Research Article

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Synthesis of prebiotic carbohydrates derived from cheese whey permeate by a combined process of isomerisation and transgalactosylation

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Abstract

BACKGROUND: Lactose from cheese whey permeate (WP) was efficiently isomerised to lactulose using egg shell, a food-grade catalyst, and the subsequent transgalactosylation reaction of this mixture with β-galactosidase from *Bacillus circulans* gave rise to a wide array of prebiotic carbohydrates derived from lactose and lactulose.

RESULTS: Lactulose obtained by efficient isomerisation of WP (16.1% by weight with respect to the initial amount of lactose) showed great resistance to the hydrolytic action of β-galactosidase from *B. circulans*, which preferentially hydrolysed lactose, acting as a galactosyl donor and acceptor. Lactulose had capacity as an acceptor, leading to the formation of lactulose-derived oligosaccharides. The enzymatic synthesis was optimised by studying reaction conditions such as pH, temperature, time, enzyme concentration and carbohydrate concentration. The maximum formation of galactooligosaccharides with degrees of polymerisation from 2 to 4 was achieved after 5 h of reaction at pH 6.5 and 50 °C with 300 g kg^{-1} carbohydrates and 3 U mL^{-1} β-galactosidase.

CONCLUSION: These findings indicate that the transgalactosylation of isomerised WP with β-galactosidase from *B. circulans* could be a new and efficient method to obtain a mixture with 50% of potentially prebiotic carbohydrates composed of lactulose, and galactooligosaccharides derived from lactose and lactulose.

Keywords: isomerisation; lactulose; galactooligosaccharides; transgalactosylation; *Bacillus circulans*; whey permeate

INTRODUCTION

Cheese whey is the most abundant by-product of the dairy industry and its disposal in the environment causes problems because of its high biochemical oxygen demand. Consequently, it is normally spray dried and used in low-value products such as animal feed or as a food supplement.[1] Alternatively, it is processed by ultrafiltration to yield whey protein concentrate and whey permeate (WP), the latter being an inexpensive by-product comprising mainly lactose and salts. Unlike whey proteins that find immediate food applications, WP has so far been of little value, probably owing to its high salt content.[2,3] Therefore its profitable use constitutes a relevant activity from the economic and environmental point of view.

The use of WP to produce lactose derivatives, including lactulose, lactitol, lactobionic acid, tagatose and sialyllactose, has long been of industrial interest.[4,5] In the last few years, increasing interest in the consumption of prebiotic carbohydrates has been observed, so the production of new bioactive oligosaccharides is currently gaining much attention for their potential use as functional ingredients.[6] Today, one of the most promising uses of WP is the synthesis of prebiotic galactooligosaccharides (GOS) via transgalactosylation of lactose catalysed by β-galactosidases (EC 3.2.1.23) of microbial origin.[7–10] Among them, β-galactosidase from *Bacillus circulans* has proved to have the ability to produce GOS in good yield from model systems consisting of lactose in buffered solutions.[11–13] However, few studies dealing with the production of GOS from cheese WP using β-galactosidase from *B. circulans* have been carried out.[14] In this sense, it is noteworthy to indicate that substantial differences in terms of yield and oligosaccharide composition between the production of GOS from model systems consisting of lactose in buffered solutions and their production from WP could be expected owing to the influence of other permeate ingredients such as mineral salts.[15–18] Furthermore, considering the relationship between the structure and prebiotic activity of oligosaccharides,[19,20] the synthesis of new

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lactulose-derived oligosaccharides has recently been reported in order to find new prebiotics with improved or complementary properties.[21–26]

The aim of this study was to develop a new approach based on the combined process of isomerisation of lactose present in cheese WP using a food-grade catalyst (egg shell) and subsequent enzymatic transgalactosylation with β-galactosidase from *B. circulans*, avoiding intermediate purification steps of lactulose, thereby contributing to the improvement of the production of a range of potential bioactive oligosaccharides. In consequence, data reported in this paper could help to broaden the use of cheese WP for the efficient production of functional carbohydrates.

MATERIALS AND METHODS

Chemical and reagents

Reagents employed for chromatographic analysis, including standards (glucose, galactose, fructose, lactose, lactulose, raffinose, stachyose and β-phenylglucoside), were obtained from Sigma (St Louis, MO, USA). Acetonitrile of high-performance liquid chromatography (HPLC) grade was purchased from Lab-Scan (Gliwice, Poland). All other chemicals were of analytical grade. Ultrapure water (18.2 MΩ cm, with levels of 1–5 ng mL^{-1} total organic carbon and <0.001 EU mL^{-1} pyrogen) produced in-house with a laboratory water purification system (Milli-Q Synthesis A10, Millipore, Billerica, MA, USA) was used throughout.

β-Galactosidase from *B. circulans* (neutral lactase) was acquired from Biocon (Barcelona, Spain). Lactase activity was 3000 U mL^{-1}, where 1 U is the amount of enzyme required to hydrolyse 1 μmol lactose min^{-1} at a working temperature of 50 °C and a lactose concentration of 300 g kg^{-1} at pH 6 in 0.05 mol L^{-1} phosphate buffer.

Egg shell powder

White egg shells were washed with tap water to remove all adhering albumen, dried at 105 °C for 24 h and ground in a ball mill (Mixer Mill MM 200, Retsch GmbH & Co. KG, Haan, Germany) at 800 rpm (13.3 Hz) for 15 min. The resulting egg shell powder had a particle size of approximately 5 μm and was stored in glass vials in a dry place at room temperature until use.

Physicochemical characterisation of cheese whey permeate

Industrial bovine cheese WP powder with a lactose content of 810 g kg^{-1} was kindly supplied by the dairy company Reny Picot (Navia, Spain). The pH of reconstituted WP was measured using a pH meter (MP 230, Mettler-Toledo, Barcelona, Spain) at a concentration of 300 g kg^{-1}.

Isomerisation reaction

The isomerisation reaction was performed according to Montilla *et al.*[27] with some modifications. A solution of WP powder in Milli-Q water at a concentration of 300 g kg^{-1} lactose was prepared. The sample was stirred at 750 rpm and 60 °C for 30 min, cooled to room temperature and then adjusted to pH 6.8 by adding 2 mol L^{-1} NaOH. Afterwards, 100 g of this sample was placed in a 250 mL round-bottom flask fitted with an additional necked sampling inlet, and 3 g of egg shell powder was added. The flask was immersed in a glycerol bath at 120 °C, stirred at 300 rpm and refluxed at 98 °C for 180 min. The start of boiling (5 min) was considered as time 0 of the reaction. Samples (20 mL) were taken at 0, 60, 90, 120, 150 and 180 min. The reaction was stopped by cooling in an ice-water bath. The egg shell was removed by centrifugation at 5000 × g for 10 min at 20 °C. The supernatant was collected, lyophilised and stored at −18 °C until further analysis. The isomerisation reaction was carried out in duplicate and analyses were performed twice for each isomerisation treatment.

Oligosaccharide synthesis

Enzymatic synthesis of oligosaccharides from isomerised whey permeate (IWP) using β-galactosidase from *B. circulans* was carried out under different reaction conditions of pH (5.5, 6.5 and 7.4), temperature (40, 50 and 60 °C), enzyme concentration (1.5, 3 and 6 U mL^{-1}), carbohydrate concentration (100, 300 and 500 g kg^{-1} reconstituted in Milli-Q water) and time (1, 3, 5, 8 and 24 h). Reactions were performed in a final volume of 1.5 mL in microtubes incubated in an orbital shaker at 300 rpm. Aliquots (250 μL) were withdrawn from the reaction mixture at the different times and immediately immersed in boiling water for 5 min to inactivate the enzyme. Samples were stored at −18 °C for subsequent analysis. Besides, another assay using WP or lactulose (300 g kg^{-1}) as substrate at 50 °C, pH 6.5 and an enzyme concentration of 3 U mL^{-1} was carried out. Enzymatic reactions were carried out in duplicate and analyses were performed twice for each enzymatic treatment.

Chromatographic determination of carbohydrates

Gas chromatography analysis

Sample preparation. A 200 μL aliquot of sample was made up to 2 mL with water in a volumetric flask and filtered using a 0.45 μm syringe filter (Symta, Madrid, Spain). Then 0.2 mg of phenyl-β-D-glucoside was added to 100 μL of the filtrate as internal standard and the mixture was dried at 38–40 °C in a rotary evaporator. These samples were analysed by two different gas chromatography systems as described below.

Gas chromatography with flame ionisation detection (GC-FID). Dried samples were treated with 100 μL of *N*-trimethylsilylimidazole to silylate the carbohydrates; the reaction was completed in 30 min at 70 °C. Silylated carbohydrates were extracted with 0.3 mL of hexane and 0.3 mL of water. A 1 μL aliquot of the organic phase containing silyl derivatives was injected into the column.

The trimethylsilyl ethers of carbohydrates were analysed as described previously using an Agilent Technologies 7890A gas chromatograph (Wilmington, DE, USA) equipped with a commercial fused silica capillary column SPB-17, bonded, crosslinked phase (50% diphenyl/50% dimethylsiloxane; 30 m × 0.32 mm i.d., 0.5 μm film thickness) (Supelco, Bellefonte, PA, USA).[28] The oven temperature was initially held at 235 °C for 9 min, then increased at a rate of 15 °C min^{-1} to 280 °C and held for 30 min. The injector and detector temperatures were set at 280 °C. Injections were carried out in split mode (1:30) using nitrogen at 1 mL min^{-1} as carrier gas. Data acquisition and integration were performed using Agilent ChemStation Rev. B.03.01 software.

To study the response factors relative to the internal standard, solutions containing glucose, galactose, lactose and lactulose were prepared over the expected concentration range in samples. The identities of carbohydrates were confirmed by comparison with relative retention times of standard samples. The amounts of lactose, lactulose, glucose and galactose remaining in the isomerisation and transgalactosylation mixtures were expressed as g kg^{-1}.

Gas chromatography/mass spectrometry (GC/MS). Selected samples of isomerised and/or transgalactosylated WP were

also analysed by GC/MS. An Agilent Technologies 7890A gas chromatograph coupled to an Agilent Technologies 5975C MSD quadrupole mass detector was employed. Trimethylsilyl oximes prepared as described by Cardelle-Cobas *et al*.[29] were separated on an HP-5 MS fused silica capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness) coated with 5% phenylmethylsilicone (J&W Scientific, Folsom, CA, USA). The helium flow rate was 1 mL min^{-1}. The oven temperature was increased from 180 to 315 °C at a rate of 3 °C min^{-1} and held for 20 min. The injector temperature was 280 °C. Injections were made in split mode (1:40). The mass spectrometer was operated in electrospray ionisation mode at 70 eV. Mass spectra were acquired using Agilent ChemStation MSD software.

Identification of trimethylsilyloxime derivatives of carbohydrates was carried out by comparison of their relative retention times and mass spectra with those of standard compounds previously derivatised.

HPLC with refractive index detection (HPLC-RID)

Samples of isomerised and transgalactosylated WP were diluted with acetonitrile/water (50:50 v/v), filtered using a 0.45 μm syringe filter (Symta) and analysed in an Agilent Technologies 1260 Series HPLC system (Böblingen, Germany). The separation of carbohydrates was carried out on a Kromasil® column (100-NH_2; 250 mm × 4.6 mm, 5 μm particle size) (Akzo Nobel, Brewster, NY, USA) using acetonitrile/water (75:25 v/v) as mobile phase and elution in isocratic mode at a flow rate of 1 mL min^{-1} for 50 min. The injection volume was 50 μL (~800 μg of total carbohydrates). Data acquisition and processing were performed using Agilent ChemStation software.

Carbohydrates in the reaction mixtures were initially identified by comparing their retention times (t_R) with those of standard sugars. Quantitative analysis was performed by the external standard method, using calibration curves in the range 0.01–10 mg for glucose (quantification of monosaccharides), lactose (disaccharides), raffinose (trisaccharides) and stachyose (tetrasaccharides). All analyses were performed in duplicate, obtaining relative standard deviation (RSD) values below 10% in all cases. The amounts of different carbohydrates present in the reaction mixtures were expressed as percentage by weight of the total carbohydrate content.

RESULTS AND DISCUSSION

Isomerisation of whey permeate using egg shell as catalyst

In order to carry out the isomerisation reaction, an industrial cheese WP was used and egg shell was chosen as catalyst instead of chemical reagents such as borates, sodium aluminate or hydroxides owing to its multiple advantages, i.e. lower quantity of catalyst required, easy removal of egg shell by centrifugation or filtration as compared with homogeneous catalysts, lower formation of products derived from side reactions, and relatively good yields of isomeric disaccharides. For this purpose, 3 g of egg shell powder was added to 100 g of reconstituted cheese WP (equivalent to 30 g of lactose) and the mixture was kept under reflux following previous studies reported by Montilla *et al*.[27] According to GC-FID analyses, the optimal production of lactulose was reached after 150 min of reaction. The carbohydrate composition of the reaction mixture after isomerisation was 7.0 g galactose, 1.0 g glucose, 1.1 g epilactose, 48.2 g lactulose and 209.1 g lactose kg^{-1} (Table 1). Thus 16.1% of lactulose by weight with respect to the initial amount of lactose was obtained under the assay conditions. A similar yield (18% of lactulose by weight with respect to the initial amount of lactose) was obtained by Montilla *et al*.[27] using concentrated milk permeate.

Transgalactosylation of isomerised cheese whey permeate using β-galactosidase from *B. circulans*

The effects of pH, temperature and enzyme concentration on the formation of GOS were studied with an initial carbohydrate concentration of 300 g kg^{-1}. The effect of substrate concentration from 100 to 500 g kg^{-1} was also assayed.

Effect of pH

Three values within the optimal pH range given by the manufacturer, i.e. pH 7.4 (pH value of permeate after isomerisation reaction), 6.5 and 5.5, were assayed at 50 °C with 3 U mL^{-1} β-galactosidase. The formation of GOS from IWP was monitored by HPLC-RID as shown in Fig. 1. As expected, the order of elution was according to the degree of polymerisation of carbohydrates: monosaccharides eluted at 7–10 min, disaccharides at 11–20 min, trisaccharides at 22–33 min and tetrasaccharides above 33 min. In addition, the presence of pentasaccharides in minor amounts cannot be ruled out, since β-galactosidase from *B. circulans* has been shown previously to be capable of producing pentasaccharides.[30] Glucose (Glc, peak 1), galactose (Gal, peak 2), lactulose (Lu, peak 3) and lactose (Lac, peak 4) were identified by comparison of their retention times with those of commercial standards, while β-D-Galp-(1→6)-D-Glu (allolactose, peak 5) was identified by comparison with a standard previously isolated in our laboratory.[31] The disaccharides formed could tentatively be assigned to galactosyl-disaccharides with links β-(1→2) and β-(1→3) according to previous studies on the transgalactosylation of lactose by β-galactosidase from *B. circulans*.[12] The principal

Table 1. Levels of galactose, glucose, epilactose, lactulose and lactose produced during heating at reflux of 300 g kg^{-1} permeate powder solution at pH 6.8 with 30 g kg^{-1} egg shell powder

Time (min)	Galactose (g kg^{-1})	Glucose (g kg^{-1})	Epilactose (g kg^{-1})	Lactulose (g kg^{-1})	Lactose (g kg^{-1})
0	0.8 ± 0.2	1.2 ± 0.2	ND	ND	293.5 ± 5.1
60	3.0 ± 0.2	1.1 ± 0.1	0.4 ± 0.1	22.4 ± 0.1	261.1 ± 4.6
90	4.5 ± 0.5	1.0 ± 0.1	0.8 ± 0.1	32.0 ± 1.4	238.8 ± 8.0
120	6.9 ± 0.3	1.2 ± 0.1	1.0 ± 0.1	38.3 ± 0.1	232.5 ± 5.8
150	7.0 ± 0.2	1.0 ± 0.0	1.1 ± 0.0	48.2 ± 0.3	209.1 ± 1.9
180	10.2 ± 0.2	1.0 ± 0.0	1.4 ± 0.0	43.9 ± 1.8	200.6 ± 3.8

Values are mean ± standard deviation ($n = 4$). ND, not detected.

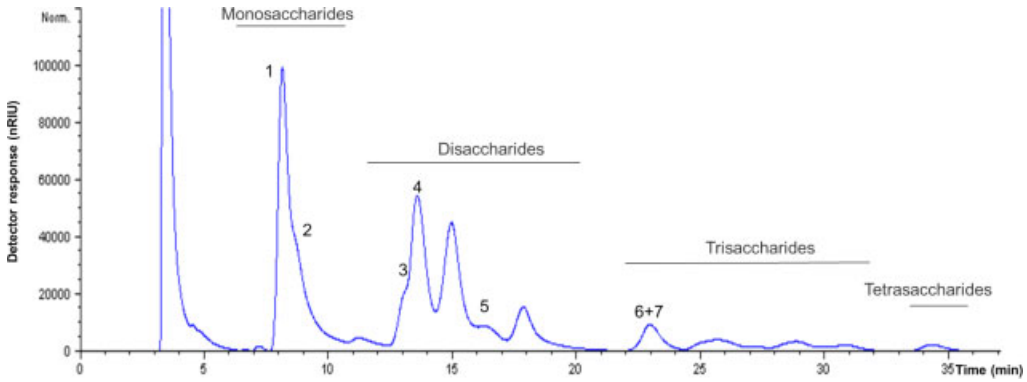

Figure 1. HPLC-RID profile of carbohydrate mixture obtained by transgalactosylation reaction of isomerised cheese whey permeate at pH 6.5 and 50 °C for 5 h with β-galactosidase from *Bacillus circulans* (3 U mL^{-1}) and initial carbohydrate concentration of 300 mg mL^{-1}. Identified peaks: 1, glucose (Glc); 2, galactose (Gal); 3, lactulose (Lu); 4, lactose (Lac); 5, β-D-Galp-(1→6)-D-Glc (allolactose); 6, β-D-Galp-(1→4)-Lac; 7, β-D-Galp-(1→4)-Lu.

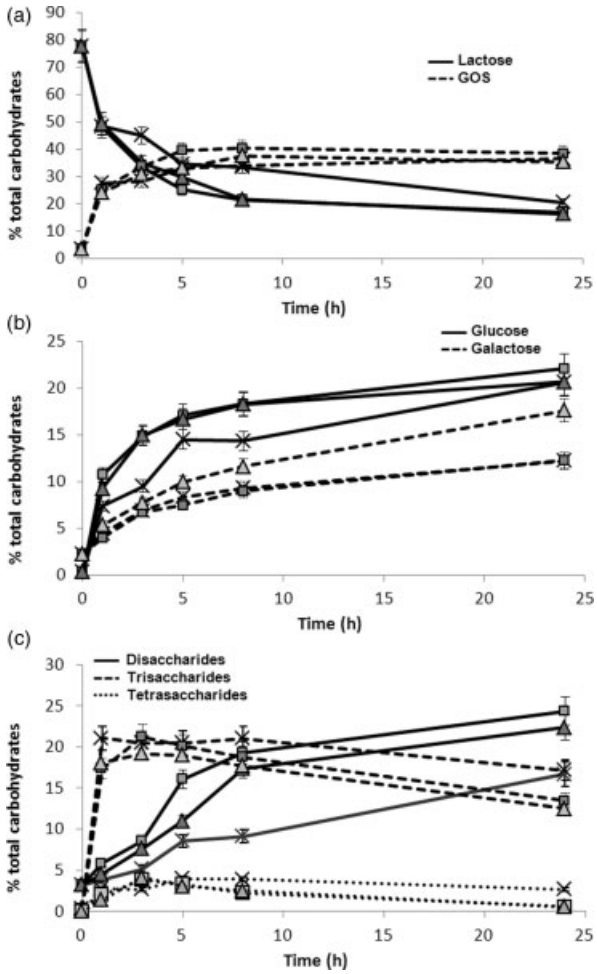

Figure 2. Effect of pH on hydrolysis of isomerised cheese whey permeate (300 mg mL^{-1} carbohydrates) and oligosaccharide production during enzymatic treatment with β-galactosidase from *Bacillus circulans* (3 U mL^{-1}) at 50 °C and pH 7.4 (crosses), pH 6.5 (squares) and pH 5.5 (triangles). Vertical bars represent standard deviation ($n = 4$).

trisaccharide, β-D-Galp-(1→4)-Lac (peak 6), was identified by comparison with a standard previously synthesised in our laboratory.[31]

Figure 2 shows the time course of the β-galactosidase-catalysed reaction at pH 5.5, 6.5 and 7.4. The lactose concentration decreased quickly from the start of the reaction to 24 h (Fig. 2a), this decrease being slowest at pH 7.4. Hydrolysis of lactose was very efficient (from 79% at 0 h to 17% after 24 h at pH 6.5 and 5.5) and gave rise to the formation of glucose and a smaller quantity of galactose, regardless of the pH value (Fig. 2b), which is indicative of the transfer of galactose to form GOS. Although the lactose hydrolysis rate was similar at pH 5.5 and 6.5, the amount of free galactose at pH 6.5 was lower and consequently the formation of GOS (tri- and tetrasaccharides) was higher and faster than at pH 5.5 (Fig. 2c). Moreover, during the first 5 h of reaction, trisaccharides were the most abundant carbohydrates formed, followed by di- and tetrasaccharides respectively. Nevertheless, after 24 h of reaction, disaccharides were the predominant saccharides formed, presumably owing to the partial degradation of tri- and tetrasaccharides as well as the continuous synthesis of disaccharides (Fig. 2c). Thus maximum formation of GOS, which led to 40% (w/w) of total sugars, was achieved after 5 h of reaction at pH 6.5, while 8 h of reaction was needed for maximum formation of GOS, i.e. 38% (w/w) of total sugars, at pH 5.5 (Fig. 2a). Other studies on the transgalactosylation of WP solutions by β-galactosidase from *B. circulans* reported yields ranging from 12 to 31%.[15,32] Cheng *et al.*,[13] using similar reaction conditions to those reported here but with lactose solution, obtained a GOS yield of 43%. Other studies carried out with lactose solution and β-galactosidase from *B. circulans* but using different reaction conditions obtained considerably lower yields ranging from 6 to 26%.[9,11,12]

Moreover, the lactulose concentration decreased moderately during the first 3 h of reaction (from 18% at 0 h to 10–11% at 3 h) and then remained fairly constant (10% at 24 h), indicating that, in the presence of both disaccharides, β-galactosidase from *B. circulans* is prone to hydrolyse lactose instead of lactulose, since GC analyses of these samples confirmed the very low presence of fructose (<0.5% at 8 h). Therefore the slight decrease in lactulose could be mainly attributed to the formation of lactulose-derived oligosaccharides. This fact was corroborated by comparing the GC/MS profiles of GOS obtained from WP and IWP treated with β-galactosidase from *B. circulans*, where an additional trisaccharide, probably corresponding to a galactosyl-lactulose derivative, was detected in the latter. This trisaccharide was also detected following the transgalactosylation of purified lactulose with β-galactosidase from *B. circulans* (chromatogram not shown) and was identified by comparison with the standard β-D-Galp-(1→4)-Lu previously synthesised in our laboratory.[31] This compound also appeared

in Fig. 1 as peak 7 and coeluted with β-D-Galp-(1→4)-Lac. The mass spectrum of the main lactulose-derived trisaccharide was characterised by the ions (in decreasing order of abundance) *m/z* 204, 73, 361, 217, 205, 147, 191, 103, 129, 169, 321, 319, 271, 305 and 448, while the ions from the lactose-derived trisaccharide β-D-Galp-(1→4)-Lac were *m/z* 204, 361, 73, 217, 205, 147, 191, 129, 103, 169, 271, 319, 451, 331 and 305. This means that ions *m/z* 321 and 448 were characteristic for the lactulose-derived trisaccharide, while ions *m/z* 451 and 331 were characteristic for β-D-Galp-(1→4)-Lac.

Effect of temperature

In addition to 50 °C, reactions at 40 and 60 °C and pH 6.5 with 3 U mL^{-1} β-galactosidase were carried out. Lactose hydrolysis was accelerated at 50 and 60 °C in comparison with 40 °C (Fig. 3a), which is in accordance with the higher levels of glucose detected throughout the reactions at 50 and 60 °C (Fig. 3b). Nevertheless, the levels of galactose were higher at 40 °C than at 50 and 60 °C (Fig. 3b), which is in good agreement with the fact that the formation of total GOS was higher and faster (maximum formation at 5 h) at 50 °C (39.5 ± 1.5%) and 60 °C (37.5 ± 2.0%) than at 40 °C (35.6 ± 2.0%), where the maximum level of GOS was obtained after 8 h of reaction (Fig. 3a). Therefore the commercial enzyme used in these assays seems to be more thermoresistant than that used by Boon *et al.*,[30] who observed inactivation of lactose hydrolysis after 90 min at 60 °C.

The different degrees of polymerisation of GOS were also studied, as shown in Fig. 3c. Similar amounts of disaccharides were formed at the end of the reactions carried out at 50 and 60 °C, i.e. 24 and 25% (w/w) of total sugars respectively, while 19% of disaccharides were found at 40 °C. The maximum levels of trisaccharides were reached after 3 h of reaction at all three temperatures assayed, then a gradual decrease with time was observed. A maximum of 21–22% of trisaccharides were quantified at 40 and 50 °C, while only 15% of trisaccharides were found at 60 °C. Similar quantities of tetrasaccharides (3–4% of total sugars) were obtained at all three temperatures assayed, although the maximum level was achieved faster in the reactions at 50 and 60 °C (3 h) than in the reaction at 40 °C (5 h).

Although similar levels of total GOS were obtained at 50 and 60 °C (Fig. 3a), the temperature selected for subsequent analyses was 50 °C, because higher amounts of tri- and tetrasaccharides were obtained at that temperature. Mozaffar *et al.*[11] reported an optimal temperature of 60 °C for two isoforms of β-galactosidase from *B. circulans*, although they only provided data on total GOS, and no differentiation of degree of polymerisation was carried out.

Effects of enzyme and substrate concentrations

To determine the effect of enzyme concentration on GOS production, in addition to 3 U mL^{-1} β-galactosidase, 1.5 and 6 U mL^{-1} enzyme levels were also assayed at 50 °C and pH 6.5 with 300 g kg^{-1} carbohydrates. Figure 4a displays the lactose content remaining during the time course of the reaction. The results showed that the lowest concentration of enzyme assayed (1.5 U mL^{-1}) led to the lowest hydrolysis of lactose (27% of lactose remaining after 24 h of reaction) and hence the lowest formation of monosaccharides (Fig. 4b). However, no differences in lactose hydrolysis rate were found between 3 and 6 U mL^{-1} enzyme. Figure 4c shows the di-, tri- and tetrasaccharide yields throughout the enzymatic reaction. Although the highest amount of trisaccharides formed was

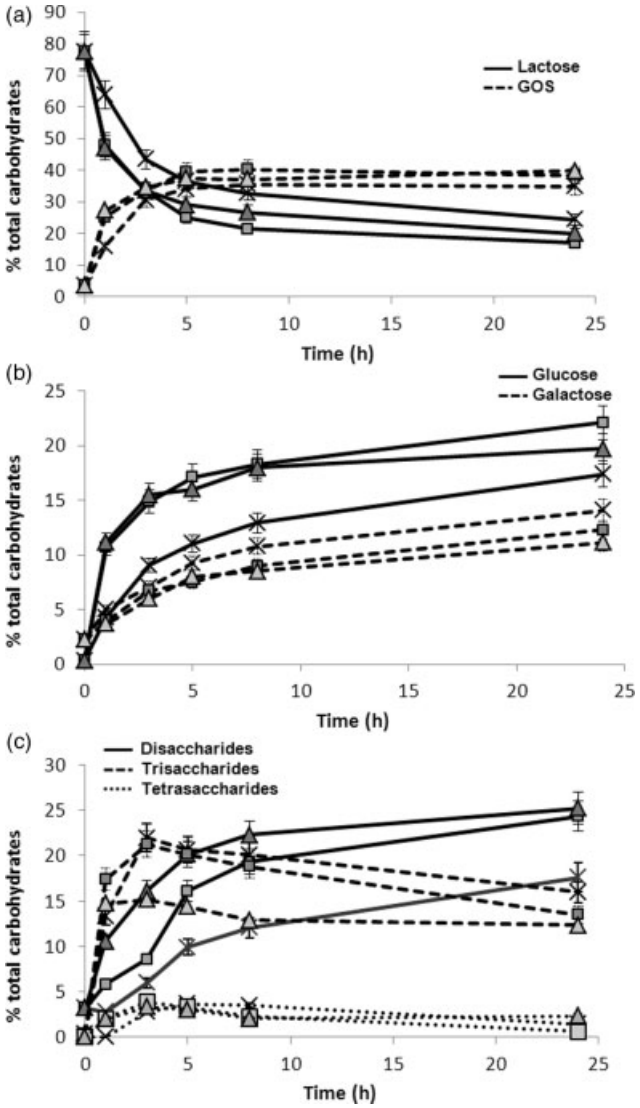

Figure 3. Effect of temperature on hydrolysis of isomerised cheese whey permeate (300 mg mL^{-1} carbohydrates) and oligosaccharide production during enzymatic treatment with β-galactosidase from *Bacillus circulans* (3 U mL^{-1}) at pH 6.5 and 40 °C (crosses), 50 °C (squares) and 60 °C (triangles). Vertical bars represent standard deviation ($n = 4$).

similar (20–21%) for all enzyme concentrations assayed, the lowest trisaccharide formation rate was observed with 1.5 U mL^{-1} enzyme. Moreover, the hydrolysis rate of oligosaccharides increased with increasing enzyme concentration. The formation of disaccharides increased steadily with increasing reaction time for all three enzyme concentrations assayed. The highest disaccharide content (27%) was found after 24 h when the synthesis was performed with 6 U mL^{-1} enzyme. Generally, in enzyme-catalysed reactions the reaction rate is directly proportional to the enzyme concentration up to a certain level, after which the proportionality is lost. This effect was found by Das *et al.*,[14] who reported that beyond a dose of 0.5% of β-galactosidase from *B. circulans* there was no further increase in GOS yield. The same effect was also observed by other authors for different enzymes and substrates.[33,34] In our assays, since the yield differences between the reactions with 3 and 6 U mL^{-1} enzyme were negligible, the lower amount of enzyme was chosen to reduce the cost of operation.

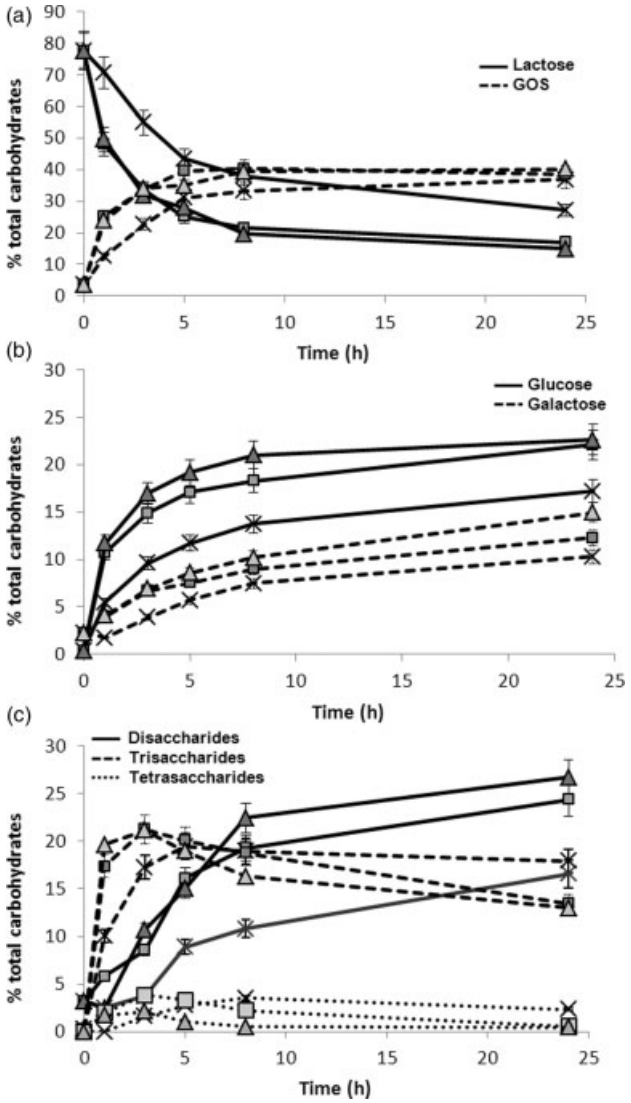

Figure 4. Effect of enzyme concentration on hydrolysis of isomerised cheese whey permeate (300 mg mL^{-1} carbohydrates) and oligosaccharide production during enzymatic treatment with β-galactosidase from *Bacillus circulans* at 1.5 U mL^{-1} (crosses), 3 U mL^{-1} (squares) and 6 U mL^{-1} (triangles) at 50 °C and pH 6.5. Vertical bars represent standard deviation ($n = 4$).

The last factor studied was the initial concentration of substrate. Reactions with 100, 300 and 500 g kg^{-1} carbohydrates at 50 °C and pH 6.5 with 3 U mL^{-1} enzyme were carried out. At the lowest substrate concentration the reaction was too fast, and after 5 h of reaction the lactose remaining was only 14%. In this condition the highest amount of trisaccharides formed was 14% after 1 h of reaction, to be then quickly hydrolysed. Nevertheless, when the highest concentration of carbohydrates (500 g kg^{-1}) was used, the lactose was hardly hydrolysed after 24 h of reaction. In assays performed with 6 U mL^{-1} enzyme and 500 g kg^{-1} carbohydrates, no improvement in GOS yields was obtained compared with those achieved using 300 g kg^{-1} carbohydrates (data not shown).

CONCLUSIONS

To summarise according to the results obtained, maximum formation of GOS was achieved after 5 h of reaction at pH 6.5 and 50 °C with 300 g kg^{-1} carbohydrates and 3 U mL^{-1} β-galactosidase, giving rise to 24% monosaccharides, 25% lactose, 11% lactulose and 40% GOS with degrees of polymerisation from 2 to 4 (whose distribution was 16% disaccharides, 21% trisaccharides and 3% tetrasaccharides). These results highlight the formation of oligosaccharides with different structures and thus with potentially different prebiotic properties.

Several studies have demonstrated that glycosidic linkages, monosaccharide composition and the degree of polymerisation of GOS contribute to the selectivity of fermentation by beneficial bacteria.[19,20] In this context the production of a mixture of prebiotics with a wide diversity of structural features might provide a value-added functional ingredient, since it could broaden its positive effects on the modulation of gut microbiota. Likewise, the presence of lactulose in addition to GOS could provide additional value to the final product, since lactulose has been shown to exert a number of biological activities such as prebiotic action[35] and improvement of intestinal transit time[36] as well as other beneficial physiological actions such as relief of chronic constipation, hepatic encephalopathy and inflammatory bowel disease.[37]

In conclusion, our results could contribute to the diversification of synthesised oligosaccharides, indicating that a novel approach, based on the combined process of isomerisation of lactose from cheese WP using a food-grade catalyst (egg shell) and subsequent enzymatic transgalactosylation with β-galactosidase from *B. circulans*, was useful to obtain a mixture composed of 50% of potentially prebiotic carbohydrates formed by lactulose, and GOS derived from lactose and lactulose. Both types of GOS have proven to be an excellent alternative to monosaccharides to support the growth of probiotics and improve their survival through the gastrointestinal tract.[26]

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REFERENCES

1 Ganzle MG, Haase G and Jelen P, Lactose: crystallization, hydrolysis and value-added derivatives. *Int Dairy J* **18**:685–694 (2008).
2 Barile D, Tao N, Lebrilla CB, Coisson J-D, Arlorio M and German JB, Permeate from cheese whey ultrafiltration is a source of milk oligosaccharides. *Int Dairy J* **19**:524–530 (2009).
3 Fox PF, Chemistry and properties, in *Advanced Dairy Chemistry* (3rd edn), Vol. **3**, ed. by McSweeney PLH and Fox PF. Springer Science+Business Media, New York, NY, pp. 1–17 (2009).
4 Lifran EV, Hourigan JA and Sleigh RW, Lactose derivatives: turning waste into functional foods. *Aust J Dairy Technol* **64**:89–93 (2009).
5 Ganzle MG, Enzymatic synthesis of galacto-oligosaccharides and other lactose derivatives (hetero-oligosaccharides) from lactose. *Int Dairy J* **22**:116–122 (2012).
6 Figueroa-Gonzalez I, Quijano G, Ramirez G and Cruz-Guerrero A, Probiotics and prebiotics – perspectives and challenges. *J Sci Food Agric* **91**:1341–1348 (2011).
7 Rustom IYS, Foda MI and Lopez-Leiva M, Formation of oligosaccharides from whey UF-permeate by enzymatic hydrolysis: analysis of factors. *Food Chem* **62**:141–147 (1998).
8 Goulas A, Tzortzis G and Gibson GR, Development of a process for the production and purification of α- and β-galactooligosaccharides

from *Bifidobacterium bifidum* NCIMB 41171. *Int Dairy J* **17**:648–656 (2007).
9 Splechtna B, Nguyen TH and Haltrich D, Comparison between discontinuous and continuous lactose conversion processes for the production of prebiotic galacto-oligosaccharides using β-galactosidase from *Lactobacillus reuteri*. *J Agric Food Chem* **55**:6772–6777 (2007).
10 Adamczak M, Charubin D and Bednarski W, Influence of reaction medium composition on enzymatic synthesis of galactooligosaccharides and lactulose from lactose concentrates prepared from whey permeate. *Chem Papers* **63**:111–116 (2009).
11 Mozaffar Z, Nakanishi K, Matsuno R and Kamikubo T, Purification and properties of β-galactosidases from *Bacillus circulans*. *Agric Biol Chem* **48**:3053–3061 (1984).
12 Yanahira S, Kobayashi T, Suguri T, Nakakoshi M, Miura S, Ishikawa H, *et al*. Formation of oligosaccharides from lactose by *Bacillus circulans* β-galactosidase. *Biosci Biotechnol Biochem* **59**:1021–1026 (1995).
13 Cheng TC, Duan KJ and Sheu DC, Application of tris(hydroximethyl) phosphine as a coupling agent for β-galactosidase immovilized on chitosan to produce galactooligosaccharides. *J Chem Technol Biotechnol* **81**:233–236 (2006).
14 Das R, Sen D, Sarkar A, Bhattacharyya S and Bhattacharjee CA, Comparative study on the production of galacto-oligosaccharide from whey permeate in recycle membrane reactor and in enzymatic batch reactor. *Ind Eng Chem Res* **50**:806–816 (2011).
15 Mozaffar Z, Nakanishi K and Matsuno R, Formation of oligosaccharides during hydrolysis of lactose in milk using β-galactosidase from *Bacillus circulans*. *J Food Sci* **50**:1602–1606 (1985).
16 Hellerová K and Čurda L, Influence of type of substrate and enzyme concentration on formation of galacto-oligosaccharides. *Czech J Food Sci* **27**:372–374 (2009).
17 Gosling A, Alftrén J, Stevens GW, Barber AR, Kentish SE and Gras SL, Facile pretreatment of *Bacillus circulans* β-galactosidase increases the yield of galactosyl oligosaccharides in milk and lactose reaction systems. *J Agric Food Chem* **57**:11570–11574 (2010).
18 Pocedicová K, Curda L, Misún D, Dryáková A and Diblíková L, Preparation of galacto-oligosaccharides using membrane reactor. *J Food Eng* **99**:479–484 (2010).
19 Rowland IR and Tanaka R, The effects of transgalactosylated oligosaccharides on gut flora metabolism in rats associated with a human fecal microflora. *J Appl Bacteriol* **74**:667–674 (1993).
20 Sanz ML, Gibson GR and Rastall RA, Influence of disaccharide structure on prebiotic selectivity *in vitro*. *J Agric Food Chem* **53**:5192–5199 (2005).
21 Cardelle-Cobas A, Martínez-Villaluenga C, Villamiel M, Olano A and Corzo N, Synthesis of oligosaccharides derived from lactulose and Pectinex Ultra SP-L. *J Agric Food Chem* **56**:3328–3333 (2008).
22 Cardelle-Cobas A, Fernandez M, Salazar N, Martınez-Villaluenga C, Villamiel M, Ruas-Madiedo P, *et al*. Bifidogenic effect and stimulation of short chain fatty acid production in human faecal slurry cultures by oligosaccharides derived from lactose and lactulose. *J Dairy Res* **76**:317–325 (2009).
23 Cardelle-Cobas A, Corzo N, Martinez-Villaluenga C, Olano A and Villamiel M, Effect of reaction conditions on lactulose-derived trisaccharides obtained by transgalactosylation with β-galactosidase of *Kluyveromyces lactis*. *Eur Food Res Technol* **233**:89–94 (2011).
24 Hernandez-Hernandez O, Montañes F, Clemente A, Moreno FJ and Sanz ML, Characterization of galactooligosaccharides derived from lactulose. *J Chromatogr A* **42**:7691–7696 (2011).
25 Cardelle-Cobas A, Corzo N, Olano A, Peláez C, Requena T and Ávila M, Galactooligosaccharides derived from lactose and lactulose: influence of structure on *Lactobacillus*, *Streptococcus and Bifidobacterium growth*. *Int J Food Microbiol* **149**:81–7 (2011).
26 Hernandez-Hernandez O, Muthaiyan A, Moreno FJ, Montilla A, Sanz ML and Ricke SC, Effect of prebiotic carbohydrates on the growth and tolerance of *Lactobacillus*. *Food Microbiol* **30**:355–361 (2012).
27 Montilla A, del Castillo MD, Sanz ML and Olano A, Egg shell as catalyst of lactose isomerisation to lactulose. *Food Chem* **90**:883–890 (2005).
28 Montilla A, Moreno FJ and Olano A, A reliable gas capillary chromatographic determination of lactulose in dairy samples. *Chromatographia* **62**:311–314 (2005).
29 Cardelle-Cobas A, Martínez-Villaluenga C, Sanz ML and Montilla A, Gas chromatographic–mass spectrometric analysis of galactosyl derivatives obtained by the action of two different β-galactosidases. *Food Chem* **114**:1099–1105 (2009).
30 Boon MA, Janssen AEM and van't Riet K, Effect of temperature and enzyme origin on the enzymatic synthesis of oligosaccharides. *Enzyme Microb Technol* **26**:271–281 (2000).
31 Cardelle-Cobas A, Synthesis, characterization and prebiotic properties of oligosaccharides derived from lactulose. *PhD Thesis*, Universidad Autónoma de Madrid (2009).
32 Bakken AP, Hill Jr CG and Amundson CH, Hydrolysis of lactose in skim milk by immobilized β-galactosidase (*Bacillus circulans*). *Biotechnol Bioeng* **39**:408–417 (1992).
33 Martınez-Villaluenga C, Cardelle-Cobas A, Corzo N, Olano A and Villamiel M, Optimization of conditions for galactooligosaccharide synthesis during lactose hydrolysis by β-galactosidase from *Kluyveromyces lactis* (Lactozym 3000 L HP G). *Food Chem* **107**:258–264 (2008).
34 Montilla, A, Olano, A, Martínez-Villaluenga C and Corzo N, Study of influential factors on oligosaccharide formation by fructosyltransferase activity during stachyose hydrolysis by Pectinex Ultra SP-L. *J Agric. Food Chem* **59**:10705–10711 (2011).
35 Méndez A and Olano A, Lactulose: a review on some chemical properties and applications in infant nutrition and medicine. *Dairy Sci Abstr* **41**:531–535 (1979).
36 EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA), Scientific opinion on the substantiation of health claims related to lactulose and decreasing potentially pathogenic gastro-intestinal microorganisms (ID 806) and reduction in intestinal transit time (ID 807) pursuant to Article 13(1) of Regulation (EC) No 1924/2006. *EFSA J* **8**(10):1806–1821 (2010).
37 Panesar PS and Kumari S, Lactulose: production, purification and potential applications. *Biotechnol Adv* **29**:940–948 (2011).

