An efficient process for obtaining of prebiotic oligosaccharides derived from lactulose using isomerised and purified whey permeate

Running title: Efficient process for obtaining prebiotics from purified isomerised permeate

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Abstract

BACKGROUND: One of the most promising uses of whey permeate (WP) is the synthesis of prebiotic oligosaccharides. Herein, commercial WP was submitted to chemical isomerisation catalysed by sodium borate at an alkaline pH and subsequent purification using anion-exchange resins to remove boron. Afterwards, purified mixtures were used to synthesise prebiotic oligosaccharides using β -galactosidase from *Bacillus circulans*.

RESULTS: Isomerisation of concentrated WP (200 g L⁻¹ lactose) gave rise to levels of lactulose up to 155.5 g L⁻¹ after 30 min of reaction (molar ratio boron/lactose 1/1; pH 12; 70 °C). Boron was removed from the isomerised WP (IWP) using the combination of a strong acid (IR-120, H⁺) and a weak base (IRA-743) anion-exchange resins reducing its level to less than 1 ppm, without loss of lactulose. During the transglycosylation reaction of purified IWP (lactose/lactulose ratio 1/2.4) maximum content of prebiotic compounds was achieved i.e. 690 g kg⁻¹ WP after 3 h of reaction.

CONCLUSION: This study shows that combined chemical-enzymatic reactions together with the purification of IWP results in an efficient synthesis of prebiotic oligosaccharides.

Keywords: whey permeate; isomerisation; anion-exchange purification; transglycosylation; galactooligosaccharides; oligosaccharides derived from lactulose

INTRODUCTION

Whey is the most abundant by-product generated from the cheese making process. Approximately 80% of the volume of milk used to make cheese is recovered as whey that contains more than half of the solids of milk. Traditionally, most of the whey produced has been treated as dairy wastewater in the industry, representing a major environmental problem (chemical oxygen demand of 50,000 mg L⁻¹, biological oxygen demand of 80,000 mg L⁻¹).^{1,2} However, since the introduction of ultrafiltration techniques in the cheese industry, to produce whey-protein concentrates, whey is more valued mainly for the nutritional benefits of the proteins recovered.^{3,4} The remaining by-product, whey permeate (WP), is composed mainly of lactose (more than 70% on a dry matter basis), and it is of interest for application in different areas, mainly in the food and pharmaceutical industries; however, a considerable percentage of WP is still treated as dairy wastewater.

In recent years, one of the most promising uses of WP is the synthesis of prebiotic oligosaccharides such as galactooligosaccharides (GOS) via transglycosylation of lactose catalysed by β -galactosidases (EC.3.2.1.23) of different microbial origin.⁵⁻⁸ Recent research has been focused on enzymatic transglycosylation using lactulose or mixtures of lactose with other carbohydrates, giving rise to new bioactive oligosaccharides with different structures.⁹ In these reactions, β -galactosidases use lactose as a galactosyl-donor to transfer the galactosyl-moiety to another lactose molecule or other suitable acceptor carbohydrate present in the mixture giving rise to new β -linked galactosyl oligosaccharides.¹⁰ However, there is no available data about oligosaccharide formation in transglycosylation of mixtures of lactose and lactulose standard.

Other prebiotic oligosaccharides obtained from WP are those derived from lactulose (OsLu), which have been synthesised by chemoenzymatic methodologies by combination of chemical isomerisation of lactose, using egg shell as catalyst, and subsequent transglycosylation of the resulting mixture using β -galactosidase from *Bacillus circulans*.¹¹ Moreover, OsLu have been produced by isomerisation, using sodium aluminates as catalyst, of transglycosylated mixtures previously obtained by hydrolysis and transglycosylation of lactose standard or from WP, using β -galactosidases from dairy *Kluyveromyces lactis* and *K. marxianus*.^{12,13}

A wide number of catalysts, such as sodium and calcium hydroxide; complexation reagents (aluminates and borates); zeolites, sepiolites, egg and oyster shell^{11,14-18} have been used to isomerize lactose. The use of aluminates or borates enables yields up to 80% of lactulose to be reached.^{19,20} However, the main drawback of the use of these two catalysts is that they must be removed before being used for human consumption. For many years, the World Health Organization (WHO) has recommended levels of boron lower than 0.5 mg L⁻¹ in drinking water.²¹

Of the many separation techniques available for removal of borate, ion-exchange chromatography is one of the most widely used.²¹ Amberlite IRA-743 resin is highly selective for borate anions and boric acid elimination. Boric acid reacts with 1-deoxy-1-methylamino-D-glucitol residues present in resin to yield a relatively stable borate-complex. The combined use of this resin together with IR-120(H⁺) has been used to remove boric acid (98%) from carbohydrate mixtures.²²

The objective of this paper was to optimize an efficient methodology for improving a chemical-enzymatic synthesis of prebiotic mixtures derived from lactose (GOS and OsLu) safe for human consumption. The chemical-enzymatic process includes chemical isomerisation of lactose from WP, using boron as a catalyst, and removal of boron by

ion chromatography using selective ion-exchange resins. The purified isomerised WP (IWP) was further used to synthesise OsLu and GOS using β -galactosidase from *B. circulans*.

MATERIALS AND METHODS

Standards and reagents

Reagents employed for chromatographic analysis, including standards (D-glucose, D-galactose, D-fructose, D-lactose, lactulose, raffinose, stachyose, boric acid and β -phenylglucoside), were obtained from Sigma (St Louis, MO, USA). All other chemicals were of analytical grade. Amberlite IR-120(H⁺) and Amberlite IRA-743 (free-base form) resins were purchased through Sigma. Ultrapure water (18.2 M Ω 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). β -galactosidase activity was 245 U mL⁻¹, where 1 U is the amount of enzyme required to hydrolyse 1 μ mol of ortho-nitrophenyl- β -galactoside (ONPG) per minute at a working temperature of 50 °C and pH 6.5 in 0.05 mM phosphate buffer. Protein content determined using the Bradford method²³ was 92.1 mg mL⁻¹. Specific β -galactosidase activity was 2660 U g⁻¹ of protein.

Physicochemical Characterisation of Cheese Whey Permeate (WP)

Industrial bovine 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 of lactose from Whey Permeate (WP)

Isomerisation of WP was carried out following the method of Zokaee *et al.*,²⁰ Solutions of WP containing 200 g L⁻¹ of lactose were prepared in water and boric acid (molar ratio boron/lactose 1/1). Reaction was performed at 70 °C and pH was adjusted at 11 and 12 by adding 2 M NaOH. After, samples (30 mL) were taken at 0, 10, 20, 30, 60, 90, 120, 150 and 180 min. The reaction was stopped by cooling in an ice-water bath and then centrifuged at 27000×g for 15 min at 4 °C. The supernatant was collected and stored at -18 °C until purification and further analysis. The isomerisation reaction was performed in duplicate and each sample was analysed twice. On the other hand, similar reactions were carried out using 2 M Ca(OH)₂ at pH 12. Similarly, aliquots of 30 mL were taken at 0, 15, 30 and 45 min.

Boron Removal

Boron was removed from the isomerised cheese whey permeate (IWP) using the combination of a strong acid (IR-120)(H⁺) and a weak base (IRA-743) anion-exchange resins.²² Weak base boron-selective resin (IRA-743) was regenerated by the following method: 14 mL of 1.2 M HCl solution were slowly percolated through a glass column containing a bed volume of 7 mL of IRA-743. Then, the resin was washed with 2 bed volumes of water, followed by 3.2 bed volumes of 1.1 M NaOH and then washed with water until neutral pH. Strong acid anion-exchange resin IR-120(H⁺) was regenerated using 2 bed volumes of 1.2 M HCl solution percolated through a glass column

containing a bed volume of 7 mL of IR-120(H^+) and washed with water until neutral pH. Columns with bed volumes of 63 mL of each resin were regenerated similarly.

Boron removal was confirmed by VIS-spectrophotometry (Azomethine H method)²⁴ and ICP-MS, indicated below.

Model systems

First, boron removal was studied in two different model systems containing 60 and 120 g L⁻¹ of total solids which were composed of boric acid (18% w/v), galactose (11% w/v), lactose (11% w/v) and lactulose (60% w/v) simulating IWP composition (Table 1). The pH of each solution was adjusted to pH 12 with 2M NaOH. An aliquot of 2 mL of each test solution was treated with IR-120 (H^+) column (resin volume 7 mL) to remove sodium ions. Samples were eluted through the column with distilled water and collected in a final volume of 4 mL. Then, these fractions were passed through IRA-743 column (resin volume 7 mL), eluted with distilled water and collected in a final volume of 11 mL. Flow rates were adjusted to 2 mL min⁻¹.

Isomerised cheese whey permeate (IWP)

IWP was purified following the procedure described above using the same resins. Aliquots of 2 ml of IWP solutions (237 g L⁻¹ carbohydrates plus boric acid) and IWP solutions diluted twice (119 g L⁻¹ carbohydrates plus boric acid) were used. At last, two samples (18 mL) of IWP (isomerised during 20 and 30 min, 237 g L⁻¹ carbohydrates plus boric acid), were passed through columns with IR-120 (H^+) and IRA-743 resins (63 mL each). These samples were eluted with distilled water and lyophilised to subsequently be used in transglycosylation reactions.

Synthesis of oligosaccharides derived from lactose (GOS) and lactulose (OsLu)

Firstly, with the objective to optimize transglycosylation reactions, different assays using model systems prepared with lactose and lactulose in 0.05 M sodium phosphate and pH 6.5 were performed. Different concentrations of lactose and lactulose were tested, 250/250, 250/500 and 250/125, respectively, expressed as g L⁻¹, leading to three different molar ratios of lactose/lactulose (1/1, 1/2, and 2/1, respectively). Hydrolysis and transglycosylation of these model systems were performed by incubation at 50°C with β -galactosidase from *B. circulans* (0.7 and 1.4 U mL⁻¹) in 0.05 M sodium phosphate buffer (pH 6.5).

Transglycosylation reactions to obtain GOS and OsLu were performed using WP (equivalent to 250 g L⁻¹ lactose), purified IWP (500 g L⁻¹ carbohydrates and lactose/lactulose ratio 1/2.4) and mixtures of WP/purified IWP (500 g L⁻¹ carbohydrates, lactose/lactulose ratio 1:1) as substrates which were reconstituted in water and adjusted the pH at 6.5. Enzymatic synthesis was performed in a final volume of 1.5 mL in microtubes incubated in an orbital shaker at 50 °C and 38×g. Samples were withdrawn at 0, 1, 3, 5, 8 and 24 h and immediately immersed in boiling water for 5 min to inactivate the enzyme. Then, samples were stored at -18 °C for subsequent analysis. Enzymatic reactions using 0.7 U mL⁻¹ of β -galactosidase from *B. circulans* were carried out in duplicate and each sample was analysed twice.

Analytical Techniques.

Gas chromatography with flame ionisation detector (GC-FID)

The carbohydrate composition of isomerisation and transglycosylation mixtures was determined by GC-FID. In isomerised samples, where boron interfered in the formation of oximes, carbohydrates were analysed as trimethyl silylated ethers (TMS). First,

supernatant aliquot equivalent to 5-6 mg of sugars was added to 0.2 mL of internal standard (I.S.) solution (0.5 mg mL⁻¹ phenyl-β-glucoside). Afterwards, the mixture was dried at 38-40 °C in a rotary evaporator (Büchi Labortechnik AG, Flawil, Switzerland). Dried samples were treated with 100 µL of N, N-dimethylformamide and incubated at 70 °C for 30 min. Then, 150 µL of N-trimethylsilylimidazole were added to obtain TMS; 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. Aliquots of 1 µL of the organic phase containing silyl derivatives were injected into the column.

GC-FID analyses of carbohydrates from boron free transglycosylation mixtures were performed using trimethyl silylated oximes (TMSO) following the method of Brobst and Lott²⁵. Sugar oximes were formed by adding 250 µL of hydroxylamine chloride (2.5%) in pyridine to dried samples and heating the mixture at 70 °C for 30 min and then silylated with hexamethyldisilazane (250 µL) and trifluoroacetic acid (25 µL) and kept at 50 °C for 30 min. Reaction mixtures were centrifuged at 6,720×g for 2 min at room temperature. Supernatants were injected or stored at 4 °C prior to analysis.

Chromatographic analysis. TMS and TMSO of carbohydrates were determined by gas chromatography (GC) 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 × 0.25 mm i.d., 0.25 µm film thickness) (Supelco, Bellefonte, PA, USA). The oven temperature was initially 200 °C increased at a rate of 4 °C min⁻¹ to 230 °C, then increased at a rate of 1 °C min⁻¹ to 250 °C, then increased at a rate of 2 °C min⁻¹ to 295 °C and held at this temperature 50 min. The injector and detector temperatures were set at 280 and 295 °C, respectively. Injections were carried out in split mode (1:20) 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 calculate the response factors relative to the I.S., solutions containing glucose, galactose, lactose, and lactulose were prepared over the expected concentration range in samples. Raffinose and stachyose were also used as standards of tri- and tetrasaccharides. The identities of carbohydrates were confirmed by comparison with relative retention times (RRT) of standard samples. Response factors were calculated after the duplicate analysis of standard solutions over the expected concentration range in samples. The amount of different carbohydrates and the yield of GOS and OsLu were expressed as % weight of the total carbohydrates content in the reaction mixtures. All analyses were carried out in duplicate and data were expressed as mean $\pm$ standard deviation (SD).

Size exclusion HPLC with refractive index detection (HPLC-SEC-RID)

Mono- and disaccharides as well as boron from purified model systems and IWP, were analysed by HPLC-SEC-RID in a thermostatised (80 °C) Shodex SUGAR KS-801 column (300 mm x 8 mm, 5 μ m particle size) in combination with a Shodex SUGAR KS-G guard column (50 mm x 6 mm, 7 μ m particle size) (Akzo Nobel, Brewster, NY, USA) using water as mobile phase in isocratic mode at a flow rate of 0.5 mL min⁻¹. Before analysis, model systems, IWP and purified IWP were diluted with water (~20 mg mL⁻¹ carbohydrates) and filtered using a 0.45 μ m syringe filter (Symta). Analyses were performed in an Agilent Technologies 1220 Infinity LC System – 1260 RID (Böblingen, Germany). The injection volume was 50 μ L (~1 mg of total carbohydrates). Data acquisition and processing were performed employing Agilent ChemStation software.

Boron and carbohydrates, in the reaction mixtures, were initially identified by comparing their retention times (RT) with those of standard compounds. Quantitative

analysis was performed by the external standard method, using calibration curves in the range 0.02–2 mg mL⁻¹ for lactose and lactulose, galactose and boron. All analyses were performed in duplicate, obtaining relative standard deviation (RSD) values below 10% in all cases. The amount of remaining carbohydrates and the yield of GOS and OsLu were expressed as % by weight of the initial carbohydrate content in the reaction mixtures.

ICP-MS analysis

The total ion composition of the WP was determined using an ICP-MS ELAN 6000 Perkin-Elmer Sciex instrument at the Servicio Interdepartamental de Investigación (SIdI-UAM) of Madrid. Either a semiquantitative or quantitative analysis of the elements of interest using the external calibration method and internal standards (I.S.) to correct instrumental drift were carried out. Values of major cations found in WP were K 30.7 mg g⁻¹, Na 7.4 mg g⁻¹, Ca 3.2 mg g⁻¹ and Mg 1.4 mg g⁻¹. Boron content of isomerised cheese whey permeates (IWP) after purification was also determined using this methodology.

MALDI-TOF-MS analysis

Before matrix-assisted laser desorption/ionisation-time-of flight-mass spectrometry (MALDI-TOF-MS) analysis, samples were submitted to a purification step following the method described by Morales *et al.*²⁶ Briefly, a total of 100 µL of reaction mixtures, containing 5 mg of carbohydrates, were dissolved in 10 mL of a 1% ethanol solution and stirred for 30 min with 300 mg of activated charcoal Darco G60, 100 mesh (Sigma, St. Louis, MO), to remove mono- and disaccharides. This mixture was vacuum-filtered through Whatman no. 1 filter paper, and activated charcoal was washed with 50 mL of

water. The oligosaccharides adsorbed onto the activated charcoal were extracted by stirring for 30 min with 10 mL of ethanol/water solution (1/1, v/v) and then vacuum-filtered. The ethanol/water solution was evaporated under vacuum at 40 °C. The sample was dissolved in 200 µL of deionised water and filtered through 0.22 µm filters (Millipore Corp., Bedford, MA) for further characterisation by mass spectrometry.

The enriched fractions containing the oligosaccharides obtained by transglycosylation were characterised by MALDI-TOF-MS on a Voyager DE-PRO mass spectrometer (Applied Biosystems, Foster City, CA) equipped with a pulsed nitrogen laser ($\lambda = 337$ nm, 3 ns pulse width, and 3 Hz frequency) and a delayed extraction ion source. Ions generated by laser desorption were introduced into a time-of-flight analyser (1.3 m flight path) with an acceleration voltage of 20 kV, 74% grid voltage, 0.001% ion guide wire voltage, and delay time of 300 ns in the reflector positive ion mode. Mass spectra were obtained over the m/z range 100-1500.

Statistical analysis

ANOVA tests and Tukey's test for $p < 0.05$ (Statistix 8.1 software package) were applied to carbohydrate contents found in IWP mixtures in order to determine differences during the isomerisation reaction.

RESULTS AND DISCUSSION

Isomerisation of lactose from whey cheese permeate

Isomerisation of WP was studied at pH values of 11 and 12. Yields near 40% were achieved at pH 11 after 240 min of isomerisation, which are lower than the 70% obtained by Zokaee *et al.*²⁰ using aqueous lactose solutions at the same pH value; in the present study, WP containing a high number of cations was utilised. This could be

attributed to an inhibitory effect of the high salts content in WP compared to buffer solutions. The influence of salts on lactose isomerisation was previously observed by Montilla *et al.*¹⁵ and Corzo-Martínez *et al.*¹¹

When isomerisation was carried out at pH 12, (Figure 1) the optimal production of lactulose was 155.5 g L⁻¹ (corresponding to 71% of total carbohydrates), and galactose and lactose contents were 6.4 and 57.1 g L⁻¹, respectively, after 20 min of reaction. No statistically significant differences ($p < 0.05$) were observed in galactose and lactulose content between 20 and 30 min of reaction. After 30 min of reaction lactulose level rapidly decreased with the time of reaction, possibly due to its degradation to α and β isosaccharinic acids and galactose. Since no glucose or fructose were detected, it may be assumed that the reducing moiety of the disaccharide was first degraded into acidic compounds, followed by its hydrolysis giving rise to galactose and isosaccharinic acids, which are further degraded into lower molecular weight organic acids.¹⁵ The results obtained herein were similar to those obtained by Zokaee *et al.*²⁰

In addition, another catalyst, calcium borate, was also assayed to isomerise lactose to lactulose. Precipitation of calcium borate, less soluble than sodium borate, would enhance the removal of boron by centrifugation or filtration.²⁷ However, low yields of lactulose were obtained. The carbohydrate composition of the reaction mixture, at the optimal conditions, was 13.9%, 64.7% and 21.4% of galactose, lactulose and lactose, respectively.

Ion-exchange removal of boron from isomerised cheese whey permeate (IWP)

To obtain a product safe for human consumption, efficient boron removal is necessary. For this purpose, IRA-743 resin has been used in combination with resin IR-120 (H⁺).²² Model systems prepared at 60 and 120 g L⁻¹ of total solids (boric acid 14%, galactose

11%, lactose 8% and lactulose 67% w/v,) and then adjusted to pH 12 with NaOH, were treated with 7 mL of each resin. According to the results obtained (Table 1), the concentration of solids was scarcely affected by the purification process. It can be observed that resin IR-120(H⁺) produced no efficient boron retention, as expected, since this resin decreases the number of cations in the medium, exchanging H⁺ for Na⁺, neutralising the reaction solution, and favouring subsequent boron elimination. Thus, when the two resins (IR-120, H⁺ and IRA-743) were used, the remaining boron level was very low, below the limit of quantification (LOQ) i.e., 0.02 mg mL⁻¹, and no important losses of lactulose were observed.

Considering these results, aliquots of IWP and IWP diluted twice (237 and 119 g L⁻¹ carbohydrates and boric acid) were purified following the same treatment. The results obtained were similar to those found in model systems.

Finally, the purification process was scaled-up. Undiluted IWP was passed through columns containing 63 mL of each resin and similar results were achieved and remaining boron levels lower than 1 ppm were found by VIS-spectrophotometry (Azomethine H method) and ICP-MS analysis. These results were similar to those reported by Hicks *et al.*²² who found a decrease of >99% of the original level in synthetic solutions of carbohydrates, with a final boron content of 216 ppm. Therefore, boron levels obtained in this study make possible the use of purified IWP as a food ingredient. Devirian and Volpe²⁸ reported that boron content of different fruits such as bananas and apples were between 21 and 43 ppm.

Transglycosylation of purified isomerised cheese whey permeate using β -galactosidase from *Bacillus circulans*

Firstly, reactions of transglycosylation were optimised using model systems of carbohydrates with different molar ratios of lactose/lactulose (such as has been described in the material and methods section). Figure 2 shows the chromatographic profile of carbohydrates found in reaction mixtures containing lactose and lactulose in a molar ratio of 1/1 after 3 h of reaction and using 0.7 U mL^{-1} of β -galactosidase from *B. circulans*. The monosaccharides released, fructose, galactose and glucose (peaks 1, 2 and 3) by hydrolysis of lactose and lactulose can be observed, as can unreacted lactose and lactulose (peaks 5 and 6). These compounds were identified by comparison of their retention times with those of commercial standards. Moreover, the formation of GOS and OsLu (tri- and tetrasaccharides) with $\beta(1\rightarrow4)$ was also detected since β -galactosidase from *B. circulans* leads the formation of oligosaccharides with this linkage. Thus, 4'-galactosyl lactulose (peak 7) and 4'-galactosyl lactose (peak 8) were identified by comparison with the standards previously synthesised in our laboratory¹¹ and 4'-digalactosyl lactulose and 4'-digalactosyl lactose (peaks 9 and 10) could only be tentatively identified. Unknown di-, tri- and tetrasaccharides were also detected (labelled with an asterisk in Figure 2).

The quantification of oligosaccharides present in hydrolysis mixtures of system models containing lactose/lactulose ratios of 1/1, 1/2 and 2/1 is depicted in Figure 3. During transglycosylation to produce GOS and OsLu, increasing amounts of glucose, galactose and fructose (Figure 3 a-c) were released as a consequence of lactose and lactulose hydrolysis. Maximum release of glucose and galactose (21.6% and 13.9% respectively) was reached with a lactose/lactulose ratio of 2/1 whereas maximum

release of fructose was produced when the lactose /lactulose ratio was 1/2 and using 1.4 U mL⁻¹ of β -galactosidase (5.3%).

The different behaviour of disaccharides is evidenced by the amount of monosaccharides released; thus, lactose is intensively hydrolysed (Figure 3 e), in all assayed conditions. After 24 h, it remained at less than 5%, whereas only about 10% of lactulose was hydrolysed. On the other hand, the minor amounts of galactose found, with respect to glucose, highlights that synthesised oligosaccharides may contain one or several galactose units.

With respect to the transglycosylation reaction, lactose and lactulose were very efficient acceptors, leading to the formation of GOS and OsLu. It could even be considered that lactulose is a more efficient acceptor than lactose, considering that the formation of 4'galactosil lactulose (14.3%) was greater than 4'galactosil lactose (6.9%) when both disaccharides were in the same proportion. Among the different lactose/lactulose ratios studied i.e. 1/1, 1/2 and 2/1, the latter produced the highest amounts of prebiotics oligosaccharides (i.e. 37.3%, GOS and OsLu) after 1 hour of reaction, using 0.7 U mL⁻¹ of β -galactosidase (Figure 3 h), mainly due to a large number of new disaccharides formed (20.1%). In these conditions, the total prebiotic content, including lactulose, was 64.5% (Figure 3 i). Reaction mixtures containing a lactose/lactulose ratio of 1/2 produced 30.7% of prebiotics after 24 h of reaction and 0.7 U mL⁻¹ of β -galactosidase, and, considering lactulose, the level of prebiotics increased up to 82.9% after 24 h of reaction and using 0.7 and 1.4 U mL⁻¹ of enzyme. Finally, a lactose/lactulose ratio of 1/1 and using 0.7 U mL⁻¹ of β -galactosidase produced high quantities of prebiotics (76.4%). These data show the influence of type and concentration of carbohydrate present in initial reaction mixtures.

Afterwards, transglycosylation reactions using 0.7 U mL⁻¹ of β -galactosidase and non-isomerised WP and purified IWP at two lactose/lactulose ratios, 1/2.4 (normal ratio of two carbohydrates after isomerisation) and 1/1, were carried out. Chromatographic profiles of oligosaccharides were similar to those obtained using standard solutions. MALDI-TOF-MS analysis of oligosaccharide-enriched fractions from non-isomerised WP and purified IWP transglycosylation mixtures enabled detection of oligosaccharides up to degree of polymerisation of 8 (m/z 1337) and 9 (m/z 1499), respectively (Figure 4).

Evolution of carbohydrates from reaction mixtures originated by transglycosylation of WP, and IWP using β -galactosidase is shown in Figure 5. In general, the evolution of disaccharides (d-e) hydrolysis and the release of monosaccharides (a-c) were similar to what is observed in reactions carried out with lactose and lactulose standards. The level of 4'-galactosyl lactose found when WP was used as a substrate for the transglycosylation was 20.1% of total carbohydrates, which is higher than that found in the case of standard solutions of lactose and lactulose or purified IWP (Figure 5 g). With respect to total oligosaccharides formed (GOS and OsLu), the WP sample showed the highest level (52.4% of total carbohydrates), after 5 h of reaction. Whereas IWP with a lactose/lactulose ratio of 1/1 showed a higher content (36.4% total carbohydrates) than IWP (17.9%) after 1 h of reaction (Figure 5 h). However, considering prebiotic sugars (lactulose, GOS and OsLu) transglycosylation mixtures of IWP presented the highest amount (83.5% of total carbohydrates), after 3 h of reaction and a lactose/lactulose ratio of 1/2.4, and IWP with a lactose/lactulose ratio of 1/1 shown of 75.0% after 5 h (Figure 5 i). These results agree with those reached in mixtures with a lactose/lactulose ratio of 1/2 and 1/1. Finally, the highest yield of prebiotic carbohydrates was reached in IWP (1:1.24) with 690 g kg⁻¹ of permeate.

CONCLUSIONS

Most commercial prebiotic carbohydrates are obtained from enzymatic reactions. However, this study shows that combined chemical-enzymatic reactions together with the purification of IWP results in an efficient synthesis of prebiotic oligosaccharides. These combined reactions could be applied more broadly to the search for novel types of products using other organic by-products as substrates of chemical-enzymatic reactions in the search of new oligosaccharide structures with improved or complementary properties.

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Figure 1. Evolution of lactose, lactulose and galactose (g L^{-1}) during the isomerization
reaction using sodium borate as catalyst (lactose/boron molar ratio 1:1, pH 12, 70°C).^{a-c}
Statistically significant differences considering individual carbohydrates ($p < 0.05$).

Figure 2. Chromatographic profile (GC-FID) of transgalactosylated lactose/lactulose model system using β -galactosidase from *Bacillus circulans* (pH 6.5, 50 °C, 3h). Peaks: (1) fructose, (2) galactose, (3) glucose, (4) internal standard, (5) lactulose, (6) lactose, (7) 4'-galactosyl-lactulose, (8) 4'-galactosyl-lactose, (9) tentatively 4'-digalactosyl-lactulose, (10) tentatively 4'-digalactosyl-lactose and (*) unknown GOS.

521 **Figure 3.** Evolution of fructose (a), galactose (b), glucose (c), lactulose (d), lactose (e), 4'-galactosyl-lactulose (f), 4'-galactosyl-lactulose (g), GOS
522 and OsLu (h) and total prebiotic carbohydrates (i) contents during the enzymatic hydrolysis of WP and IWP with different molar ratios of
523 lactose/lactulose (La/Lu) using 0.7 U mL^{-1} of β -galactosidase from *Bacillus circulans*. GOS and OsLu include unknown di-GOS, 4'-galactosyl-
524 lactulose, 4'-galactosyl-lactulose and unknown tri- and tetra-GOS. Total prebiotic carbohydrates include GOS, OsLu and lactulose.

525

526 **Figure 4.** Mass spectra obtained by MALDI-TOF-MS of transgalactosylated isomerized whey permeate (IWP) (lactose/lactulose 1:1, pH 6.5, 50
527 °C, 3 h). DP: degree of polymerization.

528

529

530 **Figure 5.** Evolution of fructose (a), galactose (b), glucose (c), lactulose (d), lactose (e), 4'-galactosyl-lactulose (f), 4'-galactosyl-lactulose (g), GOS
531 and OsLu (h) and total prebiotic carbohydrates (i) contents during the enzymatic hydrolysis of lactose/lactulose (La/Lu) model systems with
532 different molar ratios using 0.7 and 1.4 U mL⁻¹ of *Bacillus circulans*. GOS and OsLu include unknown di-GOS, 4'-
533 galactosyl-lactulose, 4'-galactosyl-lactulose and unknown tri- and tetra-GOS. Total prebiotic carbohydrates include GOS, OsLu and lactulose.

534

Table 1. Lactose, lactulose, galactose and boron contents found in model systems of 60 and 120 g L⁻¹ of total solids and IWP of 120 and 240 g L⁻¹ of total solids after successive treatment with IR-120 (H⁺) and IRA-743 resins.

		Concentration g L ⁻¹ total solids	Lactose %	Lactulose %	Galactose %	Boron %
Model systems	Before treatment		8.23 ± 0.06	67.22 ± 0.07	10.97 ± 0.19	13.59 ± 0.06
	Treatment with IR-120	60	8.65 ± 0.04	71.64 ± 0.74	8.61 ± 0.35	11.11 ± 0.43
	Treatment with IR-120 + IRA-743		11.11 ± 0.65	78.57 ± 0.01	9.84 ± 0.74	-*
	Before treatment		6.34 ± 0.24	68.66 ± 0.56	10.77 ± 0.11	14.24 ± 0.70
	Treatment with IR-120	120	7.64 ± 0.14	72.88 ± 0.47	9.00 ± 0.21	10.49 ± 0.39
	Treatment with IR-120 + IRA-743		9.81 ± 0.48	79.48 ± 0.25	10.37 ± 0.32	-*
IWP	Before treatment		20.71 ± 2.38	65.50 ± 0.96	2.62 ± 0.03	11.17 ± 1.38
	Treatment with IR-120	120	24.89 ± 0.48	70.48 ± 0.71	1.86 ± 0.03	2.78 ± 1.22
	Treatment with IR-120 + IRA-743		25.58 ± 0.42	72.91 ± 0.16	0.85 ± 0.02	-*
	Before treatment		17.35 ± 2.38	66.86 ± 0.96	2.66 ± 0.03	13.13 ± 1.38
	Treatment with IR-120	240	23.93 ± 0.39	68.39 ± 0.08	2.15 ± 0.20	5.53 ± 0.27
	Treatment with IR-120 + IRA-743		26.03 ± 0.05	72.22 ± 0.37	1.20 ± 0.09	-*
* Boron content under LOQ (0.02 g L ⁻¹) HPLC-SEC-RID and confirmed by VIS-spectrophotometry (LOQ 0.001 g L ⁻¹).						