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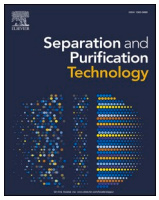

Two-step purification of epilactose produced by cellobiose 2-epimerase from *Caldicellulosiruptor saccharolyticus*

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ABSTRACT

Epilactose is a functional sugar that can be produced from lactose using cellobiose 2-epimerase and it is considered a developing prebiotic. In that sense, the development of strategies to produce and purify epilactose is key for its wider use in the food industry. The aim of this work was to establish a food-grade purification strategy suitable to be scaled-up to an industrial level. Firstly, the epilactose was produced by enzymatic epimerization of lactose in a reaction catalyzed by the recombinant cellobiose 2-epimerase from *Caldicellulosiruptor saccharolyticus* produced by *Saccharomyces cerevisiae*. Then, to remove the unreacted lactose, a screening study was performed to find a suitable β-galactosidase enzyme with high lactose hydrolysis capacity but low ability to convert the epilactose. The elimination of the generated monosaccharides was then attempted by microbial treatment using different microorganisms and using activated charcoal. The baker's yeast *S. cerevisiae* was proven to be the most suitable microorganism for glucose and galactose removal from the reaction mixture. Overall, an attractive and food-grade two-step process for epilactose recovery was established, resulting in a purity and yield of 87% and 76.4%, respectively. Additionally, the INFOGEST 2.0 static *in vitro* simulation of gastrointestinal food digestion was used, for the first time, to assess the resistance of epilactose (77% resistance) to the upper gastrointestinal tract conditions, reinforcing its potential to be used as prebiotic.

1. Introduction

The demand for functional foods has been growing since consumers are more aware of the importance of healthier eating choices and its role in the prevention of several diseases. Prebiotics are bioactive compounds that have been widely used as ingredients of functional foods, due to their reported benefits on human health, namely on the gastrointestinal tract, bones, cardiometabolism and mental health. Due to the increasing interest in prebiotics, it is expected that its market reaches 7.2 billion $ by 2024 [5].

Epilactose (4-O-β-D-galactopyranosyl-D-mannose) is an epimer of lactose composed of a molecule of mannose and galactose that is naturally found in heat-treated bovine milk. The potential prebiotic effect of epilactose was suggested based on its resistance to the intestinal enzymes of rats [12] and the ability to promote the proliferation of beneficial microorganisms [23]. Thus, it is currently considered as an "under development" prebiotic [5]. The production of epilactose is mostly based on chemical approaches that include the synthesis of the compound from mannose and galactose or its direct epimerization from lactose [18]. The need of several chemical reagents during the production results in complex downstream purification processes, which generally compromise the potential applications of epilactose. On the other hand, the enzymatic synthesis of epilactose emerge as a more suitable and sustainable approach to produce this compound. In this case, the enzyme cellobiose 2-epimerase (EC 5.1.3.11) is used to catalyze the epimerization of lactose, thus generating epilactose in a single step and single substrate reaction [5]. The cellobiose 2-epimerase enzyme from *Caldicellulosiruptor saccharolyticus* is reported as one of the most efficient in lactose epimerization and isomerization and it has been widely used to produce both epilactose and/or lactulose [6,14,22].

As a rare functional compound, epilactose needs to be produced and purified in larger amounts to expand its applications in the food industry. In that sense, the establishment of suitable purification strategies is of utmost importance. The crucial step in epilactose purification is its

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separation from the unreacted lactose, as the two disaccharides are isomers. Therefore, techniques based on the molecular size, but also enzymatic approaches, can be very tricky due to the similarity of the molecules, and need to be carefully explored. Most purification approaches rely on the use of high-performance liquid chromatography (HPLC) and ion-exchange resins which usually includes a high number of steps and results in low yields [7,16,20]. Hence, this study was focused on the development of a suitable and simple strategy to purify epilactose aiming not only to reduce the number of steps, but also to enhance the yield. Herein, we report a food-grade two-step process with improved epilactose recovery yield and high compound purity, that is also suitable for scale-up industrial purposes.

2. Materials and methods

The production and purification of epilactose using cellobiose 2-epimerase was performed according to the schematic diagram presented in Fig. 1.

2.1. Epilactose production

The production of epilactose was performed as previous reported by Cardoso and collaborators [6]. Briefly, a *S. cerevisiae* BY4741 strain carrying the gene of cellobiose 2-epimerase from *C. saccharolyticus* was used as enzyme producer. The yeast strain was grown at 30 °C and 200 rpm, and after 48 h the cells were harvested and disrupted using glass beads (425–600 μM, Sigma) and a cell disruptor Fast Prep FP120 (Thermo Fischer Scientific). The suspensions were centrifuged at 11,000 g during 10 min and the enzyme was recovered in the supernatant. The enzymatic extracts were incubated at 80 °C with 50 g/L lactose in Tris-HCl buffer (50 mM, pH 7.5) during 90 min for epilactose production.

2.2. Lactose hydrolysis

The remaining lactose present in the reaction mixture was hydrolyzed using different enzymes, namely β-glucosidase from *Agrobacterium* sp. (Megazyme E-BGOSAG) and β-galactosidase from *Aspergillus niger* (*An*Gal, DSM Maxilact® A_4) and *Kluyveromyces lactis* (*Kl*Gal, DSM Maxilact® LGX_{5000}). To evaluate the selective hydrolysis of lactose, each enzyme (25 U/mL) was incubated with the sugar's mixture at the optimum pH and temperatures reported by the suppliers: pH 6.5 and 50 °C for β-glucosidase; pH 4.5 and 37 °C for *An*Gal and pH 7.5 and 37 °C for *Kl*Gal. The reactions were conducted for 60 min and were stopped by incubating the mixture for 10 min at 100 °C. The experiments were performed in triplicate. The condition resulting in higher lactose hydrolysis and lower epilactose hydrolysis was chosen to be further optimized through experimental design.

2.3. Optimization of lactose hydrolysis by β-galactosidase from A. Niger

The Box-Behnken experimental design was used to optimize the lactose hydrolysis by β-galactosidase and to study the influence of enzyme concentration, pH and reaction time. The experimental design contained three blocks and a central point with three replicates. The range (established based on preliminary results, *data not shown*) and the levels of the independent variables were (−1, 0, 1), namely: enzyme concentration (5, 77.5, 150 U/mL), pH (3.5, 4.5, 5.5) and reaction time (5, 32.5, 60 min). The two dependent variables were the lactose hydrolysis (to be maximized) and the epilactose hydrolysis (to be minimized). The experiments were performed by adding the *An*Gal enzyme (5, 77.5 and 150 U/mL) to the sugar's mixture at the different pH values. The samples were then incubated at 37 °C during 5, 32.5 and 60 min. The reactions were stopped by incubating the mixture for 10 min at 100 °C. Three additional confirmation experiments were conducted to validate the statistical experimental strategy.

2.4. Epilactose purification by the removal of the monosaccharides

2.4.1. Microorganisms treatment

Five different microorganisms were tested to evaluate their ability to consume the monosaccharides present in the reaction mixture leading to the purification of epilactose: *Escherichia coli* BL21 (DE3), *Bacillus subtilis* DSMZ 10, *Kluyveromyces marxianus* ATCC 200963, *S. cerevisiae* and *Zymonomas mobilis* ZM4 ATCC 31821. The experiments were conducted for 48 h under the optimum conditions for each microorganism: LB medium, 37 °C and 200 rpm for *E. coli* and *B. subtilis*; YP medium (10 g/L yeast extract and 20 g/L peptone), 30 °C and 200 rpm for *K. marxianus* and *S. cerevisiae*; and RM medium (10 g/L yeast extract, 2 g/L KH_2PO_4, 1 g/L $(NH_4)_2SO_4$ and 2.04 g/L $MgSO_4$), 30 °C and without agitation for *Z. mobilis*. Pre-grown microorganisms (12 h) were used as inoculum to start the experiments at an optical density at 600 nm (OD_{600nm}) of 0.1. For each microorganism, the sugar's solution (epilactose + monosaccharides) obtained after the treatment with the *An*Gal enzyme was added to the culture medium and used as carbon source. Samples were withdrawn at specific time intervals to assess the sugars concentration by HPLC. The experiments were performed in duplicate.

2.4.2. Activated charcoal treatment

To remove the monosaccharides present in the reaction mixture as the result of the lactose hydrolysis, the sugars' solution was purified using activated charcoal in a granular form (8–20 mesh, Merck), following the method reported by Roupar and co-workers [19] with some modifications. The sugars' solution was incubated with 12.5 g of activated charcoal for 3 h at 25 °C and 165 rpm. The monosaccharides non-adsorbed to activated charcoal were removed from the solution by washing three times the charcoal with ultrapure water (10 min, 25 °C, 165 rpm). The epilactose adsorbed to the washed charcoal was then desorbed using ethanol (50% v/v) under stirring for 1 h at 25 °C and 165 rpm. A second desorption step was performed using ethanol (100% v/v). The two desorption supernatants were mixed and filtered for charcoal removal and the sugars were quantified by HPLC.

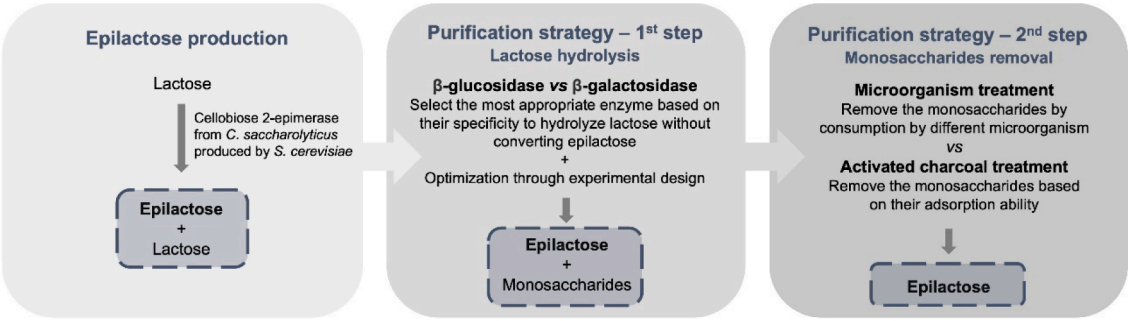

Fig. 1. Schematic representation of the designed strategy to obtain the most suitable purification process for epilactose.

2.5. Scale-up purification of epilactose

After the definition of the most suitable purification strategy, scale-up experiments were performed to validate the methodology and obtain purer epilactose. The experiments involving the *An*Gal were performed by adding the enzyme (initial concentration 5800 U/mL) to the epilactose and lactose mixture obtained as mentioned in Section 2.1. The reactions were conducted at pH 4.41 and 37 °C for 45 min. The samples were then incubated for 10 min at 100 °C to stop the reaction. The experiment was performed in triplicate.

For the yeast treatment, the scale-up experiments were performed in a 2-L DASGIP Parallel Bioreactor System (Eppendorf, Hamburg, Germany) with a working volume of 400 mL, using YP medium and the sugars' mixture (epilactose + monosaccharides) as carbon source. The experiments were conducted at 30 °C, and 250 rpm for 8 h, without aeration (sterile nitrogen was flushed through the medium for 1 h before inoculation) and the pH was automatically maintained at 6.0 by the addition of NaOH 2 M. Pre-grown yeast (24 h) was used to inoculate the reactors at an OD_{600nm} of 0.1. The experiment was performed in duplicate.

2.6. In vitro gastrointestinal digestion

The resistance and stability of the purified epilactose to gastrointestinal digestion were assessed using the harmonized INFOGEST 2.0 *in vitro* digestion method [4], which exposes the compound to conditions simulating the mouth, stomach and small intestine with the appropriate electrolyte solutions, enzymes, pH and digestion time. Briefly, at the oral phase, the sample was mixed with 1x simulated salivary fluid solution (Cl 15.1 mmol/L, KH_2PO_4 3.7 mmol/L, $NaHCO_3$ 13.6 mmol/L, $MgCl_2{\cdot}(H_2O)_6$ 0.15 mmol/L, $(NH_4)_2{\cdot}CO_3$ 0.06 mmol/L and HCl 1.1 mmol/L), 1.5 mmol/L $CaCl_2{\cdot}(H_2O)_2$ and purified water. The mixture was incubated at 37 °C in a shaking bath (B. BRAUN BIOTECH model CERTOMAT WR, Melsungen, Germany) under horizontal agitation (120 rpm) during 2 min. The gastric phase consisted in the addiction of 1x simulated gastric fluid (KCl 6.9 mmol/L, KH_2PO_4 0.9 mmol/L, $NaHCO_3$ 25 mmol/L, NaCl 47.2 mmol/L, $MgCl_2{\cdot}(H_2O)_6$ 0.1 mmol/L, $(NH_4)_2{\cdot}CO_3$ 0.5 mmol/L and HCl 15.6 mmol/L), porcine pepsin (Sigma-Aldrich P7012; final concentration 2000 U/mL) and 0.15 mmol/L $CaCl_2{\cdot}(H_2O)_2$. The pH was adjusted to 3.0 through the addition of HCl 1 M and the mixture was incubated at 37 °C for 2 h under agitation at 120 rpm. The intestinal phase was simulated by the addition of simulated intestinal fluid (KCl 6.8 mmol/L, KH_2PO_4 0.8 mmol/L, $NaHCO_3$ 85 mmol/L, NaCl 38.4 mmol/L, $MgCl_2{\cdot}(H_2O)_6$ 0.33 mmol/L and HCl 8.4 mmol/L), porcine pancreatin (Sigma-Aldrich P7545; final concentration 100 (TAME) U/mL), 10 mmol/L bile solution and 0.6 mmol/L $CaCl_2{\cdot}(H_2O)_2$. The pH was adjusted to 7.0 using NaOH 1 M and the mixture was incubated at 37 °C for 2 h under agitation (120 rpm). Samples were collected in each phase and the reaction was stopped by incubating the reaction mixture for 10 min at 100 °C. The experiment was performed in triplicate.

2.7. Analytical methods

Sugars quantification was determined by HPLC analysis. The quantification of lactose and epilactose was performed as previous described by Cardoso and collaborators [6]. The concentrations of glucose and galactose were determined using a Shimadzu chromatography equipped with a RI detector (Shimadzu) and an Aminex HPX-87H column (300 × 7.8 mm, Bio-Rad). The column was eluted at 60 °C with 5 mM H_2SO_4 at a flow rate of 0.6 mL/min.

2.8. Statistical analysis

The data analysis was performed using Prism version 7.05a (GraphPad Software Inc., California, USA). The statistical significance and differences were evaluated by one-way ANOVA. Unpaired *t*-test was used when required. Significant differences were considered when $p < 0.05$. Results are presented as mean values ± standard deviation (SD).

3. Results and discussion

3.1. Selective hydrolysis of lactose

The prebiotic mixture (11 g/L of epilactose and 31.2 g/L of lactose) was produced using the cellobiose 2-epimerase enzyme, as previously described by our group [6], resulting in a prebiotic yield of 26.2 ± 0.6%. To obtain purer epilactose for food applications as potential prebiotic ingredient, the remaining lactose (73.8 ± 0.5% of the mixture) needs to be removed. As the compounds are isomers, its separation is a complex procedure, and the lactose hydrolysis seems to be the most efficient method to eliminate this sugar [7,17]. However, due to the high similarity of the two structures, both lactose and epilactose can be substrates for microbial enzymes, thus limiting the selection of the most suitable catalyst. In that sense, three different enzymes (β-glucosidase from *Agrobacterium* sp., *An*Gal and *Kl*Gal) were tested to evaluate their specificity to hydrolyze lactose without converting epilactose. The choice of the β-galactosidases enzymes relies on the fact that they are the most used and well-known enzymes for lactose hydrolysis. Additionally, the β-glucosidase enzyme was chosen since it catalyzes the hydrolysis of terminal, non-reducing β-D-glucosyl residues, i.e., the type of bonds that are present in lactose.

On the first assay, the reactions were conducted for 60 min under the optimal conditions described for each enzyme (Section 2.2), and the results are shown in Fig. 2A. The use of the β-glucosidase enzyme from *Agrobacterium* sp. led to the lowest rates of both lactose (30.8 ± 0.9%) and epilactose (16.7 ± 0.7%) hydrolysis. The use of the *Kl*Gal resulted in the complete hydrolysis of both compounds, thus it was found to be an inadequate enzyme for the purpose of the present work. On the other hand, *An*Gal was able to completely hydrolyze the remaining lactose. Nevertheless, the epilactose hydrolysis was also high (54.6 ± 0.6%) when using this enzyme. Based on these results, *An*Gal enzyme was selected as the most promising biocatalyst at this stage to be further evaluated at shorter reaction times. As can be seen in Fig. 2B, the decrease of the reaction time resulted in a lower epilactose hydrolysis, while the lactose degradation was maintained at a high level. These results showed that, in only 20 min of reaction, the enzyme was able to hydrolyze the lactose almost completely (98.0 ± 0.1%), while the epilactose hydrolysis was considerably low (7.7 ± 0.2%). Therefore, the *An*Gal was chosen as the most suitable enzyme for the selective hydrolysis of lactose and to be used for further optimization of the reaction parameters through experimental design.

3.2. Optimization of lactose hydrolysis by β-galactosidase from A. Niger

The first step for epilactose purification involves the elimination of the lactose that was not converted when using the cellobiose 2-epimerase enzyme. In that sense, to obtain the best conditions towards a higher *An*Gal specificity for lactose hydrolysis, while minimizing the epilactose hydrolysis, the Box-Behnken design methodology was used to optimize the reaction parameters, namely the pH (X_1), reaction time (X_2) and enzyme concentration (X_3). The effects of the dependent variables are provided in Table 1. The results showed that the lactose hydrolysis was not significantly affected by the pH, while the enzyme concentration and reaction time exhibited a significant effect ($p < 0.05$). The effects promoted by the interactions between the studied variables were not significant to the model. With respect to the epilactose hydrolysis, all the three parameters significantly affect the response variable, and the same was found for the interactions between them, except for the pH × Time. The ANOVA analysis showed that the quadratic models were statically significant for both response variables (p-value = 0.0002 for lactose hydrolysis and p-value = 0.0003 for epilactose hydrolysis). The lack of fit F-value was not significant in both cases, which also demonstrates

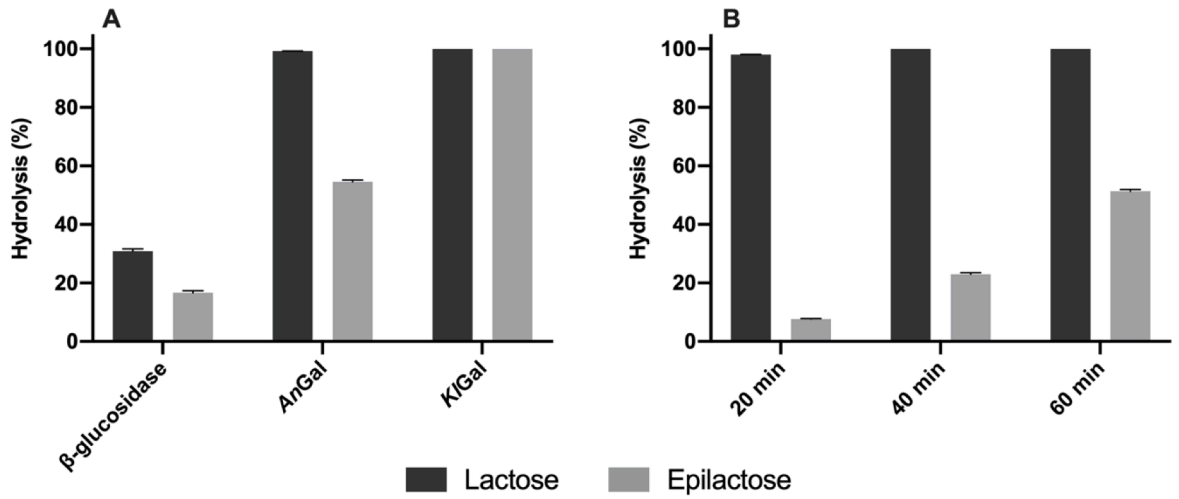

Fig. 2. Hydrolysis (%) of lactose and epilactose. A – Reactions performed by β-glucosidase from *Agrobacterium* sp. (50 °C and pH 6.5), β-galactosidase from *Aspergillus niger* (*An*Gal, 37 °C and pH 4.5) and β-galactosidase from *Kluyveromyces lactis* (*Kl*Gal, 37 °C and pH 7.5) during 60 min. B – Reactions performed by *An*Gal (37 °C and pH 4.5) during 20, 40 and 60 min. Results correspond to the mean ± SD (n = 3).

Table 1
Regression coefficients and parameters of the models for lactose and epilactose hydrolysis.

Model terms	Lactose hydrolysis (%)			Epilactose hydrolysis (%)		
	Regression coefficient	Standard error	*p*-value	Regression coefficient	Standard error	*p*-value
Constant	95.33	2.64		2.67	1.01	
pH	−0.88	1.62	0.6114	−7.12	0.62	0.0001
Time	13.50	1.62	0.0004	2.00	0.62	0.0234
Enzyme	26.13	1.62	0.0001	4.63	0.62	0.0007
pH × Time	2.25	2.28	0.3700	−5.75	0.88	0.0012
pH × Enzyme	−2.00	2.28	0.4215	1.00	0.88	0.3063
Time × Enzyme	−4.25	2.28	0.1220	4.75	0.88	0.0029
pH^2	2.33	2.38	0.3716	8.92	0.91	0.0002
$Time^2$	−12.92	2.38	0.0029	1.17	0.91	0.2578
$Enzyme^2$	−22.17	2.38	0.0002	3.92	0.91	0.0078

that the data is well adjusted to the proposed model. In addition, the obtained values of the coefficient of determination (R^2) corroborate the satisfactory adjustment to the quadratic model: 0.9692 for lactose hydrolysis and 0.9635 for epilactose hydrolysis.

Performing multiple regression analysis on the experimental data, resulted in second-order polynomial equations that describe the models for both lactose and epilactose hydrolysis:

$$Lactosehydrolysis(\%) = 95.33 - 0.88 \times X_1 + 13.50 \times X_2 + 26.13 \times X_3 + 2.25 \times X_1 \times X_2 - 2.00 \times X_1 \times X_3 - 4.25 \times X_2 \times X_3 + 2.33 \times X_1^2 - 12.92 \times X_2^2 - 22.17 \times X_3^2 \quad (1)$$

Table 2
Box-Behnken design runs to evaluate the effect of pH, reaction time and enzyme on lactose and epilactose hydrolysis and experimental responses under those conditions.

Runs	Independent variables (real and coded values)			Dependent variables			
	pH X_1	Reaction time (min) X_2	Enzyme (U/mL) X_3	Lactose hydrolysis (%)		Epilactose hydrolysis (%)	
				Experimental	Predicted	Experimental	Predicted
1	3.5 (−1)	32.5 (0)	5 (−1)	31.2	48.2	16.7	19.0
2	5.5 (1)	60 (1)	77.5 (0)	98.5	99.6	5.0	1.9
3	5.5 (1)	32.5 (0)	5 (−1)	51.7	50.5	6.1	2.8
4[a]	4.5 (0)	32.5 (0)	77.5 (0)	92.8	95.3	0.0	2.7
5	4.5 (0)	5 (−1)	150 (1)	79.7	77.1	5.9	5.6
6[a]	4.5 (0)	32.5 (0)	77.5 (0)	97.3	95.3	4.0	2.7
7	3.5 (−1)	32.5 (0)	150 (1)	100.0	104.5	27.3	26.3
8[a]	4.5 (0)	32.5 (0)	77.5 (0)	97.4	95.3	4.3	2.7
9	3.5 (−1)	5 (−1)	77.5 (0)	75.2	74.4	13.1	12.1
10	5.5 (1)	32.5 (0)	150 (1)	99.6	98.7	14.6	14.0
11	5.5 (1)	5 (−1)	77.5 (0)	60.9	68.1	10.2	9.4
12	4.5 (0)	5 (−1)	5 (−1)	14.1	16.4	6.8	5.9
13	3.5 (−1)	60 (1)	77.5 (0)	98.6	96.9	26.9	27.6
14	4.5 (0)	60 (1)	5 (−1)	44.4	51.9	0.9	0.4
15	4.5 (0)	60 (1)	150 (1)	97.3	95.6	15.9	19.1

[a] Central points.

$$Epilactosehydrolysis(\%) = 2.67 - 7.12 \times X_1 + 2.00 \times X_2 + 4.63 \times X_3 - 5.75 \times X_1 \times X_2 + 1.00 \times X_1 \times X_3 + 4.75 \times X_2 \times X_3 + 8.92 \times X_1^2 + 1.17 \times X_2^2 + 3.92 \times X_3^2 \quad (2)$$

By applying the equations to the different combinations of enzyme, pH and reaction time (as proposed by the Box-Behnken experimental design) the predicted values were obtained and compared with the experimental ones (Table 2). The results showed that in general a good agreement between the experimental data and the ones predicted by the models was achieved and so the experimental design was validated and found to be appropriate to optimize the reaction conditions. Therefore, a Design Expert subroutine for numerical optimization was used to determine the combination of conditions (within the tested ranges) that result in a higher *An*Gal specificity towards the hydrolysis of lactose while minimizing the epilactose hydrolysis. The optimal conditions were found to be pH 4.41, enzyme concentration of 128 U/mL and 45 min of reaction. Under these conditions, the model predicted a lactose hydrolysis of 98.1% and an epilactose hydrolysis of 0.5%. To confirm the prediction, three independent reactions were performed under the optimized suggested conditions, and it resulted in a 98.8 ± 0.1% of lactose hydrolysis, while the epilactose hydrolysis was 3.8 ± 0.7%. Despite the differences found on the epilactose hydrolysis values, that can be a result of the analytical procedures, the observed values are very similar to the predicted ones. The good agreement of the results confirms once more the significance of the model and so, the optimal conditions to use the *An*Gal enzyme to eliminate the remaining lactose from the mixture were established.

By using the *An*Gal enzyme, under the optimized conditions, in the first step of epilactose purification it was possible to achieve a recovery of 96.2 ± 0.7% of the epilactose while the lactose was reduced to vestigial amounts (<2%). These results are in accordance with the ones reported by Chen and collaborators that performed a 12 h reaction using 25 U/mL of β-galactosidase from *Bifidobacterium bifidum* and achieved almost a complete elimination of lactose while 94% of the epilactose was recovered from the reaction mixture [7]. Recently, a screening of β-galactosidase enzymes that could be suitable for epilactose purification showed that the most promising enzyme was the one from *Bacillus stearothermophilus* [17]. The use of this enzyme resulted in the recovery of 98.5% of the epilactose, while 87.6% of the lactose was hydrolyzed. The authors also tested the *An*Gal enzyme (3 h at 60 °C and pH 3.0) and the hydrolysis percentages were 77.5% and 20.0% for lactose and epilactose, respectively. These results suggest that the reaction conditions are crucial to obtain a good lactose hydrolysis and epilactose recovery, given that the conditions used are substantially different from the ones herein optimized for *An*Gal (pH 4.41, 37 °C, 45 min).

3.3. Epilactose purification by the removal of the monosaccharides

The use of the *An*Gal enzyme to hydrolyze the lactose leads to an accumulation of monosaccharides, thus generating a mixture of glucose, galactose and epilactose. In this sense, the second step to purify epilactose aimed at the removal of the monosaccharides from the mixture resulting from the first purification step previously discussed. For that purpose, two different strategies were evaluated, namely the use of microorganisms able to consume the monomers (so-called microorganisms treatment (Section 3.3.1)) and the use of activated charcoal as an adsorption platform (so-called activated charcoal treatment (Section 3.3.2)).

3.3.1. Microorganisms treatment

The microbial strategy to eliminate the glucose and galactose included the use of different microorganisms to evaluate their ability to consume these monosaccharides not converting/consuming the epilactose. The chosen microorganisms included three bacteria (*E. coli*, *B. subtilis* and *Z. mobilis*) and two yeast (*K. marxianus* and *S. cerevisiae*) that are considered as fast-growing strains and therefore would allow a rapid purification process. Additionally, the selected microorganisms included both natural sources and non-producers of β-galactosidases to evaluate their effect on epilactose degradation. The experiments were conducted during 48 h at the optimal conditions for each microorganism, using as substrate the sugar mixture obtained from the first step of purification (i.e., after enzymatic treatment with *An*Gal enzyme).

As Fig. 3 shows, glucose was consumed by all microorganisms under study, as expected, despite the differences in the consumption time. *K. marxianus*, *S. cerevisiae* and *Z. mobilis* depleted the glucose in just 12 h, while *B. subtilis* took 36 h and *E. coli* was not able to fully metabolize this compound. However, regarding galactose, it is possible to see that only the two yeasts were able to use this sugar, achieving its full consumption after 12 h in the case of *S. cerevisiae* and after 24 h for *K. marxianus*. Considering the three bacteria, none of them was able to consume galactose. In fact, it is well known that most microorganisms tend to use glucose as primary feedstock when both glucose and galactose are present, as for example *E. coli*, which could explain the results obtained [21]. In the case of *B. subtilis* and *Z. mobilis*, it seems that they lack the galactose transporter and therefore are not able to metabolize the galactose [9,15]. In that sense, the use of these three microorganisms would not be an adequate choice for the removal of the monosaccharides from the mixture.

Concerning the epilactose utilization, this sugar was completely depleted in 8 h for all the studied microorganisms, except for *Z. mobilis* that took 48 h. For *B. subtilis*, *E. coli* and *K. marxianus*, the results could be justified by the known presence of β-galactosidase enzymes on these species, that might have affinity to the epilactose, resulting in its total hydrolysis. Therefore, the use of these three microorganisms was found to be not suitable for the purifying epilactose. However, the results obtained for *S. cerevisiae* and *Z. mobilis* were quite unexpected as they both lack the β-galactosidase enzyme and are unable to naturally grow in a culture medium containing lactose as sole carbon source. Moreover, as epilactose is an isomer of lactose, it was firstly hypothesized that these two microorganisms would not be able to utilize/convert the epilactose.

Based on the obtained results it was found that some experimental conditions could be negatively affecting the epilactose stability, namely the pH. As showed in Section 3.2, the enzymatic reaction of lactose hydrolysis was performed at pH 4.41, which was then the pH value of the sugars' mixture used in the microbial treatment. This could mean that experiments started at an unsuitable low pH and thus some acid hydrolysis of epilactose could have occurred. Taking that into account, additional tests were performed to study the resistance of the epilactose to different pH values (4.0 and 6.0). In these experiments, the pH of the mixture containing the monosaccharides and epilactose was adjusted to the appropriate value and was then incubated during 8 h. As illustrated in Fig. 4, at pH 4.0 the epilactose hydrolysis reaches 65.6% after 8 h, while only 26.3% is hydrolyzed when a pH 6.0 was used. This result clearly shows that the pH is a crucial parameter to consider when establishing the best strategy to purify epilactose, and in further applications of this compound.

The results of the experiments with *S. cerevisiae* (Fig. 3D) showed that this microorganism was able to significantly reduce the concentrations of both glucose and galactose in only 8 h. Considering this result and the lack of the β-galactosidase enzyme, *S. cerevisiae* was chosen as the most promising microorganism to be used in the epilactose purification. Therefore, *S. cerevisiae* was used in an additional test with pH adjusted to 6.0 using the same procedure described in Section 2.4.1. The experiments were then carried out for 8 h at 30 °C and 200 rpm (Fig. 5). Under these conditions, the yeast was able to eliminate the monomers almost completely (remaining glucose 8.2 ± 1.9% and galactose 9.4 ± 0.2%), while only 28.2 ± 0.5% of epilactose was hydrolyzed. This result is in accordance with the value obtained in the acid hydrolysis assay at pH 6.0 (26%). Therefore, under these conditions, the yeast *S. cerevisiae* was proven to be the most suitable microorganism for monosaccharides

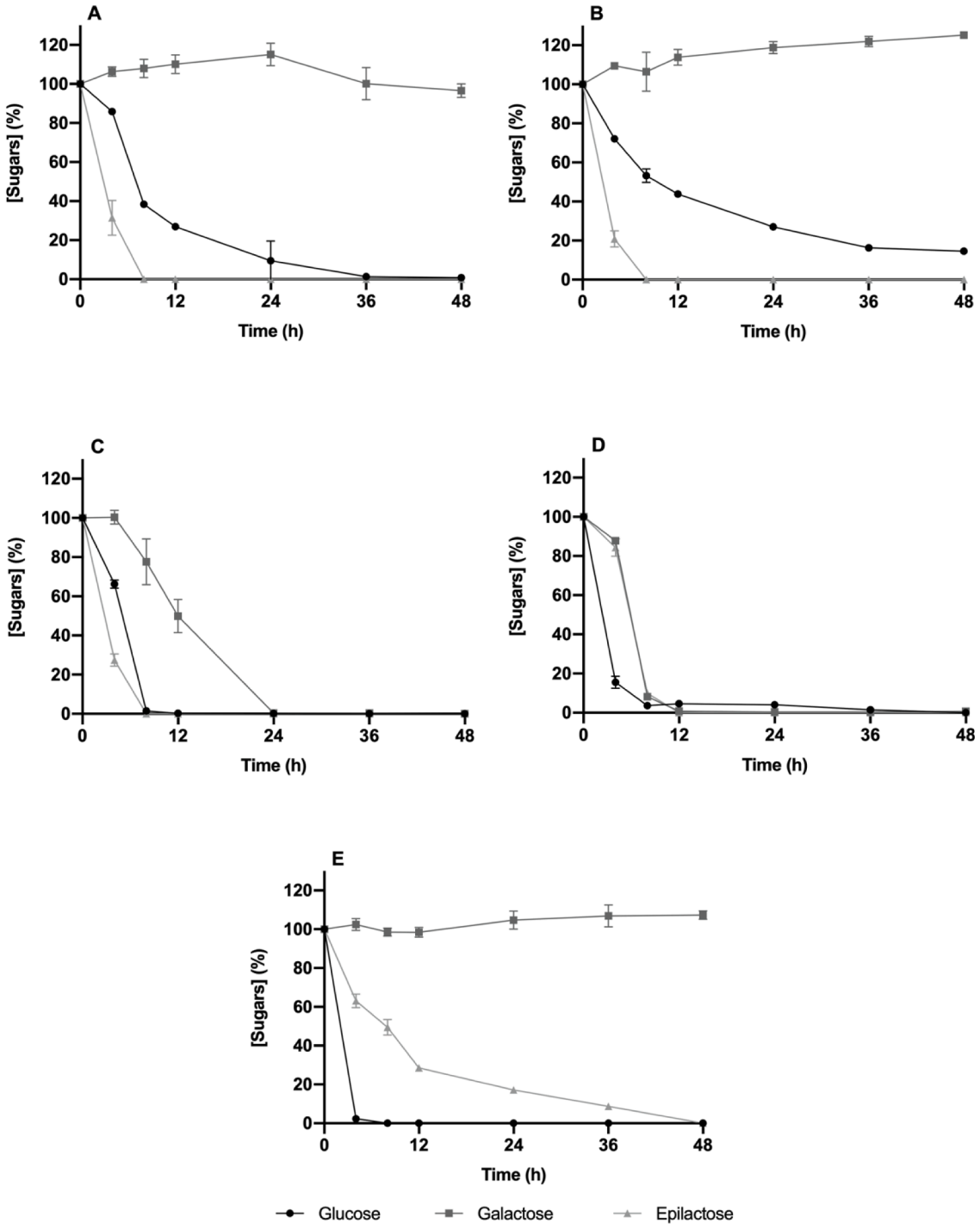

Fig. 3. Glucose, galactose and epilactose consumption by different microorganisms: A – *Bacillus subtilis*. B – *Escherichia coli*. C – *Kluyveromyces marxianus*. D – *Saccharomyces cerevisiae*. E – *Zymomonas mobilis*. The experiments were conducted at the optimal conditions for each microorganism during 48 h. Results correspond to the mean ± SD (n = 2).

removal. In fact, the use of the baker's yeast for monomers consumption and product purification is a common approach [11] and was already used in some epilactose purification strategies [7,17,20].

3.3.2. *Activated charcoal treatment*

Activated charcoal is commonly used in prebiotic purification strategies [2,1,3,13] due to its capacity to selectively adsorb compounds, resulting in the easy removal of undesired sugars or salts, while maintaining a low cost of operation [8]. In that sense, the hydrolyzed sugar mixture (obtained as mentioned in Section 3.2) was treated with activated charcoal 8–20 mesh during 3 h. After the incubation, 80.0 ± 2.2% of the epilactose and 44.1 ± 5.9% of the monosaccharides were adsorbed to the charcoal meaning that more than 50% of the initial glucose and galactose were removed. The charcoal was washed three times with water and then ethanol was used as eluent to promote the desorption of the remaining carbohydrates. The recovery of the epilactose reached 45.2 ± 12.4% and around 4% of the monosaccharides were found to be present in the supernatant. Despite the great reduction of the content of glucose and galactose (96%), only <50% of the epilactose was recovered, which represents a significant loss of the

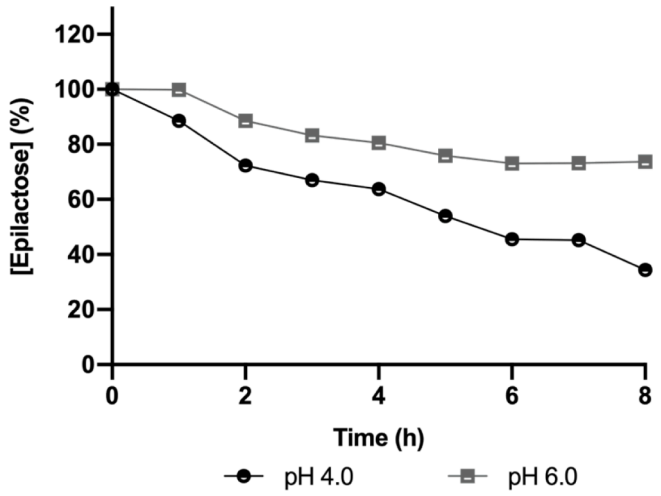

Fig. 4. Epilactose resistance to pH 4.0 and 6.0 when incubated at 37 °C for 8 h.

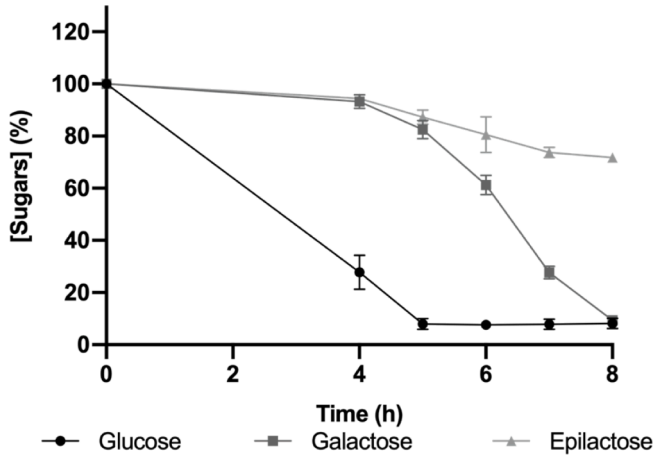

Fig. 5. Glucose, galactose and epilactose consumption by *Saccharomyces cerevisiae*. The experiment was conducted at 30 °C and 200 rpm during 48 h. Results correspond to the mean ± SD (n = 2).

compound of interest. Comparing these results with the ones obtained with the microorganism treatment it is possible to conclude that among all the strategies herein evaluated, the use of the yeast *S. cerevisiae* is the most promising approach to eliminate the monosaccharides and purify the epilactose at an industrial scale for further food applications.

3.4. Epilactose purification strategy

Considering the results herein described, the epilactose purification strategy was established using two different steps: the use of the β-galactosidase enzyme (*An*Gal) for lactose hydrolysis and the yeast treatment for the removal of the monosaccharides. Additionally, scale-up experiments (Section 2.5) were performed to validate the results and to obtain purer epilactose. For the *An*Gal step, 25 mL reactions were conducted for 45 min resulting in 3.8% and 99.5% of epilactose and lactose hydrolysis, respectively. Concerning the *S. cerevisiae* treatment, 2 L bioreactors were used (400 mL working volume) and the experiment was conducted for 8 h. At the end, 96.4% of the monosaccharides were removed. The overall process resulted in a sugar mixture containing 8.7 g/L of epilactose, 0.48 g/L of glucose, and 0.81 g/L of galactose, which represents an epilactose purity of 87.0% (total sugar basis) and a recovery yield of 74.6%.

Table 3 summarizes the reported strategies for epilactose purification, and the values obtained for its purity and yield. The methodology

Table 3
Comparison of epilactose purification strategies regarding the compound purity and yield.

Purification strategy	Purity (%)	Yield (%)	Reference
2 steps: β-galactosidase + Yeast treatment	87.0	74.6	This work
2 steps: Crystallization + Semi-preparative (Pb^{2+}) chromatography	99.0	51.0	[16]
3 steps: β-galactosidase + Yeast treatment + Cation exchange chromatography	98.5	24.0	[7]
3 steps: β-galactosidase + Yeast treatment + Amberlite CR1320 Ca^{2+} resin	95.0	69.2	[17]
4 steps: Crystallization + β-galactosidase + Yeast treatment + Ion exchange chromatography	91.1	42.5	[20]

used in this work led to the highest yield (74.6%) reported so far for epilactose recovery. Moreover, the establishment of a strategy with only two steps represents a significant improvement when compared with the reported methodologies, as it significantly reduces the time and costs of the purification process. In fact, only Kuschel and collaborators presented a two-step process, but the epilactose yield was considerably low [16]. In addition, the authors used crystallization and semi-preparative chromatography, which are more complex and expensive methodologies than the ones herein described. Concerning the purity of the compound, it was obtained a lower value than the ones reported in other studies. However, a purity of 87% can be considered a satisfactory value for food/nutritional applications. In fact, the purity is always dependent on the final desired application. For example, Amorim and collaborators reported a functional sweetening mixture containing 75% of fructo-oligosaccharides suitable for food applications [1]. Additionally, we can also find on the market products with lower contents of prebiotics, as the case of the GNC Prebiotic Galacto-oligosaccharides commercialized by GNC® that present a GOS content around 53% and several lactulose syrups that usually contain around 75% of the compound.

3.5. Digestive resistance of epilactose

To be able to modulate the gut microbiota, a potential prebiotic compound must reach the large intestine intact, meaning that it needs to be resistant to the digestion that occurs in the upper gastrointestinal tract [10]. This digestive process includes the passage through the acidic characteristics (pH 1.5–3.5) of the stomach, which can be a problem for the stability of the compound compromising its prebiotic effect on the colon. In the case of epilactose, as shown in Section 3.3.1, the pH is a parameter that can compromise the structure of the compound, strongly contributing for its hydrolysis. As epilactose is considered as an "under-development" prebiotic, there is a lack of works studying its gastric resistance. In fact, only one study reported the use of digestive enzymes using rat intestinal acetone powder to evaluate the epilactose resistance to the digestion conditions. However, the assay performed by the authors is not considered as an adequate model to simulate the human digestion, as more accurate models have been developed in the meanwhile. The INFOGEST 2.0 static *in vitro* simulation of gastrointestinal food digestion is currently the most used model to assess food and food components digestibility, as it is a standard and harmonized method [4].

Therefore, the resistance of the purified epilactose to the digestive enzymes and gastric acidity was tested using this harmonized static *in vitro* digestion model, which was already successfully used to evaluate the digestive resistance of other potential prebiotic compounds [2]. The results showed that epilactose was highly resistant to the harsh conditions present in the upper gastrointestinal system, since 77.0 ± 0.1% of the compound remained intact after 4 h of digestion at 37 °C (i.e., after exposure to oral, gastric, and small intestinal conditions). This value is in accordance with a previously reported study, that showed that 82% of the epilactose was resistant to the action of rat intestinal enzymes in a

reaction conducted at 37 °C for 2 h [12].

4. Conclusions

Overall, an effective, simple, and rapid method to obtain high purity epilactose for food applications was developed. This approach is based on two cheap and food-grade steps, namely the enzymatic hydrolysis of the unreacted lactose and the further removal of the monosaccharides from the mixture by baker's yeast treatment. The purification process herein described allows obtaining a 87% pure epilactose within one workday with a yield of 74.6%, the highest value reported so far in the literature for epilactose recovery. The pure compound has shown to be resistant to the gastric acidity and to the digestive enzymes of the upper gastrointestinal tract which reinforces the potential of epilactose to be used as prebiotic acting on the gastrointestinal tract.

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CRediT authorship contribution statement

Beatriz B. Cardoso: Conceptualization, Methodology, Investigation, Writing – original draft. **Jean-Michel Fernandes:** Conceptualization, Methodology, Investigation. **Ana C. Pinheiro:** Conceptualization, Writing – review & editing. **Adelaide Braga:** Conceptualization, Investigation, Writing – review & editing. **Sara C. Silvério:** Conceptualization, Supervision, Writing – review & editing. **Lígia R. Rodrigues:** Conceptualization, Resources, Supervision, Writing – review & editing, Funding acquisition.

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.

Data availability

Data will be made available on request.

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