Enzymatic Synthesis and Characterization of Fructooligosaccharides and Novel Maltosylfructosides by Inulosucrase from *Lactobacillus gasseri* DSM 20604

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The ability of an inulosucrase (IS) from *Lactobacillus gasseri* DSM 20604 to synthesize fructooligosaccharides (FOS) and maltosylfructosides (MFOS) in the presence of sucrose and sucrose-maltose mixtures was investigated after optimization of synthesis conditions, including enzyme concentration, temperature, pH, and reaction time. The maximum formation of FOS, which consist of β -2,1-linked fructose to sucrose, was 45% (in weight with respect to the initial amount of sucrose) and was obtained after 24 h of reaction at 55°C in the presence of sucrose (300 g liter⁻¹) and 1.6 U ml⁻¹ of IS–25 mM sodium acetate buffer–1 mM CaCl₂ (pH 5.2). The production of MFOS was also studied as a function of the initial ratios of sucrose to maltose (10:50, 20:40, 30:30, and 40:20, expressed in g 100 ml⁻¹). The highest yield in total MFOS was attained after 24 to 32 h of reaction time and ranged from 13% (10:50 sucrose/maltose) to 52% (30:30 sucrose/maltose) in weight with respect to the initial amount of maltose. Nuclear magnetic resonance (NMR) structural characterization indicated that IS from *L. gasseri* specifically transferred fructose moieties of sucrose to either C-1 of the reducing end or C-6 of the nonreducing end of maltose. Thus, the trisaccharide erlose [α -D-glucopyranosyl-(1 $\rightarrow$ 4)- α -D-glucopyranosyl-(1 $\rightarrow$ 2)- β -D-fructofuranoside] was the main synthesized MFOS followed by neo-erlose [β -D-fructofuranosyl-(2 $\rightarrow$ 6)- α -D-glucopyranosyl-(1 $\rightarrow$ 4)- α -D-glucopyranose]. The formation of MFOS with a higher degree of polymerization was also demonstrated by the transfer of additional fructose residues to C-1 of either the β -2,1-linked fructose or the β -2,6-linked fructose to maltose, revealing the capacity of MFOS to serve as acceptors.

The production of new bioactive oligosaccharides is currently attracting high interest for their potential use as functional components in the food, pharmaceutical, and cosmetic industries (1). Specifically, fructooligosaccharides (FOS) are nondigestible food ingredients that could find immediate applications, among others, as prebiotic compounds due to their ability to improve host well-being and health by allowing specific changes in the composition and/or activity of the gastrointestinal microbiota (2) when they are selectively fermented by specific genera of colon bacteria, mainly, bifidobacteria and lactobacilli (3).

Chemically, FOS are polymers (degree of polymerization, 3 to 9) consisting of a sucrose molecule that is elongated by a chain of fructosyl units, having the generic structure GF_n (where G refers to glucose molecule and F_n to the number of fructose units). They can be classified into three different types according with their linkage patterns: (i) inulin-FOS, which consist of linear nonreducing chains with β -(2 $\rightarrow$ 1) linkages, such as 1-kestose [1^F - β -D-fructofuranosylsucrose] (GF2), 1-nystose [1^F (1- β -D-fructofuranosyl)₂ sucrose] (GF3), and 1^F - β -fructofuranosylnystose (GF4); (ii) levan-FOS, which have a β -(2 $\rightarrow$ 6) linkage formed between fructose units, such as 6-kestose (6^F - β -D-fructofuranosylsucrose); and (iii) a neo-FOS series in which the D-glucose moiety of sucrose is linked directly to a fructose unit through a β -(2 $\rightarrow$ 6) linkage, such as in the case of neo-kestose (6^G - β -D-fructofuranosylsucrose), giving the possibility that chain elongation occurs on both D-fructose residues by β -(2 $\rightarrow$ 1) or β -(2 $\rightarrow$ 6) bonds (4). Nonetheless, different FOS structures have also been described as isomers, branched fructans, or mixtures of these three main types, such as bifurcose, a tetrasaccharide formed by addition of a fructosyl unit to the 6-carbon position of the internal central fructose residue of 1-kestose (5).

Although FOS are found in trace amounts as natural components in many common foods, including onions, garlic, asparagus, tomatoes, bananas, wheat, and honey (6), commercial production can be achieved using fructansucrase or fructosyltransferase (FTF) enzymes from different fungal and bacterial strains as an effective alternative to chemical synthesis (7, 8). FTFs polymerize the fructose moiety of their substrate sucrose into FOS with β -(2 $\rightarrow$ 1) and/or β -(2 $\rightarrow$ 6) linkages but also catalyze the transfer of a fructose moiety from sucrose (donor) to other carbohydrates (acceptors) upon transfructosylation reaction (9). The structure and linkage of the fructan-type oligosaccharides differ depending on the microbial source of the FTF used for the production procedure.

Among the several microorganisms capable of producing FTF enzymes that have been extensively studied, diverse species of *Lactobacillus* that naturally inhabit the human gastrointestinal tract such as *L. reuteri* (10, 11), *L. johnsonii* (12), and *L. gasseri*, associated with a variety of probiotic functions with beneficial effects on health (13), can be pointed out. While numerous levansucrases (EC 2.4.1.10) are known, only a few inulosucrase (IS) genes/enzymes (EC 2.4.1.9) with the ability to synthesize potential bioac-

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tive oligosaccharides have recently been characterized from lactic acid bacteria, including some strains of *L. gasseri* (14).

Acceptor reactions are defined as those involving sucrose and a second substrate. In the case of glucansucrase enzymes, maltose is recognized as the best acceptor, providing the synthesis of a series of potentially bioactive oligosaccharide acceptor products such as panose (6- α -D-glucopyranosylmaltose) and other isomaltooligosaccharides (15, 16). When maltose acts as an acceptor in transfructosylation reactions with FTFs, only one acceptor product, named erlose [α -D-glucopyranosyl-(1 $\rightarrow$ 4)- α -D-glucopyranosyl-(1 $\rightarrow$ 2)- β -D-fructofuranoside], produced using a levansucrase from different bacterial strains, such as *Bacillus subtilis* (17) and *Leuconostoc mesenteroides*, has been reported in the open literature to the best of our knowledge (18). This nonreducing trisaccharide, also known as maltosylfructose (17), glycosyl sucrose, or coupling sugar (19), is a carbohydrate that naturally occurs in honey (20, 21).

In this context, sucrose and maltose are present in important agroindustrial residues, such as beet and cane molasses, starch hydrolysates, or maltose syrups, being largely used as renewable raw materials (i.e., carbon sources) in bioconversion processes, including enzymatic synthesis. Therefore, new alternative ways for the valorization of these food-related by-products could attract high industrial attention (22).

It was previously shown that *L. gasseri* strain DSM 20604 synthesized both inulin poly- and oligosaccharides, products of its *inuGB* IS enzyme (14). A fragment of the recombinant IS protein lacking the cell-anchoring motif also converted sucrose or raffinose into a range of FOS (14). In the present investigation, the synthesis of FOS and novel oligosaccharides, termed maltosylfructosides (MFOS), by the *L. gasseri inuGB* fragment was studied in the presence of sucrose and maltose upon transfructosylation reaction by optimization of synthesis conditions such as IS concentration, temperature, pH, sucrose/maltose molar ratio, and reaction time. Thus, the aim of the present study was to produce new potentially bioactive MFOS starting from sucrose and maltose in order to propose new efficient ways to valorize common and cheap food-related by-products. Subsequent nuclear magnetic resonance (NMR) structural characterization of the main MFOS was accomplished in order to gain deeper knowledge on the IS mechanism in the synthesis of oligosaccharides. These newly synthesized oligosaccharides could be used as a basis for other works on prebiotics or agroindustrial applications. Potential prebiotic benefits should be further confirmed using *in vitro* and *in vivo* studies related to their digestion and fermentation.

MATERIALS AND METHODS

Chemical and reagents. Carbohydrates (fructose, glucose, sucrose, maltose, 1-kestose, erlose, and nystose) were purchased from Sigma-Aldrich (Steinheim, Germany). Acetonitrile (high-performance liquid chromatography [HPLC] grade) was obtained from Lab-scan (Gliwice, Poland). Ultrapure water (18.2 M Ω cm) with 1 to 5 ppb total organic carbon (TOC) and pyrogen levels of <0.001 endotoxin units (EU) ml⁻¹ 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.

Enzyme production and purification. A fragment of the recombinant IS protein lacking the cell-anchoring motif from *L. gasseri* DSM 20604 (14) was overproduced in *Escherichia coli* and purified. Briefly, a 1.7-kb *inuGB* DNA fragment was PCR amplified using HS Primer Start DNA polymerase (TaKaRa) and primers 806 (5'-GAACTCTAGAGGGTATTA

FIG 1 SDS-PAGE analysis of the expression and purification of recombinant inulosucrase from *Lactobacillus gasseri* strain DSM 20604. Results of analysis of a soluble cell extract of IPTG-induced *Escherichia coli* DH10B (pURI2-Cter) (lane 1) or *E. coli* DH10B (pURI2-Cter-IS) cultures (lane 2), flowthrough (lane 3), and the fractions eluted (lanes 4 to 9) from Talon chelating affinity resin are shown. The SDS-PAGE gel (10%) was stained with Coomassie blue. The positions of some molecular mass markers (Bio-Rad) are indicated on the left.

ATAatggatgcggtaaaacaagatgaaaaag) and 779 (5'-GCTATTAATGATGATGATGATGATGtggatgtggcttaagtatatcc) (the nucleotides pairing the *inuGB* gene sequence are written in lowercase letters; the start and stop codons are indicated in bold). The *inuGB* gene was amplified lacking the 137 N-terminal and the 59 C-terminal amino acid residues without affecting the catalytic domain as described previously (14). The 1.7-kb purified PCR product was inserted into pURI2-Cter vector using a restriction enzyme-free and ligation-free cloning strategy (23). The cloning of a protein into the pURI2-Cter expression vector introduced a His₆ tag on the C terminus of the recombinant protein produced. Thus, the final recombinant His₆-tagged IS protein consisted of 571 amino acid residues with a molecular mass of 70 kDa. *E. coli* DH10B cells were transformed, and recombinant plasmids containing the correct insertion (pURI2-Cter-IS) were isolated. For protein production, cells carrying pURI2-Cter-IS plasmid were grown at 37°C until they reached an optical density of 0.4 at 600 nm and induced by adding isopropyl- β -D-thiogalactopyranoside (IPTG) (0.4 mM final concentration). After induction, the cells were grown at 22°C during 20 h and collected by centrifugation. Cells were resuspended in phosphate buffer (50 mM [pH 7.0] containing 300 mM NaCl). Crude extracts were prepared by French press lysis of the cell suspension (performed three times at 1,100 lb/in²). The insoluble fraction of the lysate was removed by centrifugation at 47,000 $\times$ g for 30 min at 4°C. The supernatant was filtered through a 0.45- μ m-pore-size filter and applied to a Talon Superflow histidine affinity resin equilibrated with 50 mM sodium phosphate (pH 7.0), 300 mM NaCl, and 10 mM imidazole to improve the interaction specificity in the affinity chromatography step. The bound IS enzyme was eluted using 150 mM imidazole in the same buffer. The purity of the IS protein was determined by 10% (wt/vol) SDS-PAGE at the various stages of the purification process. Protein bands were visualized by Coomassie blue staining (Fig. 1).

Enzyme characterization—inulosucrase activity assay. The protein concentration of the purified IS was 16.2 mg ml⁻¹ according to the bicinchoninic acid (BCA) assay using as the standard a dextranucrase of *Leuconostoc mesenteroides* B-512F purchased from CRITT Bio-Industries (Toulouse, France).

Fructosidase and fructosyltransferase activities of IS were measured as a function of the amounts of glucose and fructose released from sucrose. The hydrolysis of sucrose (100 g liter⁻¹) was assayed at a working temperature of 55°C in 25 mM sodium acetate buffer (pH 5.2) supplemented with 1 mM CaCl₂. Samples of 0.4 ml were withdrawn at different times (5, 15, 30, 40, and 50 min). The reaction was stopped by heating at 100°C for 5 min, and inactivated samples were analyzed by liquid chromatography with a refractive index detector (LC-RID) using the method described below to determine the amounts of fructose and glucose formed. The fructosidase activity of IS was expressed as the amounts of free glucose. The fructose incorporated in FOS (fructosyltransferase activity) was de-

defined as the difference between the amounts of released glucose and fructose. Enzyme activity measurements were repeated three times, and the experimental error was <5%.

IS expressed a specific fructosidase activity of 17.4 units per milligram (U mg^{-1}), where 1 unit is defined as the amount of enzyme releasing 1 μmol of glucose per minute per milliliter under the assayed conditions. The fructosyltransferase activity was 10.5 U mg^{-1} , where 1 unit is defined as the amount of enzyme required to transfer 1 μmol of fructose per minute per milliliter with respect to other molecules under the assayed conditions.

Enzymatic synthesis of oligosaccharides. Initially, the optimization of reaction conditions (IS concentration, pH, temperature, and time) for the synthesis of fructooligosaccharides (FOS) and maltosylfructosides (MFOS) was carried out by using starting concentrations of 300 g liter^{-1} of sucrose and a 30:30 sucrose/maltose mixture (expressed in $\text{g } 100 \text{ ml}^{-1}$), respectively. Thus, sucrose acts both as the donor of the fructose moiety and the acceptor whereas maltose acts only as the acceptor. The reaction medium was 25 mM sodium acetate buffer (pH 5.2, 4.5, and 3.7) supplemented with 1 mM CaCl_2 , and the assayed working temperatures were 55°C and 45°C. Assayed concentrations of IS were 16.0, 8.0, and 1.6 U ml^{-1} (fructosyltransferase activity); aliquots were taken at 0, 1, 3, 8, 24, 32, and 48 h, and the reaction was stopped by heating at 100°C for 5 min. Data are shown as means $\pm$ standard deviations (SD) of the results of triplicate assays.

Furthermore, the production of MFOS was studied as a function of the concentration ratios of sucrose to maltose (40:20, 30:30, 20:40, and 10:50, expressed in $\text{g } 100 \text{ ml}^{-1}$) with an optimized inulosucrase concentration of 1.6 U ml^{-1} at pH 5.2 and 55°C. Samples were incubated in individual tubes of 1.5 ml in an orbital shaker at 1,000 rpm. Reactions were allowed to proceed for 48 h. During this time, aliquots were taken from the reaction mixture at suitable time intervals (0, 1, 3, 8, 24, 32, and 48 h). The enzyme was inactivated by heating at 100°C for 5 min, and inactivated samples were then diluted with 50:50 (vol/vol) acetonitrile/water and filtered using a $0.45\text{-}\mu\text{m}$ -pore-size syringe filter (Symta, Madrid, Spain).

Chromatographic determination of carbohydrates. (i) Liquid chromatography with refractive index detector (LC-RID). Enzymatic reactions were monitored by LC-RID on an Agilent Technologies 1220 Infinity LC system-1260 RID (Boeblingen, Germany). The separation of the synthesized oligosaccharides was carried out on a Kromasil (100- NH_2) column (Akzo Nobel, Brewster, NY) (250 by 4.6 mm ; $5 \mu\text{m}$ particle size) using 75:25 (vol/vol) acetonitrile/water as the mobile phase and elution in isocratic mode at a flow rate of 1.0 ml min^{-1} for 120 min. The injection volume was $50 \mu\text{l}$ (1 mg of total carbohydrates). Data acquisition and processing were performed using Agilent ChemStation software (Agilent Technologies, Boeblingen, Germany).

Main carbohydrates in the reaction mixtures were initially identified by comparing the retention times (t_R) with those of available commercial standard sugars. Quantitative analysis was performed by the external standard method, using calibration curves in a range of 0.01 to 10 mg for fructose (quantification of monosaccharides), sucrose and maltose (quantification of disaccharides), 1-kestose (quantification of trisaccharides), and nystose (quantification of tetrasaccharides and acceptor products of polymerization degree above 4). All analyses were carried out in triplicate. Determination coefficients obtained from these calibration curves, which were linear over the range studied, always corresponded to $R^2 > 0.999$. Reproducibility of the method was estimated on the basis of the intraday and interday precision calculated as the relative standard deviations (RSD) of concentrations of oligosaccharide standards obtained in $n \geq 5$ independent measurements, obtaining RSD values below 10% in all cases.

(ii) GC-MS. Samples were derivatized to their trimethylsilyl (TMS) oximes according to the method of Sanz et al. (24). In brief, oximes were obtained by addition of $350 \mu\text{l}$ of a solution of 2.5% hydroxylamine chloride in pyridine after 30 min at 75°C. They were then silylated with hexamethyldisilazane ($350 \mu\text{l}$) and trifluoroacetic acid ($35 \mu\text{l}$) at 45°C for 30 min. After the reaction, samples were centrifuged at $4,400 \times g$ for 10 min,

and the supernatant was subjected to analysis with a gas chromatograph coupled to a mass spectrometry detector.

Gas chromatography-mass spectrometry (GC-MS) analysis of derivatized samples was carried out using a 7890A gas chromatograph coupled to a 5975C quadrupole mass detector (both from Agilent Technologies, Palo Alto, CA). Analyses were carried out on a Rtx-65TG (Crossbond) (35% dimethyl-65% diphenyl polysiloxane; Restek, Bellefonte, PA) capillary column (30 m by 0.25-mm inner diameter by $0.1\text{-}\mu\text{m}$ film thickness), using helium at 1 ml min^{-1} as the carrier gas. The oven temperature was held at 170°C for 10 min, then programmed to 215°C at a heating rate of $15^\circ\text{C min}^{-1}$, then raised to 240°C at 1°C min^{-1} , and finally increased to 320°C at 5°C min^{-1} and held for 20 min. Injections ($1 \mu\text{l}$) were carried out in split mode (1:20) at 300°C. The mass spectrometer was operated in electron impact (EI) mode at 70 eV, scanning the m/z 35 to 700 range. The transfer line and ionization source were heated at 280 and 230°C, respectively. Acquisition was done using HP ChemStation software (Agilent Technologies).

Purification and structural characterization of maltosylfructosides by NMR spectroscopy. Considering the lack of a commercially available standard for MFOS, with the exception of erlose, the main synthesized oligosaccharides were isolated and purified by analytical LC-RID from a 30:30 sucrose/maltose mixture with 1.6 U ml^{-1} of IS after 8, 24, or 32 h of enzymatic reaction under the optimized conditions described above. An analytical column rather than a semipreparative column was employed due to the high resolution required by the complexity of the chromatographic profile. Thus, $50 \mu\text{l}$ of reaction mixture (1 mg of total carbohydrate) was repeatedly injected and eluted with 75:25 (vol/vol) acetonitrile/water using the method already described, and fractions corresponding to the main synthesized oligosaccharides were manually collected, pooled, evaporated in an R-200 rotatory evaporator (Büchi, Switzerland) at a temperature below 25°C, and freeze-dried for its subsequent characterization.

Structure elucidation of the five purified oligosaccharides was accomplished by NMR spectroscopy. NMR spectra were recorded at 298 and 313 K, using D_2O as 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 external standards of sodium: [2,2,3,3- $^2\text{H}_4$]-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 (1D) NMR experiments (^1H and ^{13}C) were performed using standard Varian pulse sequences. Two-dimensional (2D) ^1H - ^1H NMR experiments (gradient correlation spectroscopy [gCOSY] and total correlation spectroscopy [TOCSY]) were carried out with the following parameters: a delay time of 1 s, a spectral width of 1,675.6 Hz in both dimensions, 4,096 complex points in t_2 and t_4 transients for each of 128 time increments, and a linear prediction to 256. The data were zero-filled to $4,096 \times 4,096$ real points. A 2D ^1H - ^1H rotating-frame Overhauser effect spectroscopy (ROESY) NMR experiment used the same conditions, with 64 transients per increment and a mixing time of 80 ms. 2D ^1H - ^{13}C NMR experiments (gradient heteronuclear single-quantum coherence [gHSQC] and gradient heteronuclear multiple-bond correlation [gHMBC]) used the same ^1H spectral window, a ^{13}C spectral window of 30,165 Hz, a 1-s relaxation delay, 1,024 data points, and 128 time increments, with a linear prediction to 256. The data were zero-filled to $4,096 \times 4,096$ real points. Typical numbers of transients per increment were 4 and 16, respectively.

RESULTS

Enzymatic synthesis of fructooligosaccharides derived from sucrose by *L. gasseri* DSM 20604 inulosucrase (IS). First assays for the production of fructooligosaccharides (FOS) by purified IS were carried out in the presence of sucrose (300 g liter^{-1})-25 mM sodium acetate buffer-1 mM CaCl_2 (pH 4.5) at a temperature of 55°C with the aim to optimize the enzyme concentration. These initial reaction conditions were based on the data previously ob-

FIG 2 Concentrations of sucrose and total fructooligosaccharides (FOS) upon transfructosylation reaction catalyzed by inulosucrase from *Lactobacillus gasseri* DSM 20604 (IS) (1.6 U ml⁻¹) in the presence of sucrose (300 g liter⁻¹)–25 mM sodium acetate buffer–1 mM CaCl₂ (pH 5.2) at a temperature of 55°C. Vertical bars represent standard deviations ($n = 3$).

tained by Anwar et al. (14) using a different recombinant construction of the same *L. gasseri* IS enzyme. Under those conditions, different enzyme charges (16.0, 8.0, and 1.6 U ml⁻¹) were employed, allowing reaction times of up to 48 h. The optimum enzyme concentration that led to the maximum formation of FOS within 24 h of reaction was determined to be 1.6 U ml⁻¹ (data not shown). Once the optimum enzyme concentration was selected, the influence of pH (3.7 and 5.2) and the influence of a decrease in the temperature of incubation (45°C) were studied. From all these experiments, it could be concluded that the most favorable conditions included the use of 55°C and a pH of 5.2, which are in agreement with the optimum values described for other FTF enzymes (25, 26). Overall, under these optimal reaction conditions, a yield of 45% of total FOS (in weight with respect to the initial amount of sucrose) was achieved after 24 h of reaction, when the sucrose was almost consumed (Fig. 2).

A typical LC-RID chromatogram of FOS produced by IS is shown in Fig. 3. Whereas glucose (peak 2) derived from the hydrolysis of sucrose (peak 3) was released, fructose (peak 1) moieties were transferred to other sucrose molecules, producing a mixture of FOS of various degrees of polymerization (DP). By

comparing the retention times (t_R) with those of available commercial standards, it could be inferred that the synthesized FOS were mainly comprised of β -2,1-linked fructose to sucrose. In fact, the trisaccharide 1-kestose (GF2) (peak 8; $t_R = 19.5$ min) was the main FOS formed, followed by nystose (GF3) (peak 10; $t_R = 29.7$ min), fructosylnystose (GF4) (peak 14; $t_R = 45.3$ min), and fructosylfructosylnystose (GF5) (peak 18; $t_R = 67.8$ min) (Fig. 3). Trace amounts of GF6 could also be detected. Further analysis by high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) also revealed the minor presence of FOS with a higher DP up to GF11 (data not shown). These results confirmed the ability of IS to use sucrose both as a donor and as an acceptor to produce inulin-type FOS.

Moreover, two other peaks (peaks 5 and 6; $t_R = 15.2$ and 18.0 min) were detected in minor amounts in the eluting area of di- and trisaccharides. GC-MS analyses were performed to try to identify these minor carbohydrates (data not shown). The minor disaccharide showed a fragment at m/z 307 in a proportion as high as that seen with the 361 ion, which is indicative of a free ketose linked to the nonreducing unit through the hydroxyl group in position 1 or 3. The relative proportions of fragments m/z 217, 204, and 191, as well as the high ratio of fragments at m/z 437 and 451, indicate that the nonreducing unit was also fructose. This compound could be identified as inulobiose (1- β -D-fructofuranosylfructose) by comparison with a honey sample where it had been previously identified (27). The presence of this peak could indicate that fructose could also act as a minor acceptor in the FTF reaction (18). The other compound was a trisaccharide that could be an isomer of 1-kestose, such as neo-kestose, according to its characteristic m/z ratios of 217/204 and 437/451.

Enzymatic synthesis of maltosylfructosides derived from sucrose-maltose mixtures by *L. gasseri* DSM 20604 IS. The production of FOS was substantially reduced in favor of the formation of new oligosaccharides when maltose was present in the reaction mixture. This indicated the capacity of inulosucrase (IS) to transfer fructose units to another acceptor different from sucrose, such as maltose in this case, which led to the synthesis of oligosaccharides

FIG 3 Comparison between LC-RID profiles of fructooligosaccharides (FOS) and maltosylfructosides (MFOS) after 8 h of transfructosylation reaction based on sucrose (300 g liter⁻¹) (dashed line) or 30:30 sucrose/maltose (expressed in g 100 ml⁻¹) (solid line) catalyzed by inulosucrase from *Lactobacillus gasseri* DSM 20604 (IS) (1.6 U ml⁻¹) at 55°C in 25 mM sodium acetate buffer supplemented with 1 mM CaCl₂ (pH 5.2). Peak identification: 1, fructose; 2, glucose; 3, sucrose; 4, maltose; 5, inulobiose; 6, neo-kestose; 7, erlose (MFOS DP3); 8, 1-kestose (GF2); 9, neo-erlose (MFOS DP3); 10, nystose (GF3); 11 to 13, MFOS DP4; 14, fructosylnystose (GF4); 15 to 17, MFOS DP5; 18, fructosylfructosylnystose (GF5); 19, MFOS DP6.

termed maltosylfructosides (MFOS). To optimize the formation of these oligosaccharides, the same optimization experiments as those performed for the synthesis of FOS were carried out by using 30:30 sucrose/maltose mixtures (expressed in g 100 ml⁻¹). Results confirmed that the optimal IS conditions were 55°C, pH 5.2, and an enzyme charge of 1.6 U ml⁻¹ (data not shown).

Figure 3 shows a representative LC-RID profile of the synthesized oligosaccharides from a 30:30 sucrose/maltose mixture after 8 h of reaction. In addition to the previously identified FOS, resulting from the transfer of fructose to sucrose, a series of new peaks (peaks 7, 9, 11, 12, 13, 15, 16, 17, and 19) that likely correspond to a maltose molecule linked to fructosyl residues appeared as the reaction proceeded (Fig. 3). A substantial decrease of maltose, i.e., up to a 23% decrease after 32 h of reaction, was also indicative of the formation of new oligosaccharides based on the transfer of fructose moieties to maltose. Following the same pattern as that found for the synthesis of FOS, it could be expected that MFOS were mainly comprised of β -2,1-linked fructose to maltose. In good agreement, the main MFOS acceptor reaction product synthesized (peak 7; Fig. 3) corresponded to a trisaccharide identified as erlose [α -D-glucopyranosyl-(1 $\rightarrow$ 4)- α -D-glucopyranosyl-(1 $\rightarrow$ 2)- β -D-fructofuranoside] by LC-RID and GC-MS analysis by comparison with a commercial standard. Furthermore, GC-MS analyses revealed the presence of an unknown minor trisaccharide eluting before 1-kestose (characteristic m/z 437/451 ratio of 1.33) with two peaks which could correspond to *E* and *Z* isomers of a reducing trisaccharide with a characteristic fragment at m/z 451 (typical of a pyranose ring) with higher intensity than other fragments at m/z 437 (typical of cyclic ketohexoses) (m/z 437/451 ratio of 0.35). According to these data, these peaks could correspond to a trisaccharide having fructosyl-glucosyl-glucose as a monomer composition.

Moreover, due to the lack of available standards, an exhaustive NMR characterization of the most abundant MFOS (peaks 9, 12, 13, 15, and 16; Fig. 3) was further carried out to fully elucidate their structures and to gain knowledge on the mechanism in the IS synthesis of oligosaccharides from sucrose-maltose mixtures, as shown below.

Once the optimal conditions of temperature, pH, and enzyme concentration were known, the influence of the sucrose/maltose concentration ratios (10:50, 20:40, and 40:20, expressed in g 100 ml⁻¹) was studied with the main goals not only of increasing the yield obtained for the synthesis of total MFOS with 30:30 sucrose/maltose mixtures but also of modulating the formation of acceptor products with a certain DP type and the proportion of synthesized MFOS and FOS. Since an increase in the acceptor reaction efficiency is related to an increase in the concentration of dissolved solids (17), the total substrate concentration was set as high as 600 g liter⁻¹, with this value limited by the solubility of both disaccharides in the reaction buffer.

Figure 4 shows the content of total FOS and MFOS obtained using the four assayed concentration ratios. Considering the two types of oligosaccharides together, the 30:30 and 40:20 sucrose/maltose concentration ratios led to the highest yield in the synthesis of acceptor products, i.e., 226 to 236 mg ml⁻¹ ($\sim$ 39% in weight with respect to the initial amount of total carbohydrates). However, the amount of total oligosaccharides was substantially reduced when the concentration of initial sucrose was lowered to 20% and, especially, 10%, giving rise to 176 and 75 mg ml⁻¹ of total oligosaccharides, respectively. This fact was probably due to

FIG 4 Concentrations of total fructooligosaccharides (FOS) and total maltosylfructosides (MFOS) upon transfructosylation reaction at four different sucrose/maltose concentration ratios of (40:20, 30:30, 20:40, and 10:50, expressed in g 100 ml⁻¹) catalyzed by 1.6 U ml⁻¹ inulosucrase from *Lactobacillus gasseri* DSM 20604 (IS) in 25 mM sodium acetate buffer supplemented with 1 mM CaCl₂ (pH 5.2) at 55°C. Vertical bars represent standard deviations ($n = 3$).

the limited amount of fructose produced from sucrose hydrolysis in the reaction medium and, consequently, to a lower efficiency in the fructosyltransferase-catalyzed synthesis of oligosaccharides. Regarding FOS, data indicated that as the amount of sucrose in the reaction mixture increased, the level of FOS formation that occurred increased, with 40:20 sucrose/maltose mixtures giving the highest yields of these well-known prebiotic compounds (126 mg ml⁻¹ after 32 h of reaction; Fig. 4).

On the other hand, the highest yield in total MFOS (DP3 to DP6) was attained after 48 h of reaction at the 30:30 sucrose/maltose concentration ratio, i.e., 156 mg ml⁻¹ (52% in weight with respect to the initial amount of maltose), closely followed by the 20:40 sucrose/maltose concentration ratio (139 mg ml⁻¹; 35% in weight with respect to the initial amount of maltose), whereas the 10:50 sucrose/maltose concentration ratio gave rise to the smallest amount of MFOS (Fig. 4).

Table 1 summarizes the content in MFOS from DP3 to DP6 after 48 h of enzymatic reaction with all assayed sucrose/maltose concentration ratios. The trisaccharide identified as erlose (peak 7; Fig. 3) was the major synthesized MFOS acceptor reaction product followed by the trisaccharide eluting as peak 9 (Fig. 3). In general terms, while a higher ratio of sucrose to maltose resulted in the formation of MFOS with a higher DP, a lower ratio of sucrose

TABLE 1 Concentration of synthesized maltosylfructosides from DP3 to DP6 upon transfructosylation reaction at four different concentration ratios of sucrose and maltose^a

MFOS compound	Maltosylfructoside value(s) at indicated sucrose/maltose concn ratio ^b							
	40:20		30:30		20:40		10:50	
	Concn (mg ml ⁻¹)	Relative %	Concn (mg ml ⁻¹)	Relative %	Concn (mg ml ⁻¹)	Relative %	Concn (mg ml ⁻¹)	Relative %
DP3	68.1	66.5	99.9	64.2	107.5	77.6	59.1	89.2
Erllose (peak 7) ^c	37.5 ± 1.24	36.6	50.4 ± 4.34	32.4	61.4 ± 3.69	44.3	33.0 ± 0.66	49.9
Neo-erlose (peak 9) ^d	30.7 ± 2.43	30.0	49.5 ± 0.73	31.8	46.1 ± 5.21	33.2	26.0 ± 1.30	39.3
DP4	19.5	19.1	31.9	20.5	21.3	15.3	7.1	10.8
Tetrasaccharide 1 (peak 12) ^d	15.2 ± 1.62	14.8	24.0 ± 0.57	15.4	16.2 ± 0.57	11.7	4.6 ± 0.01	7.0
Tetrasaccharide 2 (peak 13) ^d	4.4 ± 0.46	4.3	7.9 ± 0.31	5.1	5.0 ± 0.48	3.6	2.5 ± 0.03	3.8
DP5	12.3	12.0	19.3	12.4	8.6	6.2		
Pentasaccharide 1 (peak 15) ^d	8.3 ± 0.70	8.1	13.1 ± 0.83	8.4	5.7 ± 0.13	4.1		
Pentasaccharide 2 (peak 16) ^d	2.5 ± 0.27	2.5	4.1 ± 0.10	2.6	1.8 ± 0.02	1.3		
Pentasaccharide 3 (peak 17)	1.5 ± 0.15	1.5	2.1 ± 0.31	1.3	1.2 ± 0.15	0.9		
DP6	2.4	2.3	4.5	2.9	1.2	0.9		
Hexasaccharide 1 (peak 19)	2.4 ± 0.16	2.3	4.5 ± 0.40	2.9	1.2 ± 0.23	0.9		
Total	102.4		155.7		138.6		66.2	

^a Reactions were catalyzed by 1.6 U ml⁻¹ inulosucrase from *Lactobacillus gasseri* DSM 20604 in 25 mM sodium acetate buffer supplemented with 1 mM CaCl₂ (pH 5.2) at 55°C. Sucrose and maltose concentrations are expressed in g 100 ml⁻¹. Standard deviations (*n* = 3) are also represented. MFOS, maltosylfructoside; IS, inulosucrase.
^b Relative % data were calculated with respect to the total MFOS compound values shown in the entries in the bottom row of the table.
^c Identified by LC-RID and GC-MS analysis.
^d Identified by NMR characterization.

to maltose resulted mainly in low DP MFOS (mostly trisaccharides). A similar behavior for the *L. mesenteroides* dextranucrase was reported by Su and Robyt (28), who indicated that as the ratio of acceptor to sucrose increased, the number of acceptor products decreased (29).
Finally, the sucrose/maltose concentration ratio also had a substantial influence on the proportion of synthesized FOS and MFOS, showing that the synthesis of MFOS was clearly favored against the formation of FOS as the ratio of sucrose to maltose decreased (Fig. 5).

Structural elucidation of novel maltosylfructosides by NMR spectroscopy. Five chromatographic peaks (peaks 9, 12, 13, 15, and 16; Fig. 3) were purified by LC-RID and subsequently successfully characterized by NMR (structures B, C, D, E, and F plus G,

respectively; Fig. 6). Panels B to G of Fig. 6 show the structures elucidated by the combined use of 1D and 2D ¹H-¹H and ¹H-¹³C NMR experiments (gCOSY, TOCSY, multiplicity-edited gHSQC, and gHMBC). The ¹H and ¹³C NMR chemical shifts observed are summarized in Table 2. The full sets of spectra are available in the supplemental material.
(i) **Trisaccharides.** The main synthesized MFOS (peak 7; Fig. 3) was the trisaccharide erlose, which was previously identified by GC-MS (Fig. 6A). For comparison, ¹H and ¹³C NMR spectral data from the literature are shown in Table 2 (30). The second-most-abundant MFOS was also a trisaccharide (peak 9; Fig. 3). The 1D ¹H NMR spectrum of structure B showed two sets of two doublets in the anomeric region due to the two anomeric forms of the reducing terminal unit. Besides, 1D ¹³C NMR spectrum showed two sets of three resonances in the anomeric region, one of them representing a quaternary carbon. Careful analysis of the 2D COSY and TOCSY spectra revealed the ¹H signals of two units of glucopyranose and a unit of fructofuranose that allowed us to correlate them to the corresponding carbon signals in the multiplicity-edited gHSQC spectra. To assign the configuration of each anomeric center, the values of the vicinal coupling constants for the anomeric protons were used. These results were consistent with the structure of a trisaccharide with the presence of a fructose residue in the form of a furanosyl ring and two glucopyranose residues, the G1 unit being α-D-glucopyranosyl and the reducing terminal G2 unit a glucose with β and α forms in a 1.5:1 ratio. The position of glycosidic linkages was analyzed as follows: for the major isomer, gHMBC showed correlations between the anomeric proton of G1 (5.30 ppm) and C-4 of the terminal glucose G2 (78.20 ppm), between the anomeric carbon of G1 (100.64 ppm) and H4 of G2 (3.58 ppm), and between C-2 of F1 (104.53 ppm) and one of the methylene H6 protons of G1 (3.95 ppm). Finally, the B structure was established as β-D-fructofuranosyl-(2→6)-α-D-glucopyranosyl-(1→4)-β-D-glucopyranose, which we have denominated “neo-erlose.”

FIG 5 Proportions of synthesized fructooligosaccharides (FOS) and maltosylfructosides (MFOS) upon transfructosylation reaction at four different sucrose/maltose concentration ratios (40:20, 30:30, 20:40, and 10:50, expressed in g 100 ml⁻¹) catalyzed by 1.6 U ml⁻¹ inulosucrase from *Lactobacillus gasseri* DSM 20604 (IS) in 25 mM sodium acetate buffer supplemented with 1 mM CaCl₂ (pH 5.2) at 55°C.

FIG 6 Structures of the products of synthesized maltosylfructosides (MFOS) (compounds A to G) based on sucrose-maltose, catalyzed by inulosucrase from *Lactobacillus gasseri* DSM 20604 (IS) in a transfructosylation reaction.

TABLE 2 ^1H (500-MHz) and ^{13}C (125-MHz) NMR spectral data for oligosaccharides A to E^a

Structure	Position	Value(s) for residue:									
		G1		G2		F1		F1'		F2'	
		δ_{H}	δ_{C}	δ_{H}	δ_{C}	δ_{H}	δ_{C}	δ_{H}	δ_{C}	δ_{H}	δ_{C}
A [α -D-glucopyranosyl-(1 $\rightarrow$ 4)- α -D-glucopyranosyl-(1 $\rightarrow$ 2)- β -D-fructofuranoside] ^b	1	5.40 (4.0)	100.65	5.41 (3.5)	92.82	3.66, 3.66	62.23				
	2	3.58	72.63	3.59	71.68		104.50				
	3	3.68	73.68	4.02	73.86	4.20	77.38				
	4	3.42	70.21	3.69	77.69	4.03	74.92				
	5	3.72	73.57	3.97	71.86	3.88	82.24				
	6	3.85, 3.76	61.37	3.82, 3.79	61.04	3.82, 3.79	63.21				
B1 [β -D-fructofuranosyl-(2 $\rightarrow$ 6)- α -D-glucopyranosyl-(1 $\rightarrow$ 4)- β -D-glucopyranose]	1	5.30 (3.8)	100.64	4.59 (8.0)	96.57			3.70, 3.62	60.90		
	2	3.53	72.40	3.22	74.70				104.53		
	3	3.62	73.54	3.70	76.94			4.13	77.50		
	4	3.39	70.17	3.56	78.20			4.07	75.26		
	5	3.77	72.60	3.55	75.31			3.82	81.95		
	6	3.95, 3.66	61.41	3.87, 3.73	61.55			3.76, 3.64	63.19		
B2 [β -D-fructofuranosyl-(2 $\rightarrow$ 6)- α -D-glucopyranosyl-(1 $\rightarrow$ 4)- α -D-glucopyranose]	1	5.30 (3.8)	100.71	5.17	92.70			3.70, 3.62	60.90		
	2	3.53	72.49	3.52	72.01				104.53		
	3	3.62	73.54	3.90	72.49			4.13	77.50		
	4	3.39	70.22	3.58	78.41			4.07	75.31		
	5	3.77	72.60	3.88	70.72			3.82	81.95		
	6	3.95, 3.66	61.41	3.78, 3.64	61.41			3.76, 3.64	63.19		
C [β -D-fructofuranosyl-(2 $\rightarrow$ 6)- α -D-glucopyranosyl-(1 $\rightarrow$ 4)- α -D-glucopyranosyl-(1 $\rightarrow$ 2)- β -D-fructofuranoside]	1	5.33 (3.9)	100.62	5.35 (3.7)	93.12	3.74, 3.67	61.73	3.77, 3.77	61.00		
	2	3.51	72.60	3.50	71.73		104.06		104.57		
	3	3.61	73.75	3.95	73.84	4.19	77.65	4.11	77.49		
	4	3.35	70.17	3.62	78.32	3.95	74.71	4.01	75.32		
	5	3.67	73.53	3.90	71.85	3.79	82.06	3.79	81.96		
	6	3.91, 3.77	61.31	3.76, 3.65	61.31	3.75, 3.73	63.00	3.75, 3.70	63.17		
D1 [β -D-fructofuranosyl-(2 $\rightarrow$ 1)- β -D-fructofuranosyl-(2 $\rightarrow$ 6)- α -D-glucopyranosyl-(1 $\rightarrow$ 4)- β -D-glucopyranose]	1	5.30 (3.9)	100.60	4.59 (7.9)	96.56			3.65	61.48	3.71, 3.63	61.09
	2	3.53	72.40	3.22	74.70				103.84		104.40
	3	3.62	73.52	3.70	76.96			4.16	78.40	4.12	77.51
	4	3.39	70.18	3.55	78.19			4.07	75.25	4.04	75.12
	5	3.76	72.54	3.54	75.34			3.81	81.95	3.80	81.83
	6	3.96, 3.63	61.35	3.87, 3.66	61.61			3.65, 3.62	63.10	3.78, 3.70	63.04
D2 [β -D-fructofuranosyl-(2 $\rightarrow$ 1)- β -D-fructofuranosyl-(2 $\rightarrow$ 6)- α -D-glucopyranosyl-(1 $\rightarrow$ 4)- α -D-glucopyranose]	1	5.30 (3.9)	100.68	5.16 (3.9)	92.68			3.65	61.48	3.71, 3.63	61.09
	2	3.53	72.49	3.52	72.01				103.84		104.40
	3	3.62	73.52	3.90	73.98			4.16	78.40	4.12	77.51
	4	3.39	70.12	3.58	78.40			4.07	75.25	4.04	75.12
	5	3.76	72.54	3.88	70.74			3.81	81.95	3.80	81.83
	6	3.96, 3.63	61.35	3.76, 3.65	61.25			3.65, 3.62	63.10	3.78, 3.70	63.04
E [β -D-fructofuranosyl-(2 $\rightarrow$ 1)- β -D-fructofuranosyl-(2 $\rightarrow$ 6)- α -D-glucopyranosyl-(1 $\rightarrow$ 4)- α -D-glucopyranosyl-(1 $\rightarrow$ 2)- β -D-fructofuranoside]	1	5.33 (3.9)	100.59	5.36 (3.9)	93.09	3.80, 3.64	61.82	3.76, 3.60	61.68	3.68, 3.60	61.18
	2	3.51	72.57	3.49	71.73		103.98		103.85		104.47
	3	3.61	73.71	3.94	73.82	4.20	77.60	4.15	78.29	4.11	77.47
	4	3.35	70.13	3.61	77.54	3.96	74.68	4.00	75.25	4.03	75.13
	5	3.67	73.50	3.90	71.82	3.79	82.03	3.79	81.88	3.79	81.88
	6	3.94, 3.64	61.28	3.76, 3.65	60.96	3.78, 3.73	62.96	3.76, 3.68	63.06	3.77, 3.68	63.04

^a Chemical shift data (δ [ppm]) and coupling constant data (J, in Hz [in parentheses]) are shown.

^b Erlöse spectral data are from the literature (30).

(ii) **Tetrasaccharides.** For structure C (peak 12; Fig. 3) the ^1H NMR spectrum showed two doublets in the anomeric region with coupling constants of 3.7 and 3.9 Hz, a pattern close to that of erlose. Besides, two doublets corresponding to H3 of fructofuranoside were observed (Fig. 7). The ^{13}C spectrum showed four signals at 104.57 (q), 104.06 (q), 100.62, and 93.12 ppm in the anomeric region. Following the same procedure, analysis of the spectra revealed the presence of two units of α -glucopyranose, according to coupling constants, and two units of fructofuranose. Interresidual gHMBC correlations were observed between the anomeric proton of G1 (5.33 ppm) and C-4 of the terminal glucose G2 (78.32 ppm), between the anomeric carbon of G1 (100.62 ppm) and H4 of G2 (3.62 ppm), between C-2 of F1' (104.57 ppm) and one of the methylene H6 protons of G1 (3.91 ppm), and between C-2 of F1 (104.06 ppm) and H1 of G2 (5.35 ppm). From these results and for comparison with NMR values of erlose for the F1 ring and neo-erlose for the F1' ring, the C structure was

established as β -D-fructofuranosyl-(2 $\rightarrow$ 6)- α -D-glucopyranosyl-(1 $\rightarrow$ 4)- α -D-glucopyranosyl-(1 $\rightarrow$ 2)- β -D-fructofuranoside.

For structure D (peak 13; Fig. 3), the ^1H NMR spectrum showed two sets of two doublets in the anomeric region due to the two anomeric forms of the reducing terminal unit, a pattern very similar to that seen with structure B (Fig. 7). Besides, in the anomeric region of the ^{13}C spectrum, four signals at 104.40 (q), 103.84 (q), 100.60, and 96.56 ppm for the major isomer and at 104.40 (q), 103.84 (q), 100.68, and 92.68 ppm for the minor one were observed. 2D-COSY, TOCSY, and gHSQC spectra revealed the presence of two units of fructofuranose and two units of glucopyranose, the G1 unit being α -D-glucopyranosyl and the reducing terminal G2 unit a glucose with β and α forms in a 1.5:1 ratio. Interresidual correlations between the anomeric proton of G1 (5.30 ppm) and C-4 of the terminal glucose G2 (78.19 for β , 78.40 ppm for α), between the anomeric carbon of G1 (100.60 for β , 100.68 ppm for α) and H4 of G2 (3.55 for β , 3.58 ppm for α),

FIG 7 One-dimensional NMR spectra of compounds A to G at 500 MHz in D_2O . Data represent 1H (left) and ^{13}C (right) spectra, showing the anomeric region.

between C-2 of F1' (103.84 ppm) and one of the methylene H6 protons of G1 (3.96 ppm), and between C-2 of F2' (104.40 ppm) and one of the methylene H1 protons of F1' (3.65 ppm) were observed. From these results and comparing with NMR values of structure B, the D compound was established as β -D-fructofuranosyl-(2 $\rightarrow$ 1)- β -D-fructofuranosyl-(2 $\rightarrow$ 6)- α -D-glucopyranosyl-(1 $\rightarrow$ 4)-D- β -glucopyranose.

(iii) Pentasaccharides. For structure E (peak 15; Fig. 3), the 1H NMR spectrum showed two doublets in the anomeric region with a coupling constant of 3.9 Hz, a pattern very close to that seen with structure C. Besides, three doublets corresponding to H3 of fructofuranoside were observed (Fig. 7). The 1D ^{13}C NMR spectrum showed five signals (104.47, 103.98, 103.85, 100.59, and 93.09 ppm) in the anomeric region, three of them quaternary carbons. 2D experiments revealed the presence of two units of α -glucopyranose, according to coupling constants, and three units of fructofuranose. Interresidual gHMBC correlations and comparison with 1H and ^{13}C spectra of tetrasaccharide C allowed us to establish structure E as β -D-fructofuranosyl-(2 $\rightarrow$ 1)- β -D-fructofuranosyl-(2 $\rightarrow$ 6)- α -D-glucopyranosyl-(1 $\rightarrow$ 4)- α -D-glucopyranosyl-(1 $\rightarrow$ 2)- β -D-fructofuranoside.

Finally, the 1H NMR spectrum corresponding to peak 16 revealed the existence of a mixture of two pentasaccharides. Due to the small quantity of sample, the 1D ^{13}C NMR spectrum could not be obtained. The 1H NMR spectrum showed the superposition of two doublets in the anomeric region with coupling constants of 3.9 Hz, very similar to the results seen with structure C, plus a pattern of two sets of two doublets very close to the pattern seen with structure D (Fig. 7). Comparison with 1H and ^{13}C spectra of tetrasaccharides C and D led us to tentatively determine that peak 16 represented a mixture of β -D-fructofuranosyl-(2 $\rightarrow$ 6)- α -D-glucopyranosyl-(1 $\rightarrow$ 4)- α -D-glucopyranosyl-(1 $\rightarrow$ 2)- β -D-fructofuranosyl-(1 $\rightarrow$ 2)- β -D-fructofuranoside (structure F) and β -D-fructofuranosyl-(2 $\rightarrow$ 1)- β -D-fructofuranosyl-(2 $\rightarrow$ 1)- β -D-fructofuranosyl-(2 $\rightarrow$ 6)- α -D-glucopyranosyl-(1 $\rightarrow$ 4)- α -D-glucopyranose (structure G).

DISCUSSION

The purified inulosucrase from *L. gasseri* DSM 20604 (IS) had the ability to efficiently synthesize either FOS or MFOS in the presence of sucrose or sucrose-maltose, respectively, following the optimization of parameters such as temperature, pH, enzyme concentration, and reaction time. The maximum formation of FOS,

consisting of β -2,1-linked fructose to sucrose, was 45% (in weight with respect to the initial amount of sucrose) and was obtained after 24 h of reaction with 300 g liter⁻¹ of sucrose and 1.6 U ml⁻¹ of IS at 55°C in 25 mM sodium acetate buffer–1 mM CaCl₂ at pH 5.2 (Fig. 2). In good agreement with this, Anwar et al. (14) previously demonstrated that growing a culture of *L. gasserii* DSM 20604 and the recombinant IS produced FOS formed by β -(2→1) linkages. Nevertheless, it should be noted that the FOS yield could probably be improved by increasing the concentration of initial sucrose, as it is well known that an increase in the concentration of substrates normally leads to higher acceptor reaction efficiency (17, 29).

In the present work, the ability of maltose to act as an acceptor in the *L. gasserii* DSM 20604 inulosucrase-catalyzed reaction to produce MFOS in the presence of sucrose-maltose mixtures was also demonstrated. The production of MFOS was also studied as a function of the ratios of sucrose to maltose (10:50, 20:40, 30:30, and 40:20, expressed in g 100 ml⁻¹), which had a considerable impact on the MFOS yield and DP distribution as well as on the proportions of synthesized MFOS versus FOS (Fig. 4 and 5 and Table 1). In fact, at low sucrose/maltose concentration ratios, MFOS production was favored over FOS formation although the yield in MFOS, mostly trisaccharides, was moderate (13% in weight with respect to the initial amount of maltose). In contrast, the highest MFOS yields (52% in weight with respect to the initial amount of maltose) were obtained when the equimolar donor/acceptor ratio was used. Likewise, high sucrose/maltose concentration ratios (40:20 and 30:30) led to the production of longer MFOS polymers.

The structural elucidation of the main MFOS accomplished by NMR, with the exception of erlose, which was identified by GC-MS, was very useful to gain knowledge about the mechanism in the synthesis of oligosaccharides by IS (Fig. 6). Erlose [α -D-Glu-(1→4)- α -D-Glu-(1→2)- β -D-Fru] (Fig. 6A) was shown to be the main synthesized MFOS (peak 7; Fig. 3) by transferring the fructosyl residue from sucrose to the anomeric carbon (C-1) of the reducing unit of maltose in an approach similar to that used for the synthesis of FOS. The second-most-abundant MFOS was also a trisaccharide (peak 9; Fig. 3) formed by the β -2,6-linked fructose to the nonreducing glucose moiety of maltose [β -D-Fru-(2→6)- α -D-Glu-(1→4)- α -D-Glu], keeping the reducing nature of maltose (Fig. 6B). We termed this trisaccharide “neo-erlose” in analogy to the nomenclature used for describing some FOS such as kestose and neo-kestose.

Furthermore, the structures of the two chromatographically resolved tetrasaccharides (peaks 12 and 13; Fig. 3) were unequivocally elucidated to be β -D-Fru-(2→6)- α -D-Glu-(1→4)- α -D-Glu-(1→2)- β -D-Fru (Fig. 6C) and β -D-Fru-(2→1)- β -D-Fru-(2→6)- α -D-Glu-(1→4)- α -D-Glu (Fig. 6D). Thus, peak 12 could be formed by the β -2,6-linked fructose to the available glucose moiety of erlose and/or by the β -2,1-linked fructose to the anomeric carbon (C-1) of the reducing unit of neo-erlose. However, peak 13 was derived exclusively from neo-erlose by the transfer of an additional fructose residue to C-1 of its fructose unit. The structural elucidation of these two tetrasaccharides unequivocally revealed the capacity of MFOS to serve as acceptors to give rise to MFOS of higher DP.

Peak 11 could not be chromatographically resolved from nystose (Fig. 3); consequently, it could not be characterized by NMR. Nevertheless, taking into account the mechanism described for

the synthesis of peaks 12 and 13, peak 11 might derive from erlose and its structure could be tentatively assigned to α -D-Glu-(1→4)- α -D-Glu-(1→2)- β -D-Fru-(1→2)- β -D-Fru.

Regarding the elution area of pentasaccharides, once again, based on the NMR elucidation of the structures of peaks 15 and 16 (Fig. 3), the mechanism of action of the enzyme was confirmed. The structure of peak 15 was clearly β -D-Fru-(2→1)- β -D-Fru-(2→6)- α -D-Glu-(1→4)- α -D-Glu-(1→2)- β -D-Fru (Fig. 6E). Therefore, it may be observed that it could be synthesized from peaks 12 and/or 13 by adding a β -2,1-linked fructose to the β -2,6-linked fructose of peak 12 and/or to the anomeric carbon of the reducing glucose moiety of peak 13. Peak 16 proved to represent a mixture of β -D-Fru-(2→6)- α -D-Glu-(1→4)- α -D-Glu-(1→2)- β -D-Fru-(1→2)- β -D-Fru (Fig. 6F) and β -D-Fru-(2→1)- β -D-Fru-(2→6)- α -D-Glu-(1→4)- α -D-Glu (Fig. 6G). The two structures could be formed by the transfer of an additional β -2,1-linked fructose residue to the F1 of peak 12 and to the F2' moiety of peak 13, respectively. Hence, structure F, in the same manner as was seen with structure E, could be derived from both erlose and neo-erlose whereas structure G is derived exclusively from neo-erlose.

Due to the small amount obtained corresponding to purified peak 17 (Fig. 3), this compound could not be characterized by NMR. However, taking into account the mechanism of action of the described enzyme, peak 17 might derive from erlose and its structure might be tentatively assignable to α -D-Glu-(1→4)- α -D-Glu-(1→2)- β -D-Fru-(1→2)- β -D-Fru-(1→2)- β -D-Fru.

To sum up, in addition to the expected inulosucrase activity shown by IS in synthesizing inulin-type FOS, this enzyme was also capable of efficiently synthesizing trisaccharide MFOS by transferring a D-fructofuranosyl residue to either the anomeric carbon (C-1) of the reducing end of maltose or C-6 of the nonreducing end of maltose to give erlose and neo-erlose, respectively. Furthermore, the formation of MFOS with a higher degree of polymerization also took place by the transfer of additional fructose residues to C-1 (via β -2,1 linkage) of either the β -2,1-linked fructose or the β -2,6-linked fructose previously transferred to maltose, highlighting the capacity of MFOS to serve further as acceptors. Nevertheless, considering the quantitative data of the synthesized MFOS (Table 1), it could be inferred that the efficiency of the acceptor reaction decreased as the size of the MFOS chain increased.

There is very limited information on the three-dimensional (3D) structure of inulosucrase enzymes, which hampers the elucidation of its catalytic mechanism. Pijning et al. (31) determined the crystal structure of an inulosucrase from *Lactobacillus johnsonii*. In addition, the high-resolution 3D structures of the glucanase from *Lactobacillus reuteri* 180 (32) and the levansucrases from *Bacillus subtilis* (33) and *Gluconacetobacter diazotrophicus* (34) have been determined in the last decade. A two-step mechanism has been proposed for catalysis by fructansucrase enzymes involving an acidic group and a nucleophilic group which are essential for transfructosylation (35). In this sense, Ozimek et al. (11) proposed a mode of action of FTF enzymes which starts with the cleavage of the glycosidic bond of the substrate sucrose, yielding a covalent enzyme-fructosyl complex. Subsequently, the fructosyl unit can be coupled to water (hydrolysis), to sucrose (to form FOS), to a growing fructan chain, or to various compounds acting as acceptors (such as in the case of maltose). Taking into account that the orientation of the incoming acceptor substrate deter-

mines the type of glycosidic bond of the product, maltose could be bound in the IS acceptor site in two different ways, considering that the enzyme is capable of transferring fructose moieties to either the nonreducing or the reducing glucose residues. A similar behavior was described by Fu and Robyt (36) for acceptor reactions of maltose and maltodextrins catalyzed by a dextranase.

According to the monomer and linkage type of the synthesized MFOS, these oligosaccharides could provide a good opportunity for a range of potential applications. As an example, erlose is a sugar that occurs naturally in honey (37) and could be used in the food industry to suppress crystal formation, the browning reaction in heat-processed foods, and retrogradation of starch. Erlose is also utilized as a substitute sweetener preventing dental caries caused by oral flora, mainly *Streptococcus mutans*, since it is approximately half as sweet as sucrose with a similar taste quality (19, 38, 39). While functional properties of neo-erlose are yet to be explored, most of the novel synthesized MFOS could be considered erlose derivatives, so they might also be successfully used as low-cariogenic sweeteners, among other uses in the food industry. Regarding potential prebiotic properties, it would be necessary to perform further studies, especially those related to digestibility, as it is well known that α -1,4 linkages are labile to digestion, so MFOS might be at least partially metabolized by human intestinal glycosidases. Nevertheless, it would be worth exploring whether the molecular size and/or degree of branching affects the digestion rate as well as the fermentation speed and the metabolites produced in the gut. In this context, MFOS with a high degree of polymerization might potentially provide prebiotic benefits even if these compounds could be partially metabolized during digestion, as they share structural features with FOS, such as β -2,1 linkages. Additionally, it has been reported that oligosaccharides with longer chains, resisting gastrointestinal digestion at least partially, are generally fermented slowly and reach more-distal regions of the colon (40, 41). It is also significant that both sucrose and maltose are present in large amounts in important agroindustrial residues, suggesting a potentially profitable means of valorization of these by-products.

Conclusion. To the best of our knowledge, the findings described in this work are the first evidence of the efficient synthesis of maltosylfructosides (MFOS) catalyzed by the inulosucrase enzyme from *Lactobacillus gasseri* DSM 20604 by transferring D-fructose residues from sucrose to the reducing or the nonreducing end of maltose. By carefully selecting the reaction conditions, the synthesis of MFOS may be favored, resulting in yields of up to 52% (in weight with respect to the initial amount of maltose). The main MFOS formed were erlose and neo-erlose, although other MFOS with higher degrees of polymerization (up to DP6) were also synthesized. NMR structural characterization was very useful for gaining knowledge of the mechanism of action of this enzyme in the production of MFOS. Such information is crucial for future studies investigating the structure-function relationship of the MFOS and their potential use in the food industry as noncariogenic agents or prebiotics, as well as for developing new alternatives for the valorization of food-related by-products.

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