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Production of a lactose-based prebiotic mixture by engineered *Saccharomyces cerevisiae*

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Background

Prebiotics are defined as 'substrates that are utilized by host microorganisms conferring a health benefit'. They have been successfully incorporated in a wide variety of food products like breads, baked goods, meet products, salad dressings, sweeteners, and yoghurts [1]. One of the most well-recognized prebiotics is lactulose, a disaccharide derived from lactose. Lactulose is not found naturally so it must be produced through different methods: chemical or enzymatic synthesis and electro-activation [2]. Recently, the production of lactulose through lactose isomerization catalyzed by cellobiose 2-epimerase (CE) was reported [3]. This strategy is gaining attention as a preferable methodology for industrial application due to its notable yields. Although cellobiose 2-epimerase exists in a wide range of microorganisms, the one produced by the bacterium *Caldicellulosiruptor saccharolyticus* is reported to be one of the most efficient in lactose isomerization [4] and epimerization [3]. Using lactose as a single substrate, CE can catalyze the epimerization of the glucose moiety to mannose and then, the isomerization of mannose to fructose through a two-step reaction. Therefore, under optimized conditions, this enzyme leads to the formation of lactulose and epilactose [3]. Epilactose is a rare functional sugar composed of a molecule of galactose and mannose that can be found in heat-treated bovine milk. This epimer of lactose was shown to be resistant to the intestinal enzymes of rats and to promote the proliferation of beneficial microorganisms, revealing its potential prebiotic effect and bifidus factor [5]. *Saccharomyces cerevisiae*, one of the most well-characterized microorganisms, is widely used for the heterologous production of several enzymes, also due to the diverse genetic manipulation tools that are currently available. In this study, we engineered *S. cerevisiae* strains to express the cellobiose 2-epimerase enzyme from *C. saccharolyticus* (*Cs*CE) and further used the enzyme to produce a prebiotic mixture with potential application in the food industry. The optimal reaction conditions, namely, pH, temperature, reaction time, and substrate concentration, toward the maximization of prebiotic production were determined [6].

Methods

The *Cs*CE gene was amplified from the genomic DNA of *C. saccharolyticus* DSM 8903 and was then cloned, using the appropriate restriction enzymes, into expression vectors p426GAP, p426TEF, and pSP-GM1. The *S. cerevisiae* BY4741 and CEN.PK2-1C yeast strains were transformed according to the LiAc/SS carrier DNA/PEG method. From the combination of the three constructed plasmids and the two yeast strains, a total of six different *S. cerevisiae* strains expressing *Cs*CE were obtained. The performance of each crude *Cs*CE was evaluated by determining its capacity to convert lactose. For the production of recombinant *Cs*CE, the yeast strains were grown in minimal synthetic YNB medium and the required amino acids were added to a final concentration of 100 mg/L. The yeast strains were cultivated in 250 mL shake flasks, with 50 mL of YNB medium, at 30 °C and 200 rpm for 48 h. For cell disruption and *Cs*CE recovery, the total biomass was resuspended in 1 mL of Tris-HCl buffer (50 mM, pH 7.5) and glass beads were added. The cells lysate was used as the source of the *Cs*CE enzyme. After the optimization of the reaction conditions (pH and temperature), the CsCE enzyme was applied in the production of the were optimized prebiotic mixture. Different parameters (reaction time and substrate concentration) were evaluated to maximize the prebiotic production.

Results

The constructed *S. cerevisiae* strains were used to express the recombinant *Cs*CE enzyme. After cell disruption, the enzyme extract was incubated with 50 g/L of lactose to evaluate the substrate conversion. As shown in **Table 1**, all the enzymes were properly active since they all exhibited the ability to convert lactose. Despite that, the *Cs*CE resulting from the combination of BY4741 yeast strain and plasmid p426GAP_*Cs*CE presented the significantly highest capacity for lactose conversion (34%). Therefore, the recombinant *Cs*CE produced by the referred combination was chosen as the most promising catalyst to maximize the production of prebiotics (lactulose and epilactose). The crude enzyme was used in all the experiments herein described.

Table 1. Lactose conversion (%) by the cellobiose 2-epimerase from *Caldicellulosiruptor saccharolyticus* (CsCE) enzyme produced by the different *Saccharomyces cerevisiae* constructed strains. Results correspond to the mean ± SD (n = 6). A different superscript letter indicates a statistically significant difference ($p < 0.05$).

***S. cerevisiae* strain**	**plasmid**		
	psP-GM1_*Cs*CE	**p426TEF_*Cs*CE**	**p426GAP_*Cs*CE**
BY4741	$24.43 \pm 1.34^{a,c}$	22.66 ± 1.39^{a}	34.47 ± 0.30^{b}
CEN.PK2-1C	26.63 ± 1.22^{c}	27.42 ± 0.98^{c}	27.26 ± 2.34^{c}

The effects of temperature, pH and metal ions on the enzymatic activity of the recombinant *Cs*CE produced by *S. cerevisiae* were investigated (**Figure 1**). The enzyme presented higher activity at 75 – 80 ºC and remained moderately active for temperatures between 60 ºC and 75 ºC. For lower temperatures, a considerable decrease on the relative activity was observed. Similarly, for temperatures higher than 80 ºC, the enzymatic activity abruptly decreased (**Figure 1A**). The optimal pH for this *Cs*CE was 7.5, but the enzymatic activity remained high (above 70%) for both more acidic or basic pH values (**Figure 1B**). The study of the effect of some metal ions and EDTA showed that, not only none of the tested metal cations promoted a significative enhancement of the enzymatic activity, but also some of the divalent and trivalent ions had a drastic negative effect on the enzyme performance, thereby proving that this *Cs*CE is a metal-independent enzyme (**Figure 1C**).

The ability of this recombinant *Cs*CE produced by *S. cerevisiae* to perform the epimerization and isomerization reactions involved in the production of epilactose and lactulose was investigated. The reactions were conducted under the optimal conditions (pH 7.5, 80 ºC) during 20 min, using lactose concentrations ranging from 10 to 270 g/L (**Figure 2**). The epilactose production increased up to 31.7 g/L with increasing lactose concentrations. Contrarywise, the prebiotic yield reached a maximum at 15 g/L of lactose (24% yield) followed by a progressive decrease between 15 – 100 g/L of substrate. For higher lactose concentrations, the yield remained almost constant at 12%. Contrarily to what was expected, lactulose was not detected in any reaction medium, possibly due to the short time of reaction (20 min). For that reason, the profile of prebiotics production was investigated over a longer period (120 min) at 80 ºC (**Figure 3**). The first detected prebiotic was epilactose (after 20 min) at a concentration of 9.7 g/L. The concentration of epilactose increased until 90 min of reaction reaching a value of 13.5 g/L and then it stabilized. With a delay of 10 min, lactulose was finally detected in the reaction medium at a concentration of 0.82 g/L, reaching its maximum concentration (1.3 g/L) after 120 min.

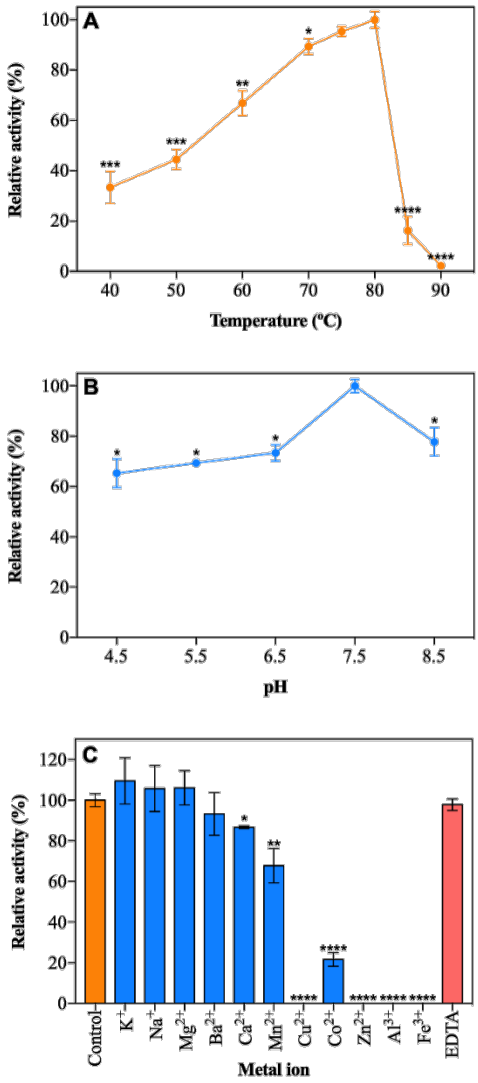

Figure 1. Effect of (A) temperature, (B) pH, and (C) metal ions and EDTA on crude cellobiose 2-epimerase from *C. saccharolyticus* (*Cs*CE) produced by *S. cerevisiae*. Tris-HCl buffer (50 mM, pH 7.5) was used as a control when necessary. Results correspond to means ± SD (n = 3). Asterisks indicate a statistically significant difference from 100% relative activity or the control condition (*p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001).

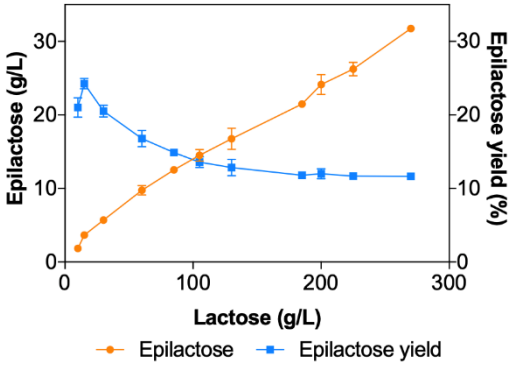

Figure 2. Effect of substrate concentration on the production of epilactose from lactose using the crude recombinant cellobiose 2- epimerase from *C. saccharolyticus* (*Cs*CE) produced by *S. cerevisiae*. Results correspond to means ± SD (n = 3).

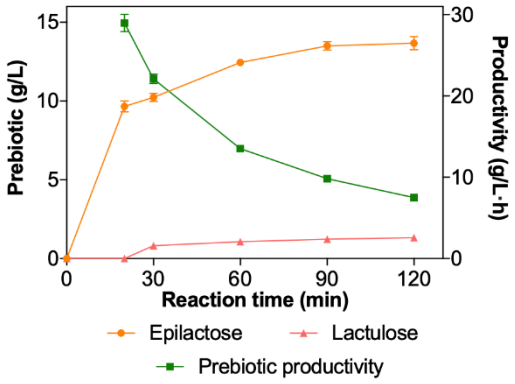

Figure 3. Time-course production of prebiotics from lactose (50 g/L) using the crude recombinant cellobiose 2-epimerase from *C. saccharolyticus* (*Cs*CE) produced by *S. cerevisiae*. Results correspond to means ± SD (n = 3). A different letter within the same line indicates a statistically significant difference ($p < 0.05$).

Conclusion

S. cerevisiae was proven, for the first time, to be a suitable host to produce *Cs*CE towards further applications in prebiotics production. The maximum epilactose production (13.5 g/L) was achieved after 90 min, representing a yield of 27%. This is the highest yield reported so far using *Cs*CE and a GRAS microorganism as enzyme producer. Less than 3% yield was obtained for lactulose, which suggests that this *Cs*CE is more suitable to produce epilactose-enriched prebiotic mixtures. Despite the promising results, longer enzymatic reactions using lower temperatures and purified enzyme should be evaluated in the future towards an improved prebiotic production.

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