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Efficient Synthesis and Characterization of Lactulosucrose by *Leuconostoc mesenteroides* B-512F Dextransucrase

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Supporting Information

ABSTRACT: This work describes an efficient enzymatic synthesis and NMR structural characterization of the trisaccharide β-D-galactopyranosyl-(1→4)-β-D-fructofuranosyl-(2→1)-α-D-glucopyranoside, also termed as lactulosucrose. This oligosaccharide was formed by the *Leuconostoc mesenteroides* B-512F dextransucrase-catalyzed transfer of the glucosyl residue from sucrose to the 2-hydroxyl group of the reducing unit of lactulose. The enzymatic reaction was carried out under optimal conditions, i.e., at 30 °C in 20 mM sodium acetate buffer with 0.34 mM $CaCl_2$ at pH 5.2, and the effect of factors such as reaction time (0−48 h), enzyme charge (0.8, 1.6, and 2.4 U mL^{-1}), and sucrose:lactulose concentration ratios (20:40, 30:30, and 40:20, expressed in g/100 mL) on the formation of transfer products were studied. The highest formation in lactulosucrose was attained at 8 and 24−32 h by using 20%:40% and 30%:30% sucrose:lactulose mixtures, respectively, with 1.6 or 2.4 U mL^{-1} dextransucrase, leading to lactulosucrose yields of 27−35% in weight respect to the initial amount of lactulose. Furthermore, minor tetra- and pentasaccharide, both probably derived from lactulose, were also detected and quantified. Likewise, the capacity of lactulosucrose to act as D-glucosyl donor once the sucrose was consumed, could explain its decrease from 16 to 24 h when the highest charge of dextransucrase was used. Considering the chemical structure of the synthesized oligosaccharides, lactulosucrose and its derivatives could potentially be excellent candidates for an emerging prebiotic ingredient.

KEYWORDS: *lactulosucrose, lactosucrose, Leuconostoc mesenteroides B-512F dextransucrase, transglycosylation*

INTRODUCTION

Lactose (β-D-Gal-(1→4)-D-Glu) is the precursor for a number of bioactive compounds derived by chemical, physical, or enzymatic conversion that have an established and expanding place in the pharmaceutical and food industries.[1] Lactulose, lactosucrose, lactobionic acid, lactitol, and galacto-oligosaccharides are showing interesting new application opportunities.[2] Likewise, trends supporting annual growth rate increases of some of these lactose-derivative ingredients include a greater health awareness/illness prevention and willingness to demand ingredients exerting beneficial effects on gastrointestinal tract health.

The development of simple and convenient methods for the enzymatic synthesis of novel oligosaccharides with biological activities is attracting high interest over the current years.[3] In this context, lactosucrose (β-D-Gal-(1→4)-α-D-Glu-(1→2)-β-D-Fru) is a trisaccharide produced from the transfer of the fructosyl moiety of sucrose to lactose as an acceptor molecule catalyzed by β-fructofuranosidases[4] or levansucrases.[5] Lactosucrose is recognized as a prebiotic on the basis that is not readily digested by intestinal enzymes in the stomach and small intestine and is selectively fermented by beneficial bacteria in the human colon according to different studies including human feeding trials.[6−9] Moreover, an enhancement of intestinal calcium absorption has been observed after lactosucrose intake by young women.[10] These data warrant its use as a prebiotic ingredient in a range of food products which have attained FOSHU (Foods for Specified Health Use) status in Japan, as well as its recent introduction into the USA market.[1]

On the other hand, ingredients derived from lactulose (β-D-Gal-(1→4)-D-Fru) could have enhanced bioactive properties compared to those derived from lactose because lactulose has been shown to exert a series of biological activities, such as prebiotic action,[11−15] improvement of the intestinal transit time,[16−20] and other beneficial physiological actions.[21] In good agreement with this, novel bioactive galacto-oligosaccharides derived from lactulose instead of lactose by using β-galactosidases of microbial origin[22−24] have shown the ability to reach the large intestine of rats in physiologically relevant doses due to their lower digestibility and to exert a stronger in vivo bifidogenic effect than the conventional GOS derived from lactose.[25]

Nonetheless, despite the number of studies carried out on lactosucrose, scarce data are available for the efficient production of lactulosucrose (β-D-Gal-(1→4)-β-D-Fru-(2→1)-α-D-Glu). Formation of lactulosucrose was first described by Suzuki and Hehre[26] using cultures of *Leuconostoc mesenteroides* strain NRRL B-1299 grown in an autoclaved solution of 2% of sucrose and 10% of lactose. These authors indicated that lactulosucrose was produced from the transfer of the glucosyl moiety of sucrose to lactulose which was indirectly formed

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(around 1%) from lactose during autoclaving. Later on, these same authors demonstrated that the enzyme responsible for the lactulosucrose synthesis was the dextransucrase by replacing growing cultures by dried cells and 10% of lactose by 3% of lactulose.[27] In this sense, taking into account that lactulosucrose yields described in these works were rather moderate, and considering the dramatic effect of optimizing (i) the concentration of the substrates, (ii) the concentration of the enzyme, and (iii) the molar ratio of lactulose to sucrose, as well as the use of highly purified enzymes on the yield of acceptor-reaction products,[28] the present work describes an efficient enzymatic synthesis and the nuclear magnetic resonance spectroscopy (NMR) structural characterization of lactulosucrose using a dextransucrase from *Leuconostoc mesenteroides* B-512F.

MATERIALS AND METHODS

Chemical and Reagents. Dextransucrase (E.C. 2.4.1.5) from *Leuconostoc mesenteroides* B-512F was purchased from CRITT Bio-Industries (Toulouse, France). Specific activity was 0.4 U mg^{-1}, where 1 unit is the amount of enzyme required to transfer 1 μmol of glucose per minute, with 100 g of sucrose per liter as the substrate in 20 mM sodium acetate buffer (pH 5.2) with 10 mg L^{-1} of $CaCl_2$ at a working temperature of 30 °C. Fructose, sucrose, lactulose, and leucrose were purchased from Sigma-Aldrich (Steinheim, Germany), and lactosucrose was purchased from Wako Pure Chemical Industries (Osaka, Japan). Acetonitrile (HPLC grade) was obtained from Lab-scan (Gliwice, Poland). Ultrapure water quality (18.2 MΩ cm) with 1−5 ppb total organic carbon (TOC) and <0.001 EU mL^{-1} pyrogen levels was produced in-house using a laboratory water purification Milli-Q Synthesis A10 system from Millipore (Billerica, MA). All other chemicals were of analytical grade and commercially available.

Enzymatic Synthesis of Oligosaccharides. Oligosaccharide synthesis catalyzed by dextransucrase from *Leuconostoc mesenteroides* B-512F was carried out in the presence of sucrose (donor) and lactulose as acceptor, in 20 mM sodium acetate buffer with 0.34 mM $CaCl_2$ (pH 5.2) at 30 °C. In order to study the effect of synthesis conditions such as enzyme charge and donor:acceptor ratio on the formation of transfer products, the reactions were done at three different concentrations of enzyme (0.8, 1.6, and 2.4 U mL^{-1}) and at three different concentration ratios of sucrose:lactulose (20:40, 30:30, and 40:20, expressed in g/100 mL, leading in all cases to a concentration of total sugar of 60 g/100 mL). The reactions were allowed to proceed up to 48 h, and within this time samples were repeatedly taking from the reaction mixture at suitable time intervals (1, 3, 8, 16, 24, 32, and 48 h). The enzyme was inactivated by heating at 100 °C for 5 min, and inactivated samples were then diluted with acetonitrile:water (50:50, v:v), filtered using a 0.45 μm syringe filter (Symta, Madrid, Spain), and analyzed by liquid chromatography with refractive index detector (LC-RID). The dilution factor depended on the type of chromatographic column (i.e., analytical or semi-preparative) used.

Chromatographic Determination of Carbohydrates by Liquid Chromatography with Refractive Index Detector (LC-RID). The synthesized oligosaccharides were analyzed by liquid chromatography with refractive index detector (LC-RID) on an Agilent Technologies 1220 Infinity LC System 1260 RID (Boeblingen, Germany). The separation of carbohydrates was carried out with a Kromasil (100-NH_2) column (250 × 4.6 mm, 5 μm particle size) (Akzo Nobel, Brewster, NY) using acetonitrile:water (75:25, v:v) as the mobile phase and eluted in isocratic mode at a flow rate of 1.0 mL min^{-1} for 70 min. Injection volume was 50 μL (1 mg of total carbohydrates). Data acquisition and processing were performed using the Agilent ChemStation software (Agilent Technologies, Boeblingen, Germany).

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 fructose (quantification of monosaccharides), sucrose, lactulose, and leucrose (disaccharides), and lactosucrose (quantification of acceptor products of degree of polymerization ≥3). All analyses were carried out in triplicate. Determination coefficients obtained from these calibration curves, which were linear over the range studied, were high (R^2 > 0.999). Reproducibility of the method was estimated on the basis of the intraday and interday precision, calculated as the relative standard deviation (RSD) of concentrations of oligosaccharide standards obtained in $n \geq 5$ independent measurements, obtaining RSD values below 10% in all cases.

Purification and Structural Characterization of Lactulosucrose by Nuclear Magnetic Resonance (NMR). Considering the absence of commercially available standard for lactulosucrose, this trisaccharide, the main synthesized oligosaccharide, was isolated and purified by LC-RID from 20%:40% sucrose:lactulose mixture with 2.4 U mL^{-1} dextransucrase, after 16 h of enzymatic reaction and using a semipreparative column Kromasil (100-NH_2) column (250 × 10 mm, 5 μm particle size) (Akzo Nobel, Brewster, NY). Five hundred microliters of reaction mixtures (30 mg of total carbohydrates) were repeatedly injected and eluted with acetonitrile:water (75:25, v:v) as the mobile phase at a flow rate of 4 mL min^{-1}, and fractions corresponding to the main synthesized oligosaccharide were manually collected, pooled, evaporated in a rotatory evaporator R-200 (Büchi, Switzerland) below 25 °C, and freeze-dried for subsequent characterization.

Structure elucidation of the purified oligosaccharide was accomplished by nuclear magnetic resonance (NMR) spectroscopy. NMR spectra were recorded at 298 and 313 K, using D_2O as the solvent, on a Varian SYSTEM 500 NMR spectrometer (^1H 500 MHz, ^{13}C 125 MHz) equipped with a 5 mm HCN cold probe. Chemical shifts of ^1H (δ_H) and ^{13}C (δ_C) in ppm were determined relative to an external standard of sodium [2,2,3,3-$^2H_4$]-3-(trimethylsilyl)propanoate in D_2O (δ_H 0.00 ppm) and 1, 4-dioxane (δ_C 67.40 ppm) in D_2O, respectively. One-dimensional NMR experiments (^1H and ^{13}C) were performed using standard Varian pulse sequences. Two-dimensional [^1H,^1H] NMR experiments (gCOSY and TOCSY) were carried out with the following parameters: a delay time of 1 s, a spectral width of 1675.6 Hz in both dimensions, 4096 complex points in t2 and 4 transients for each of 128 time increments, and linear prediction to 256. The data were zero-filled to 4096 × 4096 real points. The 2D [^1H,^1H] ROESY NMR experiment used the same conditions with 64 transients for increment and a mixing time of 80 ms. Two-dimensional [^1H−^{13}C] NMR experiments (gHSQC and gHMBC) used the same ^1H spectral window, ^{13}C spectral windows of 30165 Hz, 1 s of relaxation delay, 1024 data points, and 128 time increments, with a linear prediction to 256. The data were zero-filled to 4096 × 4096 real points. Typical numbers of transients per increment were 4 and 16, respectively. A full set of spectra are collected in the Supporting Information.

RESULTS

Optimization of the Enzymatic Synthesis of Lactulosucrose. Oligosaccharide synthesis catalyzed by dextransucrase from *L. mesenteroides* B-512F in the presence of a wide variety of carbohydrate acceptors, lactulose being not included among them, has previously been determined under optimized reaction conditions.[29−32] These previous works allowed determining the optimum pH (5.2) and temperature (30 °C) of *L. mesenteroides* B-512F dextransucrase for glucansucrase activity.

Figure 1 shows two representative chromatograms at the beginning of the reaction and after 24 h. Major peaks were initially identified by comparing the retention times (t_R) with those of available standards as follows: fructose (peak 1, t_R 7.4 min), glucose (peak 2, t_R 8.5 min), sucrose (peak 3, t_R 10.9 min), leucrose (peak 4, t_R 12.0 min), lactulose (peak 5, t_R 12.8 min), and acceptor-reaction products (peak 6, t_R 20.8 min; peak 7, t_R 34.6 min, peak 8, t_R 63.8 min). As expected, sucrose and

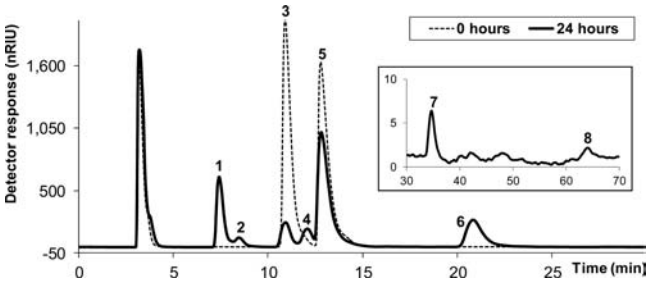


Figure 1. LC-RID profile of transglycosylation reaction based on sucrose:lactulose (30%:30%) catalyzed by dextransucrase from *Leuconostoc mesenteroides* B-512F (2.4 U mL^{-1}) at 30 °C, in 20 mM sodium acetate buffer at pH 5.2, for 0 and 24 h. Labeled peaks are as follows: 1 (fructose); 2 (glucose); 3 (sucrose); 4 (leucrose); 5 (lactulose); 6 (lactulosucrose); 7 and 8 (minor acceptor products of degree of polymerization 4 and 5, respectively). The inset shows a zoom area of the minor acceptor products eluting between 30 and 70 min.

lactulose were present at the initial time, whereas fructose and glucose were appearing as sucrose hydrolysis progressed. Leucrose (α-D-Glu-(1→5)-D-Fru) formation was attributed to the minor capacity of free fructose to act as acceptor in the dextransucrase-catalyzed reactions.[28] Unidentified peak 6, which represented the main acceptor-reaction product, eluted in the trisaccharide area, being likely to correspond to lactulosucrose (β-D-Gal-(1→4)-β-D-Fru-(2→1)-α-D-Glu). This hypothesis was supported by the facts that (i) lactulose decreased during the first 24 h of reaction and (ii) glucose levels were dramatically lower than fructose levels, which was clearly indicative of the efficient transfer of glucose moieties to lactulose. Lastly, minor peaks 7 and 8 eluted in the tetra- and pentasaccharide areas, respectively.

Concerning quantitative data, enzymatic reactions were initially carried out at the three sucrose:lactulose concentrations studied (20:40, 30:30, and 40:20, expressed in g/100 mL) with 0.8 U mL^{-1} dextransucrase charge. Since an increase in the acceptor reaction efficiency is observed with increased concentration of dissolved solids,[33] the total substrate concentration was set as high as 60% (w/v), this value being limited by the solubility of both disaccharides in the reaction buffer. Figure 2 shows the total amount in the main acceptor-reaction product (the trisaccharide labeled as peak 6 in Figure 1 and tentatively assigned to lactulosucrose) throughout the enzymatic reaction. The lowest trisaccharide formation took place with the sucrose:lactulose concentration 40%:20% at every time point, followed by 30%:30% and 20%:40%. Likewise, the maximum formation of the trisaccharide was found at the end of the reaction (48 h) for the three assayed ratio concentrations. Consequently, the sucrose:lactulose concentration 40%:20% was discarded for further assays, and higher enzyme concentrations (1.6 and 2.4 U mL^{-1}) were used with the aim to enhance the formation in the main acceptor-reaction product and/or shorten the reaction time.

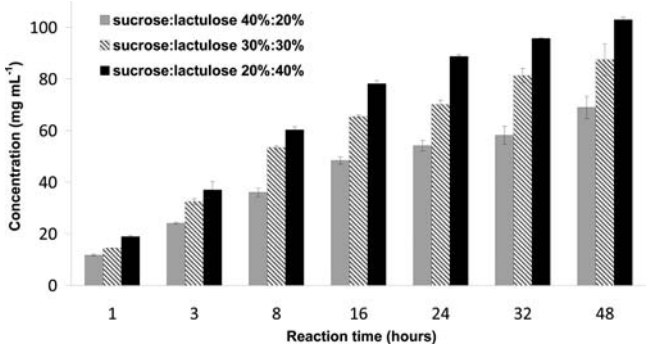


Figure 2. Concentration of lactulosucrose upon transglycosylation reaction at three different concentration ratios of sucrose:lactulose (20:40, 30:30, 40:20, expressed in g/100 mL) catalyzed by 0.8 U mL^{-1} dextransucrase from *Leuconostoc mesenteroides* B-512F at 30 °C in 20 mM sodium acetate buffer at pH 5.2. Vertical bars represent standard deviations (n = 3).

Figures 3 and 4 show the concentration of lactulose, sucrose, leucrose, and the main acceptor-reaction product upon the enzymatic reaction time at sucrose:lactulose concentrations of 20%:40% and 30%:30%, with either 1.6 or 2.4 U mL^{-1} dextransucrase concentration.

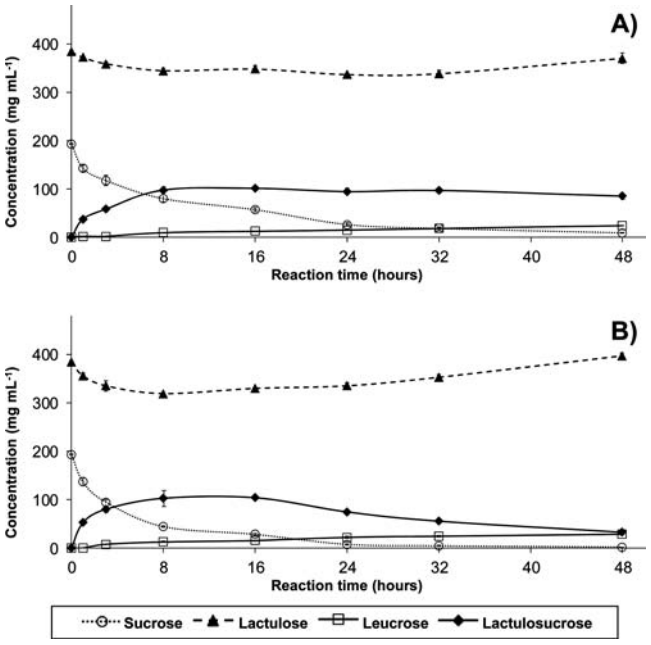


Figure 3. Concentrations of sucrose, lactulose, leucrose, and lactulosucrose upon transglycosylation reactions based on sucrose:lactulose (20:40, expressed in g/100 mL) catalyzed by dextransucrase from *Leuconostoc mesenteroides* B-512F at 30 °C in 20 mM sodium acetate buffer at pH 5.2. (A) 1.6 U mL^{-1} dextransucrase; (B) 2.4 U mL^{-1} dextransucrase. Sucrose (···○···), lactulose (- -▲- -), leucrose (—□—), lactulosucrose (—◆—). Vertical bars represent standard deviations (n = 3).

A progressive decrease in lactulose with a concomitant increase in the levels of the main acceptor-reaction product was found during the first 8 h of reaction at the sucrose:lactulose concentration 20%:40% with both enzyme charges (Figure 3). Likewise, the maximum amount of formed trisaccharide was rather similar in both cases (101−104 mg mL^{-1}), as well as the reaction time at which this was achieved (8 h). Also, similar maximum levels for leucrose (24−28 mg mL^{-1}) were obtained at 48 h of reaction for both enzyme concentrations, and this was probably due to the fact that similar amounts of free fructose were present in both reaction mixtures (data not shown). The most remarkable difference was the substantial decrease in the main acceptor-reaction product which coincided with an increase in the lactulose content between 16 and 48 h of reaction when 2.4 U mL^{-1} enzyme charge was used (Figure

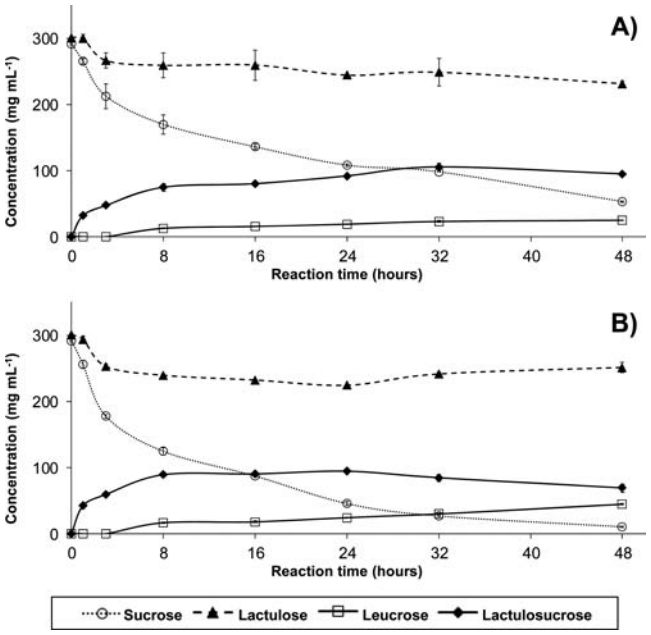


Figure 4. Concentrations of sucrose, lactulose, leucrose, and lactulosucrose upon transglycosylation reactions based on sucrose:lactulose (30:30 expressed in g/100 mL) catalyzed by dextransucrase from *Leuconostoc mesenteroides* B-512F at 30 °C in 20 mM sodium acetate buffer at pH 5.2. (A) 1.6 U mL^{-1} dextransucrase; (B) 2.4 U mL^{-1} dextransucrase. Sucrose (···○···), lactulose (- -▲- -), leucrose (—□—), lactulosucrose (—◆—). Vertical bars represent standard deviations (n = 3).

3B). This is indicative of a degradation of the trisaccharide which could revert in the lactulose content, which would further corroborate the involvement of lactulose in the main acceptor-reaction product. Finally, maximum levels of the minor acceptor-reaction products, i.e., tetra- and pentasaccharide labeled as peaks 7 and 8 in the inset of Figure 1, were quantified to be 6.1 mg mL^{-1} and 3.0 mg mL^{-1}.

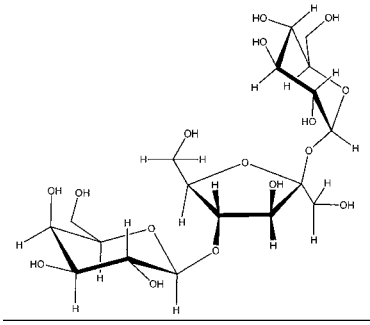
The maximum levels of the main acceptor-reaction product achieved at the sucrose:lactulose concentration of 30%:30% were also similar at both assayed enzyme concentrations, i.e., 106 mg mL^{-1} at 32 h of reaction for 1.6 U mL^{-1} dextransucrase and 95 mg mL^{-1} at 24 h of reaction for 2.4 U mL^{-1} (Figure 4), in comparison to those found for the sucrose:lactulose concentration of 20%:40%. However by comparing both sets of reactions it could be inferred that a low concentration of starting lactulose led to a longer reaction time in achieving the maximum formation of the trisaccharide. As above, a partial degradation of the main acceptor-reaction product in accordance with an increase in lactulose was detected between 24 and 48 h of reaction when 2.4 U mL^{-1} enzyme was used (Figure 4B). Likewise, the maximum leucrose levels obtained at 48 h were noticeably superior at the highest enzyme charge (44 mg mL^{-1}) than when 1.6 U mL^{-1} dextransucrase was used (25 mg mL^{-1}). This behavior can be explained because the formation of leucrose by action of dextransucrase is favored at high fructose concentration,[34] and a higher sucrose hydrolysis was obtained with 2.4 U mL^{-1} enzyme (Figure 4). The maximum levels of the minor acceptor-reaction products, tetra- and pentasaccharide, were 7.7 and 4.1 mg mL^{-1}, respectively.

Overall, the optimum reaction conditions led to the main acceptor-reaction product, which could correspond to lactulosucrose, yields of 27% and 35% (in weight respect to the initial amount of lactulose) for the sucrose:lactulose concentrations 20%:40% and 30%:30%, respectively. Furthermore, minor tetra- and pentasaccharide, both probably derived from lactulose, were also quantified to yield 2.5% and 1.4% (in weight respect to the initial amount of lactulose), respectively, at the sucrose:lactulose concentration 30%:30%, and 1.6% and 0.8%, respectively, at the sucrose:lactulose concentration 20%:40%.

Structural Characterization of the Main Acceptor-Reaction Product by Nuclear Magnetic Resonance (NMR). To structurally characterize the main synthesized acceptor-reaction product, the enzymatic reaction mixture at 16 h (sucrose:lactulose, 20%:40%) with 2.4 U mL^{-1} dextransucrase was repeatedly analyzed by LC-RID using a semi-preparative column, and peak 6 (Figure 1) was manually collected to be further analyzed by NMR. Then, unequivocal structural elucidation of this compound was carried out by the combined use of 1D and 2D [^{1}H,^{1}H] and [^{1}H−^{13}C] NMR experiments (gCOSY, TOCSY, ROESY, multiplicity-edited gHSQC and gHMBC). ^{1}H and ^{13}C NMR chemical shifts are given in Table 1.

The ^{13}C spectrum showed a major set of signals corresponding to 18 carbons including three anomeric carbons (δ104.30, δ102.87, and δ92.08), indicating the presence of a trisaccharide with three hexose sugars in the structure. A multiplicity-edited gHSQC spectrum was used to link the carbon signals to the corresponding proton resonances. So, the

Table 1. ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR Spectral Data for Lactulosucrose[a]

Structure	Position	β-Gal		β-Fru		α-Glu	
		δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
	1	4.29 (7.8)	102.87	3.54	61.24	5.29 (3.9)	92.08
	2	3.43	71.00	---	104.30	3.42	70.63
	3	3.52	72.42	4.27	75.31	3.62	72.47
	4	3.78	68.36	4.07	82.87	3.32	69.12
	5	3.58	75.22	3.92	80.50	3.73	72.35
	6	3.64	60.98	3.72	62.31	3.67	60.04

[a]Chemical shift (δ, ppm) and coupling constants (J in Hz, in parentheses).

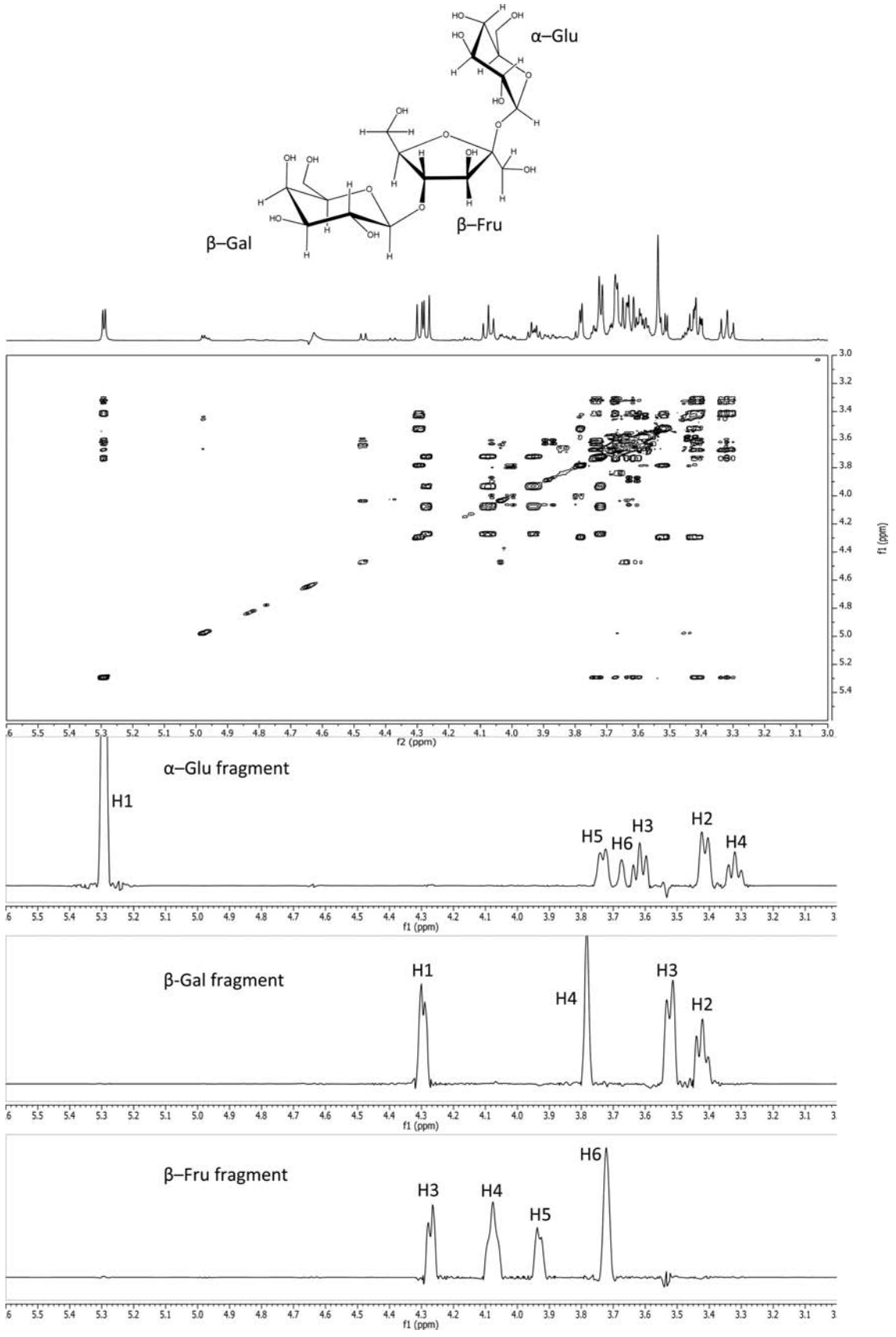


Figure 5. Two-dimensional TOCSY NMR spectrum with water suppression corresponding to lactulosucrose obtained at 500 MHz in D_2O.

anomeric carbon at δ102.87 correlated with a beta anomeric proton at δ4.29 (J(H1,H2) = 7.8 Hz) and the anomeric carbon at δ92.08 correlated with an alpha anomeric proton at δ5.29 (J(H1,H2) = 3.9 Hz). The anomeric carbon at δ104.30 was a

quaternary carbon. In addition, four methylene carbons at δ62.31, δ61.24, δ60.98, and δ60.04 were identified.

The $^{1}H-^{1}H$ COSY and $^{1}H-^{1}H$ TOCSY experiments revealed the ^{1}H signals of galactopyranose, glucopyranose, and fructofuranose residues (Figure 5). From these data, it could be inferred that the trisaccharide consisted of a unit of β-galactopyranose, a unit of α-glucopyranose, and a unit of β-fructofuranose.

The position of glycosidic linkages was analyzed as follows: gHMBC showed correlations between the α-Glu-H1 anomeric proton (5.29 ppm) and the β-Fru anomeric carbon (104.30 ppm), between the β-Gal-C1 anomeric carbon (102.87 ppm) and the β-Fru-H4 proton (4.07 ppm), and between the β-Gal-H1 anomeric proton (4.29 ppm) and the β-Fru-C4 carbon (82.87 ppm). Consequently, the major compound was identified as β-D-galactopyranosyl-(1→4)-β-D-fructofuranosyl-(2→1)-α-D-glucopyranoside.

To the best of our knowledge, these data provide the first evidence of ^{1}H and ^{13}C NMR full assignments for lactulosucrose.

DISCUSSION

In the presence of efficient carbohydrate acceptors and under optimized reaction conditions, glucansucrases (E.C. 2.4.1) catalyze the synthesis of low-molecular weight oligosaccharides instead of high molecular weight polymers.[35] Lactulose has rarely been used as acceptor in reactions with glucansucrases. Côté et al.[36] showed that lactulose was a poor acceptor with alternansucrases (E.C. 2.4.1.140). Hehre and Suzuki[27] tested lactulose as acceptor in the presence of several strains from *L. mesenteroides* (not including the B-512F strain) with dissimilar results. This behavior is in good agreement with the fact that the glucansucrase stereoselectivity and regioselectivity is highly strain-dependent.[37] In an elegant study, Robyt and Eklund[29] studied the reaction of seventeen acceptors with dextransucrase (E.C. 2.4.1.5) from *L. mesenteroides* B-512F, although lactulose was not included.

To the best of our knowledge, results reported in the present work are the first evidence for the efficient capacity of lactulose to act as acceptor using a highly purified dextransucrase from *L. mesenteroides* B-512F under consistent conditions of temperature, pH, substrate, and acceptor concentrations. Furthermore, the complete elucidation of the structure of lactulosucrose has been successfully accomplished by NMR, indicating that the dextransucrase-catalyzed synthesis of lactulosucrose was carried out by the transfer of the glucosyl residue from sucrose to the 2-hydroxyl group of the reducing unit of lactulose (Figure 5 and Table 1).

Suzuki and Hehre[26] demonstrated the formation of lactulosucrose using cultures of *L. mesenteroides* strain NRRL B-1299 grown in an autoclaved solution of 2% of sucrose and 1% of lactulose (derived from lactose during autoclaving). The use of cultures instead of highly purified enzymes, as well as the low concentration of initial sucrose and lactulose, could explain the moderate yields obtained in that work. Likewise, these authors stated that cultures of *L. mesenteroides* B-512F were not able to synthesize lactulosucrose. However, our results clearly indicated that the dextransucrase purified from *L. mesenteroides* B-512F efficiently catalyzed the formation of lactulosucrose under optimized reaction conditions with yields ranging between 27 and 35% in weight respect to the initial amount of lactulose.

Reaction parameters, such as the concentration ratio of donor (sucrose) to acceptor (lactulose) and the concentration of dextransucrase, had a considerable impact on the yield of lactulosucrose. In addition, although very minor acceptor-reaction products susceptible to correspond to lactulose-based tetra- and pentasaccharide could be detected, the predominant formation of lactulosucrose confirms the pattern described by Robyt,[28] who indicated that only one acceptor product is formed when D-galactose composes part of the acceptor structure. This behavior was observed for raffinose[38] or lactose[32,39,40] acting as acceptors, and, according to our results, this could be extended to lactulose.

Regarding the ratio of lactulose to sucrose, from our results it can be inferred that, using the same concentration of dextransucrase, the higher the ratio, the higher the amount of synthesized lactulosucrose (Figure 2). Furthermore, in most cases, as the concentration of enzyme was increased, the levels of lactulosucrose were higher and achieved at shorter reaction times (Figures 3 and 4). These results are in agreement with previous findings reported for reaction of sucrose:maltose mixtures using *L. mesenteroides* B-512F dextransucrase.[28]

On the other hand, an important decrease in the content of lactulosucrose with a concomitant increase in lactulose was observed from 16 to 24 h of reaction when the highest concentration of enzyme was used (2.4 U mL^{-1}), this effect being more remarkable with the sucrose:lactulose concentration of 20:40 (expressed in g/100 mL) (Figures 3B and 4B). Strikingly, lactulosucrose can also act as a D-glucosyl donor for dextransucrase,[27] being one of the few saccharides with this ability in addition to sucrose.[41] In consequence, the capacity as a donor of lactulosucrose could explain its decrease when the enzymatic reaction is well-advanced and its concentration is relatively high. Considering that sucrose is by far the best donor, with the lowest Michaelis constant (K_m), for glucansucrases,[28] it is very plausible that, once the availability of sucrose is very limited, lactulosucrose could then act as donor. This could explain that the degradation of lactulosucrose occurred earlier at the sucrose:lactulose concentration of 20%:40% (i.e., from 16 h of reaction) than at 30%:30% (i.e., from 32 h of reaction), as the levels of sucrose were consumed within a shorter reaction time with the former.

Although further in vitro and in vivo bioactivity studies should be conducted, lactulosucrose could be an excellent candidate for an emerging bioactive oligosaccharide with potential pharmaceutical and food applications. On the one hand, having lactulose as a core structure, lactulosucrose might possess the beneficial properties attributed to lactulose, such as the selective stimulation of beneficial bacteria in the large intestine and the regulation of intestinal transit time, among others. On the other hand, considering that recent findings pointed out galacto-oligosaccharides derived from lactulose having enhanced functional properties as compared to galacto-oligosaccharides derived from lactose,[25,42] lactulosucrose could exert new or improved biological activities as compared to lactosucrose.

Focusing on prebiotic properties, a large part of the prebiotic carbohydrates available up to the present undergo fermentation rapidly in the proximal zones of the colon as it could the case of lactulose.[43] However, it has been proven that difference in structure, as well as the chain length, may influence the speed of fermentation. Thus, longer carbohydrate chains are normally fermented slower, so their action could take place in the distal parts of the colon, where many chronic gut disorders

originate.[44,45] Considering the chemical structure of the synthesized oligosaccharides, lactulosucrose and its derivatives could potentially be employed as prebiotic oligosaccharides that could effectively combine the bioactive properties attributed to lactulose and the possibility to possess lower fermentation rates. This latter property would increase its interest as prebiotic by increasing its capacity to reach the distal parts of the colon. Finally, the transferred glucose moiety is attached to lactulose through a α-(1→2) linkage which has shown high resistance to in vitro and in vivo gastrointestinal digestion.[46−48]

■ ASSOCIATED CONTENT

Supporting Information

^{1}H and ^{13}C NMR, gCOSY, TOCSY, gHSQC, gHMBC, and ROESY spectra of lactulosucrose. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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