overnight. The effect of substrate concentration for lactulose and epilactose production was evaluated at lactose concentrations of 1, 10, 30, and 50 g/L. The reactions were set up in 100 mM MOPS at pH 7.0 containing either 1 μM of *Cb*CEP or *Rf*CEP and incubated at 37 or 70 °C for up to 48 h. Time-course studies were performed to determine the lactulose and epilactose production by *Cb*CEP over 24 h. The reactions were set up in 100 mM MOPS at pH 7.0 containing 0.2 g/L lactose and 1.5 μM of enzyme at 70 °C and aliquots were withdrawn at specific intervals. The effect of product concentration on the activity of *Cb*CEP was evaluated using 0.2 g/L carbohydrates consisting of different proportions of lactose and epilactose. The reactions were set up in 100 mM MOPS at pH 7.0 containing 1 μM of *Cb*CEP and incubated at 30 °C for 1 h. All enzymatic reactions were incubated in a thermomixer (Eppendorf, Germany) with shaking at 800 rpm. The pH of all buffers was adapted to different temperatures to give the pH indicated above in the reactions.

2.4. Thermal stability

The thermal stability of *Cb*CEP and *Rf*CEP was assessed by measuring the residual activity of the enzymes on 0.2 g/L lactose after 40 min of pre-incubation in 100 mM phosphate buffer at pH 7.0. The pre-incubation was performed at 70, 75, 80, 85 and 90 °C. The pre-incubated samples were then mixed with lactose and incubated at 70 °C for 1 h in a thermomixer (Eppendorf, Germany) with shaking at 800 rpm.

2.5. Effect of buffers and high temperature on production of lactose-derivatives

To determine the effect of buffers and high temperature on lactulose and epilactose production, reactions were set up using 0.2 g/L lactose in various buffers and 1 μM of *Cb*CEP. The used buffers were 50 mM sodium acetate buffer pH 5.0, 100 mM Bis-Tris buffer pH 6.5, 100 mM MOPS buffer pH 7 and pH 7.8, 100 mM sodium phosphate buffer pH 5.8, pH 7.0 and pH 8.0. Samples were incubated at temperatures ranging from 20 to 90 °C for up to 1 h in a thermomixer (Eppendorf, Germany) with shaking at 800 rpm.

2.6. Large scale production of lactose-derivatives

Two vessels (5 L Duran glass bottles, DWK Life Sciences, Germany) containing 5 L reaction mixtures with 50 g/L lactose in 100 mM MOPS buffer at pH 7.0 were supplemented with 1 μM of purified *Cb*CEP and maintained under stirring at 180 rpm for 24 h inside an incubator at either 70 °C, to maximize the production of lactulose, or 30 °C, to maximize the production of epilactose. In addition, two control vessels, in 1 L Duran glass bottles, were incubated at either 30 °C or 70 °C for 24 h without *Cb*CEP under the same conditions described above. Samples were taken at designated time intervals, boiled for 20 min to stop the reactions and subsequently stored at −20 °C until analysis of product formation.

2.7. Analytical methods

Unless otherwise stated, all the enzymatic reactions were stopped by boiling the samples for 20 min before analysis of product formation by High-Performance Anion Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD). The disaccharides were separated using a Dionex ICS 3000 system (Thermo Scientific, USA) equipped with a CarboPac PA1 2 × 250 mm analytical column. The system was run isocratically with 5 mM or 10 mM KOH at a flow rate of 0.25 mL/min for 50 min. To analyze the assays on the effect of product concentration on *Cb*CEP's activity, lactose and epilactose were separated using a Dionex ICS 6000 Capillary HPIC system (Thermo Scientific, USA) equipped with a CarboPac PA210-fast-4 μm 2 × 150 mm analytical column and a PA210-fast-4 μm 2 × 30 mm guard column. The system was run isocratically with 12 mM KOH at a flow rate of 0.18 mL/min for 18 min. Commercial mannose, glucose, fructose, galactose, lactose, epilactose and lactulose were used as external standards. Data analysis was performed using the Chromeleon 7.2 software.

2.8. Statistical analysis

Data are presented as means ± standard deviation of the mean. Statistical analysis of the data was performed using a paired Student's *t* test. Differences were considered significant at p-values of <0.05.

3. Results

3.1. Optimal operating conditions and thermal stability

To determine if *Cb*CEP and *Rf*CEP have the ability to epimerize lactose, the full-length proteins were expressed as histidine-tagged fusion proteins in *E. coli* and purified by His-Trap affinity chromatography. Both enzymes were produced in soluble form and the final yield was approximately 100 mg of purified protein per liter of *E. coli* culture in both cases. Both *Cb*CEP and *Rf*CEP showed epimerization activity of lactose at 37 °C overnight in 100 mM sodium phosphate buffer (pH 7.0) as determined by HPAEC-PAD. At this temperature, both enzymes seemingly produced epilactose as the only product from lactose epimerization.

The optimal operating conditions of *Cb*CEP and *Rf*CEP on lactose-epimerization/isomerization activity were assessed using commercial lactose. *Cb*CEP exhibited a broad pH spectrum and was most active at pH 7.5 (Fig. 1A). Interestingly, epilactose was the main product when the reactions were conducted at temperatures between 20 and 60 °C (Fig. 1B). *Cb*CEP converted lactose into both lactulose and epilactose at temperatures between 60 °C and 80 °C, after which the production of both compounds declined. A time-course experiment was conducted to observe the dynamic of product formation of *Cb*CEP over 24 h at 70 °C. Results showed that *Cb*CEP initially favors the production of epilactose that is likely converted to the more stable product lactulose over time (Fig. 1C), as indicated by the increase of the concentration of lactulose in the reaction mixture. A similar progression of the sugar ratio has been observed before for other epimerases and linked to the fact that the epimerization reaction is faster than the isomeration (Kuschel et al., 2016). The maximal activity of *Cb*CEP for lactulose and epilactose production was observed at pH 7.5 (Fig. 1A) and 70 °C (Fig. 1B)

The pH optimum of *Rf*CEP was 8, but the enzyme maintained more than 60% of its activity at pH 7 and 7.5 (Fig. 1E). *Rf*CEP was able to produce epilactose within a 3-h reaction at temperatures ranging from 20 to 90 °C. However, the activity quickly dropped above 50 °C (Fig. 1F). The highest activity was obtained in reactions at pH 8 and 50 °C.

The thermal stability of *Cb*CEP and *Rf*CEP was assessed and compared by measuring epimerization of lactose to epilactose after pre-incubation of the enzymes in sodium phosphate buffer at optimal pH and temperatures ranging from 70 to 90 °C. *Cb*CEP retained its full activity after pre-incubation up to 80 °C (Fig. 1D), while no activity remained for *Rf*CEP (Fig. 1F). At temperatures higher than 85 °C, no enzymatic activity was observed for *Cb*CEP (Fig. 1D).

Notably, a chemical isomerization occurs at higher temperatures and pHs and needs to be carefully taken into account by the research

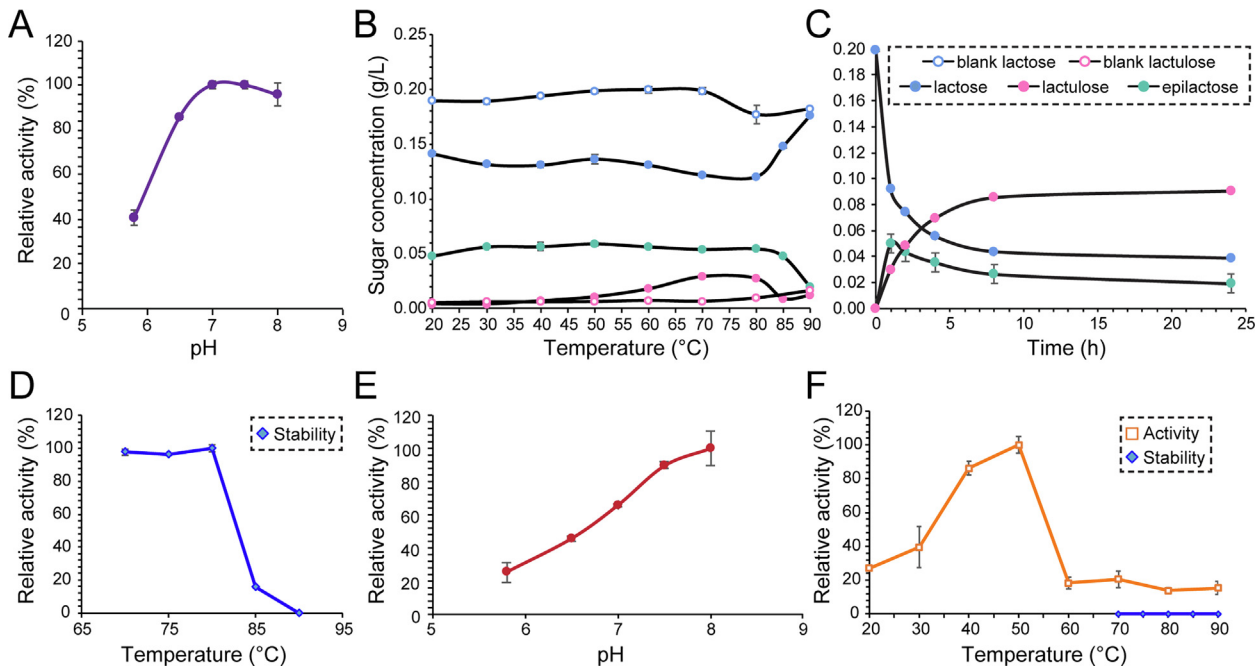

Fig. 1. (A) pH optimum for *Cb*CEP. (B) Temperature-dependent conversion of lactose into epilactose and lactulose by *Cb*CEP. Sample blanks were without enzyme and used to monitor the non-enzymatic formation of lactulose as the temperature increased. Considering the temperature dependency of the pH of MOPS, the buffer was adapted to different temperatures to give pH 7 in all reactions. (C) Pattern of conversion of lactose into epilactose and lactulose by *Cb*CEP over time. (D) Temperature stability of *Cb*CEP. (E) pH optimum for *Rf*CEP. (F) Temperature optimum and stability of *Rf*CEP. In all panels, data are expressed as mean ± standard deviation of the mean from triplicate experiments.

community when evaluating enzymatic activities. Indeed, when lactose was incubated at temperatures higher than 80 °C, we observed that chemical conversion of lactose to lactulose occurs. The effect of buffer composition and temperature to this phenomenon was evaluated in reactions set up with lactose in BisTris, MOPS, sodium acetate and sodium phosphate buffers at temperature ranging from 50 to 90 °C (Fig. 2). Lactulose chemical conversion increased with increasing pHs in MOPS buffer (p-value < 0.05) and drastically increased at pH 8 in sodium phosphate buffer at 90 °C (p-value < 0.05). This phenomenon is likely due to the Lobry de Bruyn-Alberda van Ekenstein transformation in basic solution (Aider and de Halleuxc, 2007). Given that sodium phosphate buffer greatly affected the chemical lactulose conversion, all subsequent reactions were conducted in MOPS buffer. Notably, the chemical conversion of lactose to lactulose decreased when *Cb*CEP was present in the solution (Fig. 2). It is important to note that although Fig. 2 shows that lactose can be converted to lactulose at high pH and high temperatures without the use of an enzyme, with almost more than 25% conversion, the use of a buffered system is not applicable in a dairy industry setting for the direct generation of lactulose-enriched dairy products. Here, the use of epimerases is being sought for the development of new approaches to obtain lactulose-containing milk products with the potential to extend the array of functional foods in the near future (Sitanggang et al., 2016). Furthermore, application of an epimerase as biocatalyst is required for synthesis of epilactose, as only negligible amounts can be obtained via chemical conversion.

3.2. Optimal enzyme and lactose concentrations for maximum production of lactulose and epilactose

The effect of lactose concentration on the conversion yields of lactulose and epilactose was tested with 1 μM of enzyme by varying the concentrations of lactose from 1 to 50 g/L (Fig. 3A). Increases in incubation time resulted in a proportional increase of lactulose and epilactose production (p-value < 0.05). At a lactose concentration of 50 g/L and 48 h incubation, *Cb*CEP converted 51% of lactose to lactulose and epilactose, at approximately 29.8% and 21.6%, respectively. Decreases in lactose concentration shifted the reaction towards lactulose production. When the reactions were carried out at lower temperatures (50 and 40 °C), the proportional amount of epilactose converted from lactose increased by 10 and 30%, respectively (Fig. 3B, p-value < 0.05). For *Rf*CEP, the lactose concentration to obtain the highest amount of epilactose after 48 h incubation at 37 °C was 30 g/L, obtaining a conversion of 19.3% (Fig. 3A). Both for *Cb*CEP and *Rf*CEP, a lower amount of lactose in the reaction led to higher total conversion (p-value < 0.05). However, the conversion yield of epilactose by *Rf*CEP (19.3%) was significantly lower than the yield produced by *Cb*CEP at 30 °C (26%) (p-value < 0.05). Building on these results, the effects of different enzyme loadings at the optimal temperature and lactose concentrations were next assessed (Fig. 3B). When the enzyme concentration was doubled from 1 μM to 2 μM, results showed that *Cb*CEP-mediated lactulose conversion significantly increased with a concomitant decrease in epilactose conversion at 70 °C (p-value < 0.05). A 1.6-fold increase in the amount of epilactose was observed when the concentration of *Rf*CEP was doubled (p-value < 0.05).

To further investigate the effect of substrate and product on *Cb*CEP activity, a range of experiments with different concentrations of lactose and epilactose were carried out. Fig. 3C shows that at concentrations of epilactose higher than 30%, *Cb*CEP epimerizes epilactose back to lactose (p-value < 0.05). These results demonstrate that *Cb*CEP reaches an equilibrium at around 30% epilactose.

3.3. Large scale production of lactose-derivatives from lactose-enriched whey permeate

Based on the results shown in Fig. 3B, the conditions for maximum lactulose production by *Cb*CEP were pH 7.0, 70 °C and 50 g/L lactose. The maximum epilactose production was achieved when *Cb*CEP was used at pH 7.0, 30 °C and 50 g/L lactose. Whey permeate powder containing 85% (w/w) lactose was dissolved in 15 mM MOPS, pH7, and

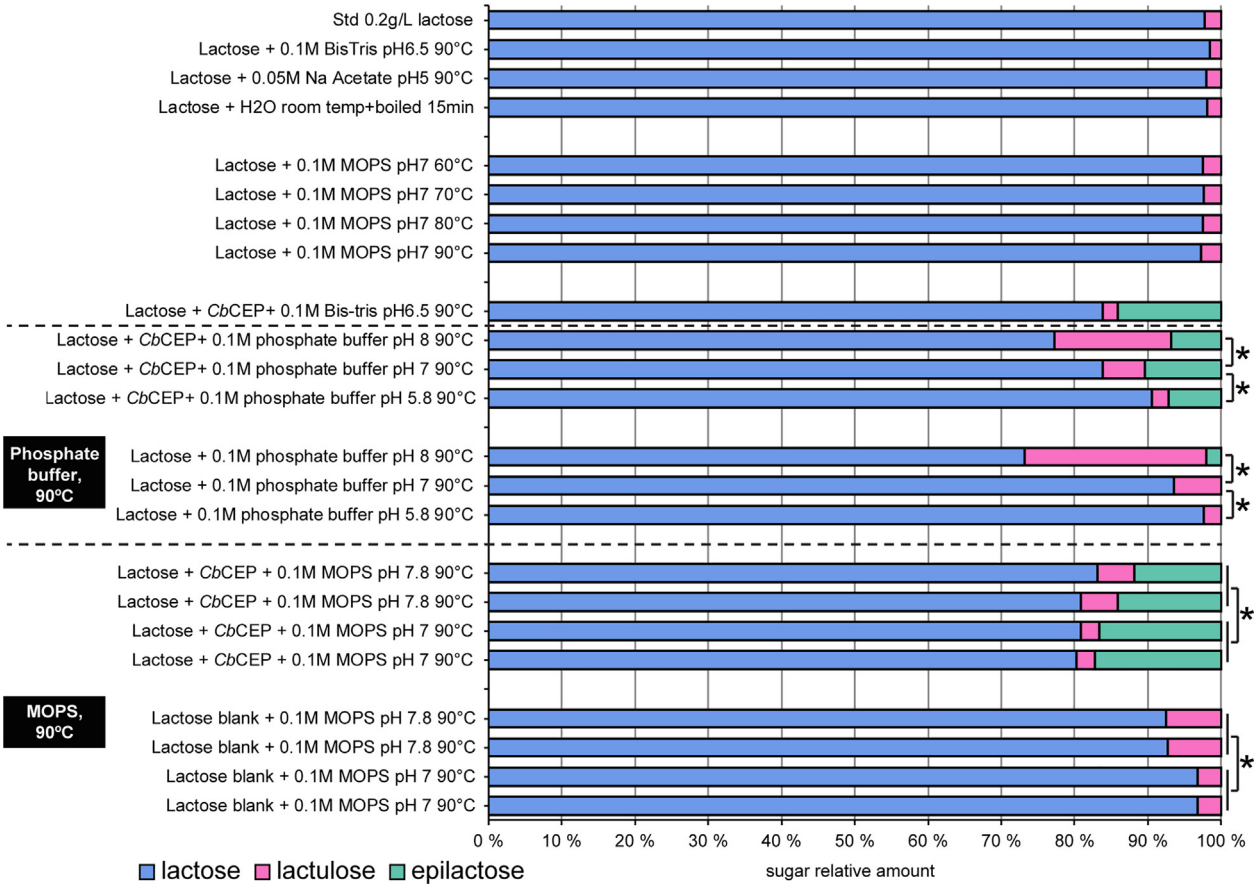

Fig. 2. Buffer and temperature-dependency of lactose conversion into lactulose and epilactose. An asterisk (*) indicates a statistically significant difference (p-value < 0.05) in the lactulose relative amount when comparing reactions that were conducted at the same temperature (90 °C) but different pHs.

incubated for 24 h according to the conditions described above. At 30 °C, 14.9 g/L of epilactose and 1.8 g/L of lactulose were produced with yields of 35% and 4.3%, respectively (Fig. 4A). The total yield and productivity were 39.3% and 16.7 g/L, respectively. The yields of lactulose and epilactose from lactose at 70 °C were 30% and 24.7% (Fig. 4B), respectively, producing 12.8 g/L of lactulose and 10.6 g/L of epilactose. The yield and productivity of both lactulose and epilactose from lactose were 54.7% and 23.4 g/L, respectively.

4. Discussion

The development of commercially viable technologies that convert abundantly available bioresources into value-added products is a critical step for a more sustainable and profitable utilization of these waste residues (Galanakis, 2012). Hence, the application of CEases to produce lactulose and epilactose from cheese-whey lactose is a promising approach that not only enhances the value of this milk by-product but also to reduces the environmental problems associated with whey disposal (Lappa et al., 2019).

Here, we biochemically characterize and compare the activity of two CEases, *Cb*CEP and *Rf*CEP, for enzymatic lactose conversion. *Cb*CEP displayed higher activity than *Rf*CEP at pH 7 (Fig. 1A and E), which represents a suitable pH for a potential later utilization of the enzyme directly in dairy products. *Cb*CEP was the most efficient enzyme for maximum lactulose and epilactose production from pure lactose (Fig. 3B) with a conversion of 29.8% and 21.6%, respectively, while *Rf*CEP produced only epilactose with a yield of 19.3% (Fig. 3B). By tuning the enzymatic activity of *Cb*CEP through temperature, this enzyme could effectively produce lactose and epilactose when directly added to cheese whey permeate. At high temperature (70 °C), *Cb*CEP's activity resulted in increased lactulose production (Fig. 4A). Epilactose was the predominant product of *Cb*CEP when the reaction was run at a lower temperature (30 °C) (Fig. 4A).

In recent years, several authors have reported that common CEases typically catalyze the epimerization reaction of lactose only to epilactose. In contrast, only three CEases are able to catalyze an epimerization and an isomerization reaction to generate both lactulose and epilactose (Ito et al., 2008; Chen et al., 2015; Kim and Oh, 2012; Kim et al., 2012; Park et al., 2013). Among these, *Cs*CE from *C. saccharolyticus* has been shown to be the most efficient lactulose-producing enzyme described so far (Kim and Oh, 2012). Indeed, *Cs*CE produced both lactulose and epilactose at yields of 58% and 15%, respectively, from pure lactose in a synthetic buffer system operated at 80 °C for 2 h (Kim and Oh, 2012) while generated exclusively 58.5% lactulose from cheese whey permeate when used in an enzymatic membrane reactor system ran at 70 °C for 2 h (Wu et al., 2017). Compared to *Cs*CE, the epimerase *Cb*CEP characterized in this study was less efficient in generating lactulose when utilized in thermophilic conditions. However, the remarkable feature of *Cb*CEP is that this enzyme can be directed via temperature changes to favor either the isomerization (at 70 °C) or epimerization (at 30 °C) activity towards lactose. Given that epimerization and isomerization are equilibrium reactions (Seeholzer, 1993), it is likely that the temperature and use of the catalyst (*Cb*CEP) reduces the time to reach the equilibrium, without altering the final yield. Although the structural basis for the two reactions remains to be explained, this property represents a significant advantage in the application of *Cb*CEP in an industrial setting as one enzyme can produce two different end-products from the same substrate. Further

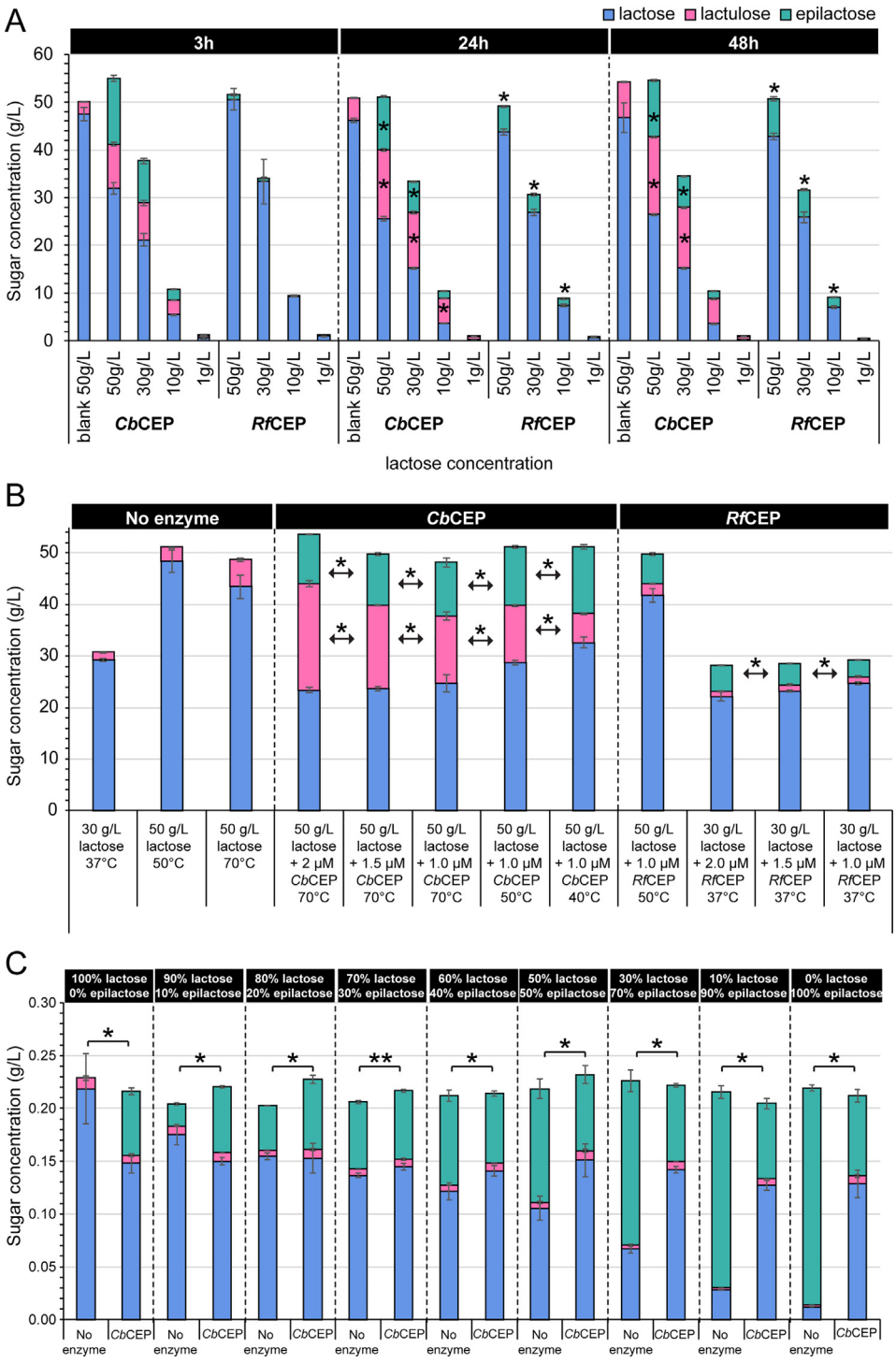

Fig. 3. (A) Effect of varying concentration of lactose (from 1 to 50 g/L) on the enzyme activities. Notably, the blank (no enzyme addition) contained a small amount of lactulose, but no epilactose was detected. An asterisk (*) indicates a statistically significant difference (p-value < 0.05) in the sugar relative amount when compared to the amount detected in the previous time point. (B) Effect of varying enzyme concentrations on lactulose and epilactose production from either 30 or 50 g/L lactose. An asterisk (*) indicates a statistically significant difference (p-value < 0.05) in the lactulose or epilactose relative amount when comparing reactions either conducted with different enzyme loadings (2, 1.5 and 1 μM) or the same enzyme loading (1 μM) but different temperatures (40, 50, 70 °C). (C) Substrate-product equilibrium on *Cb*CEP. An asterisk (*) indicates a statistically significant difference (p-value < 0.05) in the epilactose concentration when compared to the amount in the control reaction. ** p-value > 0.05. In all panels, data are expressed as mean ± standard deviation of the mean from triplicate experiments.

immobilization of the enzyme could bring the benefits of continuous process operations and the generation of different products in a single, whey-fed reactor as a function of temperature (Basso and Serban, 2019).

When used at 30 °C, *Cb*CEP's activity can be directed to improve the utilization rate of lactose to yield more epilactose (35%) than *Cs*CE and other characterized enzymes (Chen et al., 2018; Wu et al., 2017; Chen et al., 2020). The previous highest concentration and productiv-

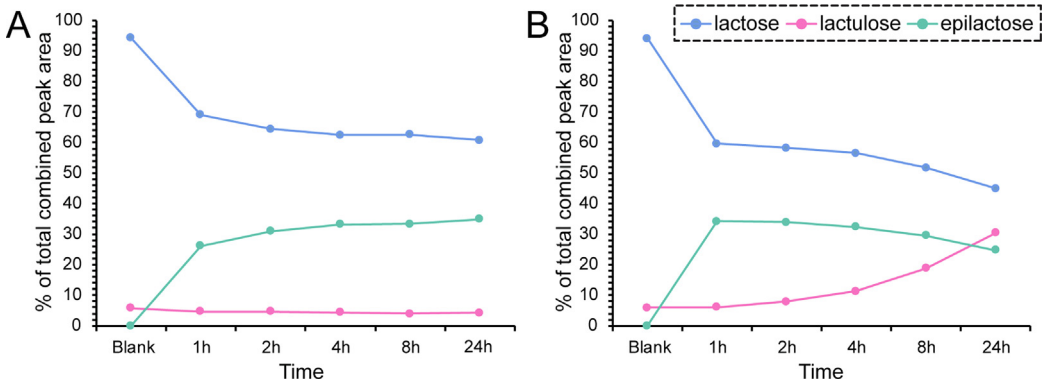

Fig. 4. Time course study of enzymatic conversion of lactose in whey permeate by *Cb*CEP at (A) 30 ˚C and (B) 70 ˚C. The "blank" refers to values determined from a reference sample incubated at either (A) 30 ˚C or (B) 70 ˚C for 24 h without *Cb*CEP.

ity of epilactose was reported in a system based on the activity of a *T. saccharolyticum* CEase, expressed in the food-grade *Bacillus subtilis* 1A751, followed by a β-galactosidase-based hydrolysis of residual lactose, yeast treatment and cation exchange chromatography, eventually yielding 24% pure epilactose (with a purity > 98%) (Chen et al., 2018). Similar to the approach reported in (Chen et al., 2018), removal of residual lactose and *Cb*CEP-derived lactulose by selective hydrolysis with a β-galactosidase followed by yeast fermentation has the potential to generate 35% high purity epilactose. In addition, while the enzymatic reaction with *T. saccharolyticum* CE is performed at 60 ˚C (Chen et al., 2018), lactose conversion with *Cb*CEP is carried out at 30 ˚C (Fig. 3A). This property is favorable in applications of *Cb*CEP to milk products, as it allows reduced thermal impact during food-processing (due to increased risk of the Maillard reactions), avoiding detrimental side effects, such as off-flavors and off-coloring, on the product (Jaeger et al., 2010). Furthermore, given that *Cb*CEP shows an apparent equilibrium distribution of substrate-product, a continuous product removal (epilactose) may in future be utilized to obtain even higher conversion yields.

Besides its application to enhance the value of by-products as whey, *Cb*CEP demonstrates a set of industrially relevant properties and has the potential to become part of the enzymatic toolbox for the production of lactulose and/or epilactose directly in milk products. Enzymatic synthesis of lactose derivatives could be integrated into a variety of dairy processes that are conducted at different temperatures, such as pasteurization or milk fermentation, to expand the array of foods and drinks in the constantly growing number of functional/enriched food products. However, in order to be feasible at industrial level, the commercial production of *Cb*CEP needs to be established and the enzyme preparation should receive a GRAS (generally recognized as safe) status (Sitanggang et al., 2016). Future studies are needed to address these aspects.

In conclusion, this proof-of-concept study demonstrates a possible way of obtaining functional lactose derivatives at high yields as a product of the enzymatic activity of *Cb*CEP from low value side streams of the dairy industry (such as whey). To the best of our knowledge, this is the first study to report high yield epilactose and lactulose production by merely controlling the enzymatic activity of a CEase through temperature. This information has implication for designing industrial processes to reduce the cost and time needed for commercial scale manufacture of lactulose and epilactose using cheese whey as a feedstock.

CRediT authorship contribution statement

John Kristian Jameson: Conceptualization, Methodology, Investigation, Data curation, Writing - review & editing. **Geir Mathiesen:** Methodology, Supervision, Writing - review & editing. **Phillip B. Pope:** Methodology, Funding acquisition, Supervision, Writing - review & editing. **Bjørge Westereng:** Conceptualization, Methodology, Investigation, Funding acquisition, Supervision, Writing - review & editing. **Sabina Leanti La Rosa:** Conceptualization, Methodology, Data curation, Investigation, Funding acquisition, Supervision, Visualization, Writing - original draft.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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