"Regioselective synthesis of neo-erlose by the β-fructofuranosidase from Xanthophyllomyces dendrorhous"

Authors: M. Gimeno-Perez, P. Santos-Moriano, L. Fernández-Arrojo, A. Poveda, J. Jiménez-Barbero, A.O. Ballesteros, M. Fernández-Lobato, F.J. Plou*

Published in:

Process Biochemistry, 49, 423-429 ***(2014)***

http://dx.doi.org/10.1016/j.procbio.2013.12.018

Regioselective synthesis of neo-erlose by the β-fructofuranosidase from *Xanthophyllomyces dendrorhous*

M. Gimeno-Perez[a], P. Santos-Moriano[b], L. Fernández-Arrojo[b], A. Poveda[c], J. Jiménez-Barbero[d], A.O. Ballesteros[b], M. Fernández-Lobato[a,*], F.J. Plou[b,*]

[a] *Centro de Biología Molecular Severo Ochoa, UAM-CSIC, 28049 Madrid, Spain*

[b] *Instituto de Catálisis y Petroleoquímica, CSIC, 28049 Madrid, Spain*

[c] *Servicio Interdepartamental de Investigación, UAM, 28049 Madrid, Spain*

[d] *Centro de Investigaciones Biológicas, CSIC, 28040 Madrid, Spain*

***Corresponding authors**:

Francisco J. Plou, Instituto de Catálisis y Petroleoquímica, CSIC, Marie Curie 2, 28049 Madrid, Spain. Phone: +34-91-5854869. Fax: +34-91-5854760. E-mail: fplou@icp.csic.es.

María Fernández-Lobato, Centro de Biología Molecular Severo Ochoa, UAM-CSIC, 28049 Madrid, Spain. Phone: +34-91-1964492. Fax: +34-91-1964420. E-mail: mfernandez@cbm.uam.es.

ABSTRACT

The β-fructofuranosidase from the yeast *Xanthophyllomyces dendrorhous* (Xd-INV) catalyzes the synthesis of neo-fructooligosaccharides (neo-FOS of the ^{6}G-series), which contain a β(2→6) linkage between a fructose and the glucosyl moiety of sucrose. In this work we demonstrate that the enzyme is also able to fructosylate other carbohydrates that contain glucose, in particular disaccharides (maltose, isomaltulose, isomaltose, trehalose) and higher oligosaccharides (maltotriose, raffinose, maltotetraose), but not monosaccharides (glucose, fructose, galactose). With maltose as acceptor, the reaction in the presence of Xd-INV proceeded with high regioselectivity; the product was purified and chemically characterized, and turned out to be 6'-*O*-β-fructosylmaltose (neo-erlose). Using 100 g/L sucrose as fructosyl donor and 300 g/L maltose as acceptor, the maximum concentration of neo-erlose was 38.3 g/L. Thus, novel hetero-fructooligosaccharides with potential applications in the functional food and pharmaceutical industries can be obtained with Xd-INV.

Keywords: fructooligosaccharides; food oligosaccharides; maltosylfructosides; neo-erlose; *Xanthophyllomyces dendrorhous*.

1. INTRODUCTION

The *Xanthophyllomyces dendrorhous* yeast (formerly *Phaffia rhodozyma*) produces an extracellular β-fructofuranosidase of 160-200 kDa (Xd-INV, formerly described as an INVertase) with heterogeneous degree of glycosylation [1;2]. The crystallization and preliminary X-ray diffraction analysis of this enzyme after a deglycosylating treatment was performed [3]. The enzyme hydrolyzes efficiently fructosyl-β-(2→1) linked carbohydrates such as sucrose and 1-kestose. The level of glycosylation affects the thermal behaviour of Xd-INV but not to its transglycosylation/hydrolysis ratio [2]; the synthesis of fructooligosaccharides (FOS) reaches its maximum at 60-70ºC. Besides Xd-INV, an intracellular β-fructofuranosidase of 33 kDa with an optimal temperature of 45ºC and a similar activity profile has been described in *X. dendrorhous* [4;5].

To our knowledge, Xd-INV is the most efficient enzyme for the synthesis of FOS of the ^{6}G-series (neoFOS, e.g. neokestose and neonystose), which contain a β-(2→6) linkage between a fructose and the glucosyl moiety of sucrose. Several studies indicate that such neoFOS possess enhanced properties and chemical stability compared to ^{1}F-FOS typically used as prebiotics (e.g. 1-kestose or nystose) [6-9].

Undoubtedly, the distinctive property of Xd-INV is its ability to transfer a fructosyl moiety to the glucose unit of sucrose. This contrasts with the behaviour of other fructosylating enzymes, including levansucrases [10], inulosucrases [11]

and most β-fructofuranosidases [12-15], which commonly form β-(2→1) or β-(2→6) linkages between fructose units.

Some β-fructofuranosidases and fructosyltransferases also catalyze the transfer of the fructose moiety from sucrose to other carbohydrates (acceptors), thus forming hetero-fructooligosaccharides (hetero-FOS) with potential applications in functional foods [16], pharmaceutical [17] and cosmeceutical [18] industries. Examples of hetero-FOS include: lactosylfructoside (lactosucrose) that is obtained with *Zymomonas mobilis* levansucrase [19] or dextransucrase B-512F from *Leuconostoc mesenteroides* [20] and that selectively promotes the growth of bifidobacteria [21]; cellobiofructose synthesized with *Bacillus subtilis* levansucrase, which is employed as a low-calorie sweetener [22]; fructosylxyloside produced by fructosyltransferase from *Bacillus macerans* [23]; maltosylfructosides synthesized by inulosucrase from *Lactobacillus gasseri* [24].

In this work we have assessed the ability of Xd-INV to fructosylate a series of carbohydrates that contain a glucose moiety in its structure. The aim of this study was thus to obtain novel hetero-FOS that are difficult to produce by chemical synthesis.

2. MATERIALS AND METHODS

2.1. Materials

Sucrose, glucose and fructose were from Merck. 1-Kestose and nystose were from TCI Europe. Maltose, palatinose, isomaltose, maltotriose, maltotetraose, raffinose, trehalose, cellobiose, galactose, lactose, lactulose and turanose were from Sigma-Aldrich. All other reagents were of the highest available purity.

2.2. Organism, culture conditions and protein purification

The *Xanthophyllomyces dendrorhous* strain ATCC MYA-131 was grown at 24°C on Maltose Minimal Medium (MMM) [0.7% yeast nitrogen base without aminoacids (Difco), 2% (w/v) maltose] with shaking at 200 rpm. Growth was monitored spectrophotometrically at 660 nm (A_{660}). The extracellular β-fructofuranosidase activity produced by one liter of *X. dendrorhous* culture (7.2 U/mL; A_{660} = 2.4) was purified as previously described [2]. Briefly, the extracellular medium was concentrated through a 30,000 MWC PES membrane by using a VivaFlow 50 system (Vivascience), dialyzed in 20 mM sodium phosphate buffer pH 7.0, applied to a DEAE-Sephacel chromatography column and eluted at 0.1 M NaCl.

2.3 Activity assay

The enzymatic activity towards sucrose was determined at 60ºC in 0.1 M sodium phosphate buffer (pH 5.0) by measuring the release of reducing sugars from 20 g/L sucrose solution using the dinitrosalicylic acid (DNS) method [25].

The assay was adapted to 96-well microplates as described in our previous work [26]. A calibration curve was performed with a 5 g/L glucose solution. One unit (*UI*) of activity was defined as that catalyzing the formation of 1 µmol of reducing sugars per minute under the above conditions.

2.4. Acceptor reaction with Xd-INV

The pure **Xd-INV** was added to a solution containing sucrose (100 g/L, 0.29 M) in the presence of the sugar acceptor (100 g/L), in 100 mM sodium acetate buffer, pH 5.6. The final activity in the mixture was adjusted to 3 U/mL. Reaction mixtures were incubated at 60ºC and 100 rpm in a Vortemp 56 shaker. At different reaction times (0-240 min), 50 µL aliquots were withdrawn, boiled for 10 min to inactivate the enzyme and conveniently diluted with water (1:5 or 1:20). The samples were filtered through 0.45 µm cellulose centrifuge filters (National Scientific) for 5 min at 3500 rpm and analyzed by HPLC. In order to elucidate the optimum donor/acceptor molar ratio in the production of fructosylmaltose, different concentrations of sucrose and maltose were assayed to a final sugar concentrations of 200 g/L (1:1) or 400 g/L (3:1; 1:3) under the above conditions. Each reaction was performed in duplicate.

2.5. HPLC analysis

The analysis and quantification of the different carbohydrates present in the transfructosylation reactions was carried out by HPLC with a quaternary pump (Delta 600, Waters) coupled to a 4.6 x 250 mm Luna-NH_2 column (5 µm, 100 Å)

from Phenomenex. Detection was performed using a refractive index detector (model 2410, Waters) equilibrated at 30ºC. Acetonitrile/water 82:18 (v/v), degassed with helium, was used as mobile phase at 1.0 mL/min for 25 min for the reactions with disaccharides as acceptors, and 80:20 (v/v) for higher saccharides. The column temperature was kept constant at 30ºC. The data obtained were analysed using the Varian Star 4.0 Software. Each analysis was performed in duplicate. The transfer/hydrolysis ratio for each acceptor molecule was calculated as the ratio between the chromatographic areas of all the fructosylated products generated in the reaction (neokestose, 1-kestose and derivatives for the new acceptors) and the free fructose.

2.6. Purification and characterization of the acceptor product with maltose

Purification. For the isolation of the acceptor product with maltose, the biocatalytic reaction was scaled to 3 mL. At the point of maximum concentration of the unknown compound, the enzyme was inactivated at 100ºC for 10 min. The carbohydrates in the mixture were purified by semi-preparative HPLC using a system equipped with a Waters Delta 600 pump coupled to a 5 µm Kromasil-NH_2 column (10 x 250 mm; Analisis Vinicos). A three-way flow splitter (model Accurate, Dionex) and a refraction index detector (Varian, model star 9040) equilibrated at 30ºC were used. Acetonitrile/water 75:25 (v/v), degassed with helium, was used as mobile phase at 5 mL/min for a total analysis time of 25 min. The column temperature was kept constant at 30ºC. After collection of the different

oligosaccharides, the mobile phase was eliminated by rotary evaporation in a R-210 rotavapor (Buchi).

Analysis of purity. The purity of the acceptor product with maltose was analyzed by high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) on a ICS3000 Dionex system (Dionex Corp., Sunnyvale, CA) consisting of a SP gradient pump, an AS-HV autosampler and an electrochemical detector with a gold working electrode and Ag/AgCl as reference electrode. All eluents were degassed by flushing with helium. A pellicular anion-exchange 4 x 250 mm Carbo-Pack PA-1 column (Dionex) connected to a CarboPac PA-1 guard column at 30°C was used. For eluent preparation, MilliQ water and 50% (w/v) NaOH (Sigma-Aldrich) were used. The flow rate was 1.0 mL/min during the analysis. The initial mobile phase was 20 mM NaOH for 13 min. A mobile phase linear gradient from 20 mM to 100 mM NaOH and from 0 to 40 mM sodium acetate was performed in 7 min, and the latter composition was kept constant for 10 min. Then a linear gradient from 40 to 100 mM sodium acetate in 5 min maintaining 100 mM NaOH was programmed, and the mobile phase composition was kept constant for 2 min. The peaks were analyzed using Chromeleon software. The identification of the different carbohydrates was done based on commercial standards and purified FOS as described elsewhere [1;12].

Mass spectrometry. The molecular weight of the unknown carbohydrate was analyzed by MALDI-TOF mass spectrometry (Bruker, model Ultraflex III

TOFTOF) using 2,5-dihidroxybenzoic acid doped with sodium iodide as matrix, in positive reflector mode.

Nuclear magnetic resonance (NMR). The structure of the purified carbohydrate was elucidated using a combination of 1H, 13C- and 2D-NMR (COSY, TOCSY, NOESY, HSQC) techniques. The spectra of the sample (ca. 10 mM), dissolved in deuterated water, was recorded on a Bruker AVANCE DRX500 spectrometer equipped with a tuneable broadband 1H/X probe with a gradient in the Z axis, at a temperature of 298 K. Chemical shifts were expressed in ppm with respect to the 0 ppm point of DSS, used as internal standard. COSY, TOCSY, NOESY and HSQC sequences were provided by Bruker. COSY, TOCSY (80 ms mixing time), and NOESY (500 ms mixing time) experiments were performed with 16, 8, and 48 scans, respectively, with 256 increments in the indirect dimension and with 1024 points in the acquisition dimension. The spectral widths were 9 ppm in both dimensions. The HSQC experiment (16 scans) also used 256 increments in the indirect dimension and with 1024 points in the acquisition dimension. The spectral width was 120 ppm in the indirect dimension and 9 ppm in the acquisition one.

3. RESULTS AND DISCUSSION

3.1. Hetero-oligosaccharides synthesis with Xd-INV

Neokestose and 1-kestose are the main transglycosylation products from sucrose obtained with Xd-INV, a yeast extracellular glycoprotein characterized by its high thermostability [1]. The level of glycosylation of Xd-INV does not affect its hydrolase and transferase activities, both optimal in the range of 60-70ºC [2].

When other saccharides coexist with sucrose as acceptors, some glycosidases and transglycosidases may synthesize novel heterooligosaccharides [27;28]. In order to assess the transferase activity of Xd-INV towards different carbohydrates, purified enzyme (a mixture of 160-200 kDa glycoforms) was incubated at 60ºC in presence of 100 g/L sucrose as the fructosyl donor and different sugar acceptors (maltose, isomaltose, trehalose, isomaltulose, lactose, lactulose, turanose, cellulose, maltotriose, raffinose or maltotetraose) at the same concentration (100 g/L).

Fig. 1 shows some representative HPLC profiles of the transfer reaction using trehalose, isomaltulose and maltose. Using maltose as acceptor, a new peak (***10***) appeared at the end of the chromatogram in addition to the neokestose and 1-kestose (peaks ***7*** and ***8***) previously described [1;2]. New peaks were also obtained in the acceptor reactions with isomaltulose (peak ***9***), trehalose (peak ***11***), isomaltose, maltotriose, maltotetraose and raffinose (chromatograms not shown). However, the fructosyl moiety of sucrose was not transferred to cellobiose, lactulose, lactose or turanose and neither to the monosaccharides fructose, glucose or galactose.

We followed the concentration of total biosynthetic products (neokestose, 1-kestose and acceptor derivatives) in the reaction mixture during 240 min with the different acceptor sugars (Fig. 2). In the case of maltose, isomaltose and isomaltulose, acceptor products reached about 8% (w/w), referred to the total amount of sugars in the reaction mixture. The lowest concentration of acceptor product was obtained with trehalose, not surpassing 2% (w/w). In a control reaction by using 200 g/L of sucrose as the sole substrate, only neokestose and 1-kestose were obtained (15.6 g/L, 7.8% of total sugars).

Considering that the response factor of the refraction index detector is very similar for most carbohydrates, we defined the acceptor efficiency of the different sugars as the ratio between the area of the main acceptor product –at the point of maximum concentration– and the area of FOS (neokestose plus 1-kestose) at the same point of the reaction. As shown in Table 1, isomaltulose, maltotetraose, maltotriose, maltose and isomaltose displayed efficiencies higher than 1.0, which indicated that they were better acceptors than sucrose itself. In addition, these acceptors gave rise to transfer/hydrolase ratios (calculated as described in section 2.5) in the range 1.2-1.6, which were at least 4-fold higher than those obtained using monosaccharides, cellobiose, lactulose or turanose (sugars unable to act as fructose acceptors; ratio of 0.2-0.3) and even trehalose (poor fructose acceptor; Table 1). The transfer/hydrolase ratio was 0.96 in the reaction based exclusively on sucrose (200 g/L), which suggests that the monosaccharides and disaccharides not

acting as acceptors possibly displayed an inhibitory effect towards the transfer reaction.

3.2. Structural characterization of the acceptor product with maltose

The new fructosyl derivative obtained in the reaction with maltose as acceptor was purified by semipreparative HPLC. The purity of the fructosyl-maltose was evaluated by HPAEC-PAD due to its higher sensitivity compared with conventional HPLC. Fig. 3A illustrates the typical HPAEC-PAD chromatogram of the acceptor reaction with maltose, and Fig. 3B shows the chromatogram of the purified oligosaccharide. The purity of the maltose derivative was only 75% (the main contaminants were maltose and neokestose) but allowed its chemical characterization.

The mass spectrum of compound ***10*** confirmed that it was a trisaccharide. The 1D and 2D ^{1}H NMR spectra displayed three anomeric signals. Two of them were identified as arising from an α/β equilibrium. From the combination of the signals from COSY, TOCSY, NOESY, HSQC and HMBC, the signal pattern for fructose and three glucose units were readily identified. In fact, the sequential assignment was completed from the individual the 1H and 13C resonance signals (Fig. 4). Thus, it was possible to identify the compound as the trisaccharide 6'-*O*-β-fructosylmaltose [Fru-β(2→6)-Glc-α(1→4)-Glc] (Fig. 5), which was recently named neo-erlose [24]. This trisaccharide was not a good acceptor for the Xd-INV because no tetrasaccharides were observed by HPLC and HPAEC-PAD; in contrast, the

inulosucrose from *Lactobacillus gasseri* produces a mixture of maltosylfructosides with different polymerization degree, including erlose and neo-erlose [24].

When the acceptor is a reducing sugar such as maltose, most fructosylating enzymes, especially levansucrases, transfer the fructosyl moiety of sucrose to the anomeric carbon of the acceptor. Thus, levansucrase from *Microbacterium laevaniformans* transfers the fructosyl moiety of sucrose to the C1-OH position of the glucose residue of melibiose, maltose and cellobiose [29]. Recently, Tian and Karboune synthesized a variety of hetero-FOS using the levansucrase from *Bacillus amyloliquefaciens* with sucrose-maltose mixtures [10]; the major transfructosylation product was identified to be the non-reducing trisaccharide erlose [Glc-α(1→4)-Glc-α(1→2)-β-Fru]. The same product (erlose) was obtained with the levansucrase from *Bacillus subtilis* [30].

Most β-fructofuranosidases such as that from *Aspergillus niger* are only able to transfer the fructosyl moiety of sucrose to the 1-OH group of terminal fructofuranosides such as sucrose, 1-kestose, inulobiose and raffinose [31]. Muramatsu and Nakakuki reported that the good acceptors for β-fructofuranosidase from *Aspergillus sydowi* were the saccharides having a furanose ring in their chemical structure, such as D-xylose, L-arabinose and raffinose, except for melibiose and trehalose [32]. In contrast with all these enzymes, Xd-INV fructosylates specifically the glucose at the non-reducing end producing neo-erlose as the only hetero-fructooligosaccharide with maltose as acceptor. To analyze in detail the peculiar behaviour of Xd-INV, the complete protein structural

characterization (work in progress) will certainly provide light on the basis of its regiospecificity. The reaction profile of Xd-INV in the absence or presence of maltose is represented in Fig. 5. On the basis of the enzyme specificity, the proposed structures obtained with the different acceptors are included in Table 1.

3.3. Kinetics of formation of 6′-*O*-β-fructosylmaltose (neo-erlose)

The transfructosylating activity of Xd-INV towards maltose was assayed in detail during 240 min using the conditions described above. Fig. 6 shows the evolution of different sugar content in the reaction mixture. The amount of fructose detected was slightly lower than that of glucose, a finding clearly indicative of the fructosyltransferase activity of the enzyme even at moderate sucrose concentration (100 g/L). At the point of maximum neo-erlose production (240 min), the reaction mixture contained 26.8 g/L of fructose, 43.7 g/L of glucose, 11.1 g/L of sucrose, 86.2 g/L of maltose, 3 g/L of neokestose, 1.3 g/L of 1-kestose and 16.8 g/L of neo-erlose. The new linkage formed between the fructosyl unit and maltose appears to be stable under the reaction conditions, because the concentration of neo-erlose increased continuously until at least 80% sucrose conversion. This contrasts with the typical behaviour of FOS formation, whose maximum concentration is commonly achieved at lower sucrose conversions; after that, product hydrolysis becomes the major process [33;34].

3.4. Effect of donor/acceptor ratio in the synthesis of neo-erlose

In order to increase the yield of synthesized neo-erlose, several concentrations of sucrose and maltose were assayed. Lowering the molar ratio donor (sucrose)/acceptor (maltose), a significant enhancement in the concentration of neo-erlose was observed (Table 2). Thus, a 2.3-fold improvement in the synthesis of neo-erlose was obtained using a molar ratio donor/acceptor 1:3 compared with 1:1. In this situation only 5.2 g/L of neokestose plus 1-kestose were obtained, which indicates that maltose clearly competes with sucrose for the fructosyl residue. Using a molar ratio of sucrose/maltose of 3:1 the yield of neokestose plus 1-kestose was twice that of neo-erlose.

4. CONCLUSIONS

Xd-INV is able to fructosylate maltose and other carbohydrates containing a glucose moiety. Compared with other fructosyl-transferring enzymes such as levansucrases, inulosucrases and most β-fructofuranosidases, the enzyme displays high regiospecificity towards the 6-OH hydroxyl group of the glucose at the non-reducing end. No further fructosylation of the synthesized hetero-FOS was observed with this enzyme. The Xd-INV may become an appropriate tool for the single fructosylation of glucooligosaccharides, glucosides or glucoconjugates.

ACKNOWLEDGEMENTS

Projects BIO2010-20508-C04-01 and BIO2010-20508-C04-04 from Spanish Ministry of Science and Innovation supported this research. We thank Fundación Ramon Areces for an institutional grant to the Centro de Biología Molecular Severo Ochoa.

Reference List

[1] Linde D, Macias I, Fernandez-Arrojo L, Plou FJ, Fernandez-Lobato M. Molecular and biochemical characterization of a beta-fructofuranosidase from *Xanthophyllomyces dendrorhous*. Appl Environ Microbiol 2009;75:1065-1073.

[2] Linde D, Rodriguez-Colinas B, Estevez M, Poveda A, Plou FJ, Fernandez-Lobato M. Analysis of neofructooligosaccharides production mediated by the extracellular β-fructofuranosidase from *Xanthophyllomyces dendrorhous*. Bioresour Technol 2012;109:123-130.

[3] Polo A, Linde D, Estevez M, Fernandez-Lobato M, Sanz-Aparicio J. Crystallization and preliminary X-ray diffraction analysis of the fructofuranosidase from *Xanthophyllomyces dendrorhous*. Acta Crystallogr F 2010;66:1441-1444.

[4] Chen J, Chen X, Xu X, Ning Y, Jin Z, Tian Y. Biochemical characterization of an intracellular 6G-fructofuranosidase from *Xanthophyllomyces dendrorhous* and its use in production of neo-fructooligosaccharides (neo-FOSs). Bioresour Technol 2011;102:1715-1721.

[5] Sheu DC, Chang JY, Chen YJ, Lee CW. Production of high-purity neofructooligosaccharides by culture of *Xanthophyllomyces dendrorhous*. Bioresour Technol 2013;132:432-435.

[6] Hayashi S, Yoshiyama T, Fujii N, Shinohara S. Production of a novel syrup containing neofructo-oligosaccharides by the cells of *Penicillium citrinum*. Biotechnol Lett 2000;22:1465-1469.

[7] Kilian S, Kritzinger S, Rycroft C, Gibson G, du Preez J. The effects of the novel bifidogenic trisaccharide, neokestose, on the human colonic microbiota. World J Microb Biot 2002;18:637-644.

[8] Lim JS, Lee JH, Kang SW, Park SW, Kim SW. Studies on production and physical properties of neo-FOS produced by co-immobilized *Penicillium citrinum* and neo-fructosyltransferase. Eur Food Res Technol 2007;225:457-462.

[9] Fernandez-Arrojo L, Rodriguez-Colinas B, Gutierrez-Alonso P, Fernandez-Lobato M, Alcalde M, Ballesteros AO, Plou FJ. Dried alginate-entrapped enzymes (DALGEEs) and their application to the production of fructooligosaccharides. Process Biochem 2013;48:677-682.

[10] Tian F, Karboune S. Enzymatic synthesis of fructooligosaccharides by levansucrase from *Bacillus amyloliquefaciens*: Specificity, kinetics, and product characterization. J Mol Catal B-Enzym 2012;82:71-79.

[11] Rodriguez-Alegria ME, Enciso-Rodriguez A, Ortiz-Soto ME, Cassani J, Olvera C, Munguia AL. Fructooligosaccharide production by a truncated *Leuconostoc citreum* inulosucrase mutant. Biocatal Biotransform 2010;28:51-59.

[12] Alvaro-Benito M, de Abreu M, Fernandez-Arrojo L, Plou FJ, Jimenez-Barbero J, Ballesteros A, Polaina J, Fernandez-Lobato M. Characterization of a β-fructofuranosidase from *Schwanniomyces occidentalis* with transfructosylating activity yielding the prebiotic 6-kestose. J Biotechnol 2007;132:75-81.

[13] Kurakake M, Masumoto RYO, Maguma K, Kamata A, Saito E, Ukita N, Komaki T. Production of fructooligosaccharides by β-fructofuranosidases from *Aspergillus oryzae* KB. J Agric Food Chem 2010;58:488-492.

[14] Plou FJ, Alcalde M, Ghazi I, Fernandez-Arrojo L., Ballesteros A. Efficient fructooligosaccharide synthesis with a fructosyltransferase from *Aspergillus aculeatus*. In: Fessner WD, Anthonsen T editors. Modern Biocatalysis: Stereoselective and enviromentally friendly reactions. Weinheim, Germany: Wiley-VCH; 2009. p. 153-171.

[15] Alvaro-Benito M, Sainz-Polo MA, Gonzalez-Perez D, Gonzalez B, Plou FJ, Fernandez-Lobato M, Sanz-Aparicio J. Structural and kinetic insights reveal that the amino acid pair Gln-228/Asn-254 modulates the transfructosylating specificity of *Schwanniomyces occidentalis* β-fructofuranosidase, an enzyme that produces prebiotics. J Biol Chem 2012;287:19674-19686.

[16] Roberfroid M. Prebiotics: The concept revisited. J Nutr 2007;137:830S-837S.

[17] Shibata R, Kimura M, Takahashi H, Mikami K, Aiba Y, Takeda H, Koga Y. Clinical effects of kestose, a prebiotic oligosaccharide, on the treatment of atopic dermatitis in infants. Clin Exp Allergy 2009;39:1397-1403.

[18] Kim KH, Chung CB, Kim YH, Kim KS, Han CS, Kim CH. Cosmeceutical properties of levan produced by *Zymomonas mobilis*. J Cosmet Sci 2005;56:395-406.

[19] Han WC, Byun SH, Kim MH, Eun HS, Jung DL, Byung HU, Chul HK, Soon AK, Jang KH. Production of lactosucrose from sucrose and lactose by a levansucrase from *Zymomonas mobilis*. J Microbiol Biotechnol 2009;19:1153-1160.

[20] Diez-Municio M, Herrero M, Jimeno ML, Olano A, Moreno FJ. Efficient synthesis and characterization of lactulosucrose by *Leuconostoc mesenteroides* B-512F dextransucrase. J Agric Food Chem 2012;60:10564-10571.

[21] Rastall RA. Bacteria in the gut: Friends and foes and how to alter the balance. J Nutr 2004;134:2022S-2026S.

[22] Biton J, Michel JM, Le Beller D, Pelenc V, Paul F, Monsan PF, Gellf G. Enzymatic synthesis of low-calorie sugar substitutes- Cellobiofructose and gentiobiofructose. Ann N Y Acad Sci 1995;750:321-324.

[23] Nam SW, Yun HJ, Ahn JH, Kim KH, Kim BW. Production of fructosylxyloside by the new fructosyltransferase from *Bacillus macerans* EG-6. Biotechnol Lett 2000;22:1243-1246.

[24] Diez-Municio M, De las Rivas B, Jimeno ML, Muñoz R, Moreno FJ, Herrero M. Enzymatic synthesis and characterization of fructo-oligosaccharides and novel maltosyl-fructosides by inulosucrase from *Lactobacillus gasseri* DSM 20604. Appl Environ Microbiol 2013;79:4129-4140.

[25] Sumner JB, Howell SF. A method for determination of saccharase activity. J Biol Chem 1935;108:51-54.

[26] Ghazi I, Gomez de Segura A, Fernandez-Arrojo L, Alcalde M, Yates M, Rojas-Cervantes ML, Plou FJ, Ballesteros A. Immobilisation of fructosyltransferase from *Aspergillus aculeatus* on epoxy-activated Sepabeads EC for the synthesis of fructo-oligosaccharides. J Mol Catal B-Enzym 2005;35:19-27.

[27] Martin MT, Cruces MA, Alcalde M, Plou FJ, Bernabe M, Ballesteros A. Synthesis of maltooligosyl fructofuranosides catalyzed by immobilized cyclodextrin glucosyltransferase using starch as donor. Tetrahedron 2004;60:529-534.

[28] Sanz ML, Cote GL, Gibson GR, Rastall RA. Prebiotic properties of alternansucrase maltose-acceptor oligosaccharides. J Agric Food Chem 2005;53:5911-5916.

[29] Park HE, Park NH, Kim MJ, Lee TH, Lee HG, Yang JY, Cha J. Enzymatic synthesis of fructosyl oligosaccharides by levansucrase from *Microbacterium laevaniformans* ATCC 15953. Enz Microb Technol 2003;32:820-827.

[30] Canedo M, Jimenez-Estrada M, Cassani J, Lopez-Munguia A. Production of maltosylfructose (erlose) with levansucrase from *Bacillus subtilis*. Biocatal Biotransform1999;16:475-485.

[31] Hirayama M, Sumi N, Hidaka H. Purification and properties of a fructooligosaccharide-producing beta-fructofuranosidase from *Aspergillus niger* ATCC-20611. Agric Biol Chem 1989;53:667-673.

[32] Muramatsu M, Nakakuki T. Enzymatic synthesis of novel fructosyl and oligofructosyl trehaloses by *Aspergillus sydowi* beta-fructofuranosidase. Biosci Biotech Bioch 1995;59:208-212.

[33] De Abreu M, Alvaro-Benito M, Plou FJ, Fernandez-Lobato M, Alcalde M. Screening β-fructofuranosidases mutant libraries to enhance the transglycosylation yield of β-(2→6) fructooligosacharides. Comb Chem High T Scr 2011;14:730-738.

[34] Gutierrez-Alonso P, Fernandez-Arrojo L, Plou FJ, Fernandez-Lobato M. Biochemical characterization of a beta-fructofuranosidase from *Rhodotorula dairenensis* with transfructosylating activity. FEMS Yeast Res 2009;9:768-773.

Figure legends

Fig. 1. HPLC chromatograms of the reaction of sucrose with Xd-INV using different acceptors. The chromatograms correspond to the reaction mixture after 240 min. Experimental conditions: 100 g/L sucrose, 100 g/L acceptor, 3 U/mL, 100 mM sodium acetate buffer (pH 5.6), 60°C. Peak assignation: (***1***) fructose; (***2***) glucose; (***3***) sucrose; (***4***) isomaltulose plus sucrose; (***5***) maltose; (***6***) trehalose; (7) neokestose; (*8*) 1-kestose; (***9***) fructosyl-isomaltulose plus 1-kestose; (***10***) fructosyl-maltose; (***11***) fructosyl-trehalose.

Fig. 2. **Formation of acceptor products *vs.* FOS.** The percentage (w/w) of acceptor products (black bars) and neokestose plus 1-kestose (grey bars) refers to the total amount of sugars in the mixture. Experimental conditions: 100 g/L sucrose, 100 g/L acceptor, 3 U/mL, 100 mM sodium acetate buffer (pH 5.6), 60°C. Standard deviations were lower than 5%.

Fig. 3. HPAEC-PAD analysis of the reaction with maltose as acceptor. **(A)** Chromatogram of the reaction mixture with Xd-INV; **(B)** Chromatogram of the purified fructosyl-maltose. Peak assignation: (***1***) fructose; (***2***) glucose; (***3***) sucrose; (***5***) maltose; (7) neokestose; (*8*) 1-kestose; (***10***) fructosyl-maltose; (*) unknown compound.

Fig. 4. 2D-NMR DEPT-HSQC spectrum of the trisaccharide Fru-β(2→6)-Glc-α(1→4)-Glc (neo-erlose). The signals are assigned and labelled. The key points for identifications are also shown.

Fig. 5. **Scheme of the reactions catalyzed by Xd-INV.** (***1***) fructose; (***2***) glucose; (***3***) sucrose; (***5***) maltose; (7) neokestose; (***8***) 1-kestose; (***10***) neo-erlose; (***12***) neonystose.

Fig. 6. Kinetics of neo-erlose and FOS synthesis. Conditions: 100 g/L sucrose, 100 g/L maltose, 3 U/mL of Xd-INV, 100 mM sodium acetate buffer (pH 5.6), 60°C. Standard deviations were lower than 5%.

Table 1. Acceptor specificity of Xd-INV.

Acceptor	**Acceptor efficiency** [a]	**Synthesized product**
Isomaltulose	5.2	Fru-β(2→6)-Glc-α(1→6)-Fru [b]
Maltotetraose	2.6	Fru-β(2→6)-[Glc-α(1→4)]$_3$-Glc [b]
Maltotriose	2.4	Fru-β(2→6)-[Glc-α(1→4)]$_2$-Glc [b]
Maltose	1.8	Fru-β(2→6)-Glc-α(1→4)-Glc
Isomaltose	1.2	Fru-β(2→6)-Glc-α(1→6)-Glc [b]
Raffinose	0.8	Fru-β(2→6)-Gal-α(1→6)-Glc-α(1→2)-β-Fru [b]
Trehalose	0.4	Fru-β(2→6)-Glc-α(1→1)-α-Glc [b]

[a] Defined as the ratio between the areas of the main acceptor product and the formed FOS (neokestose plus 1-kestose). Standard deviations were lower than 5%.

[b] Proposed structure based on the specificity of this enzyme.

Table 2. Effect of the ratio sucrose/maltose in the synthesis of neo-erlose and FOS catalyzed by Xd-INV. [a]

[Sucrose] (g/L)	**[Maltose] (g/L)**	**[Neo-erlose] [b] (g/L)**	**[Neokestose + 1-Kestose] [b] (g/L)**
100	100	16.8	9.1
100	300	38.3	5.2
300	100	8.9	17.5

[a] Conditions: 3 U/mL, 100 mM sodium acetate buffer (pH 5.6), 60°C.

[b] The concentration of the products corresponds to 240 min reaction. Standard deviations were lower than 5%.

Fig. 1

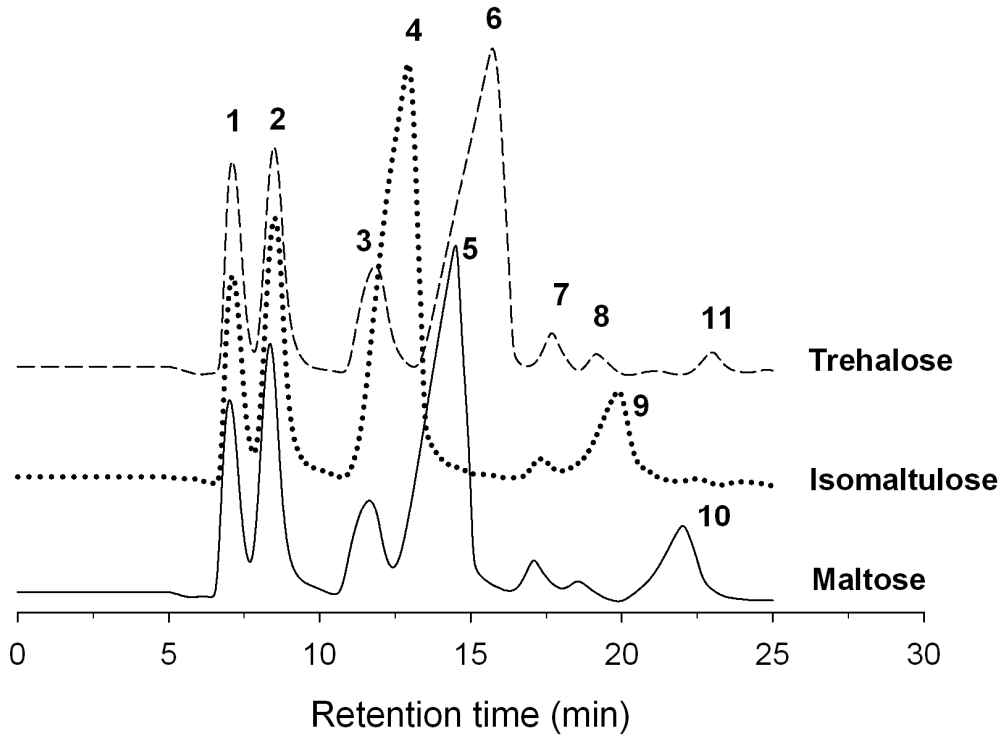

Fig. 2

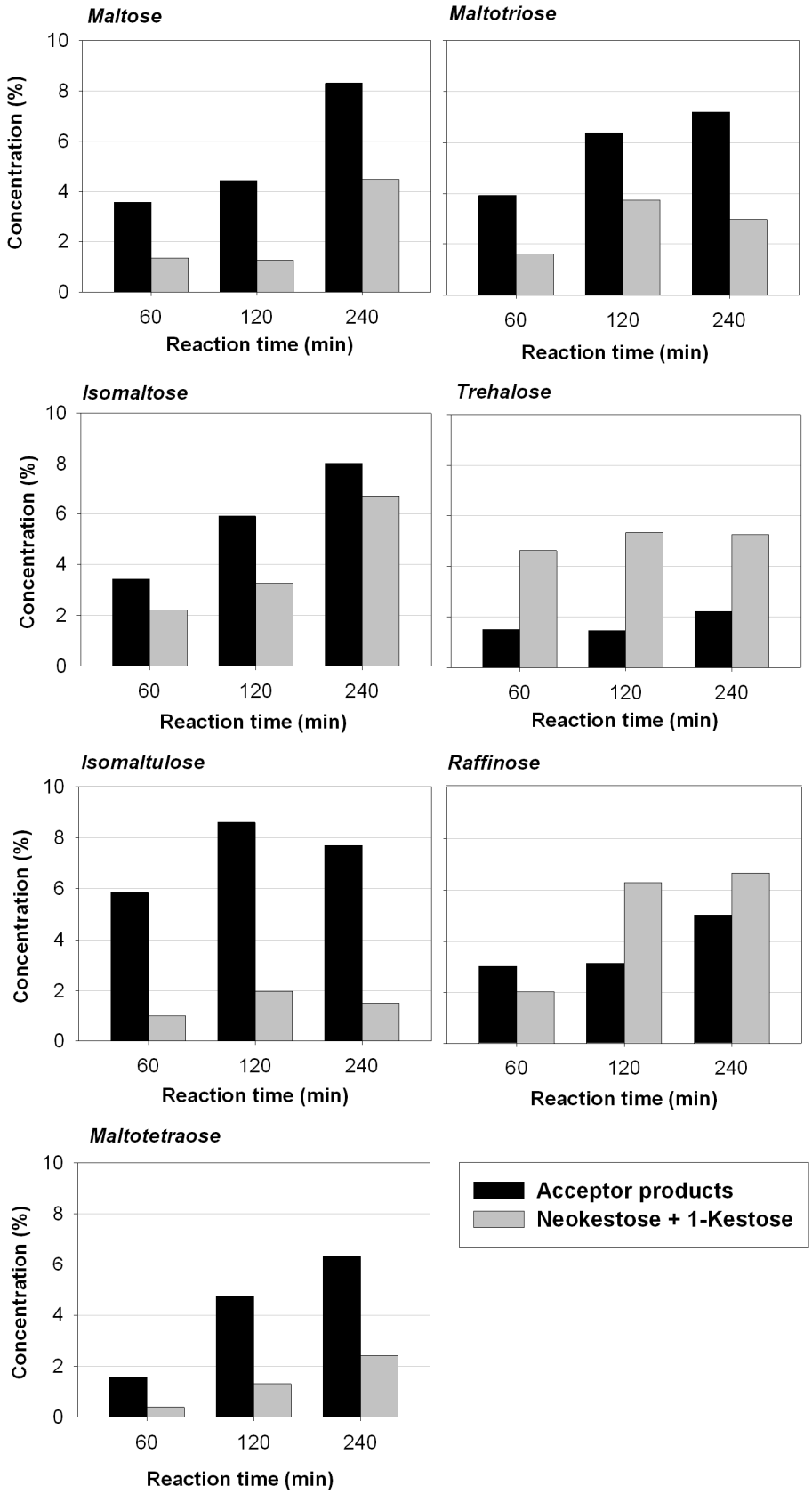

Fig. 3

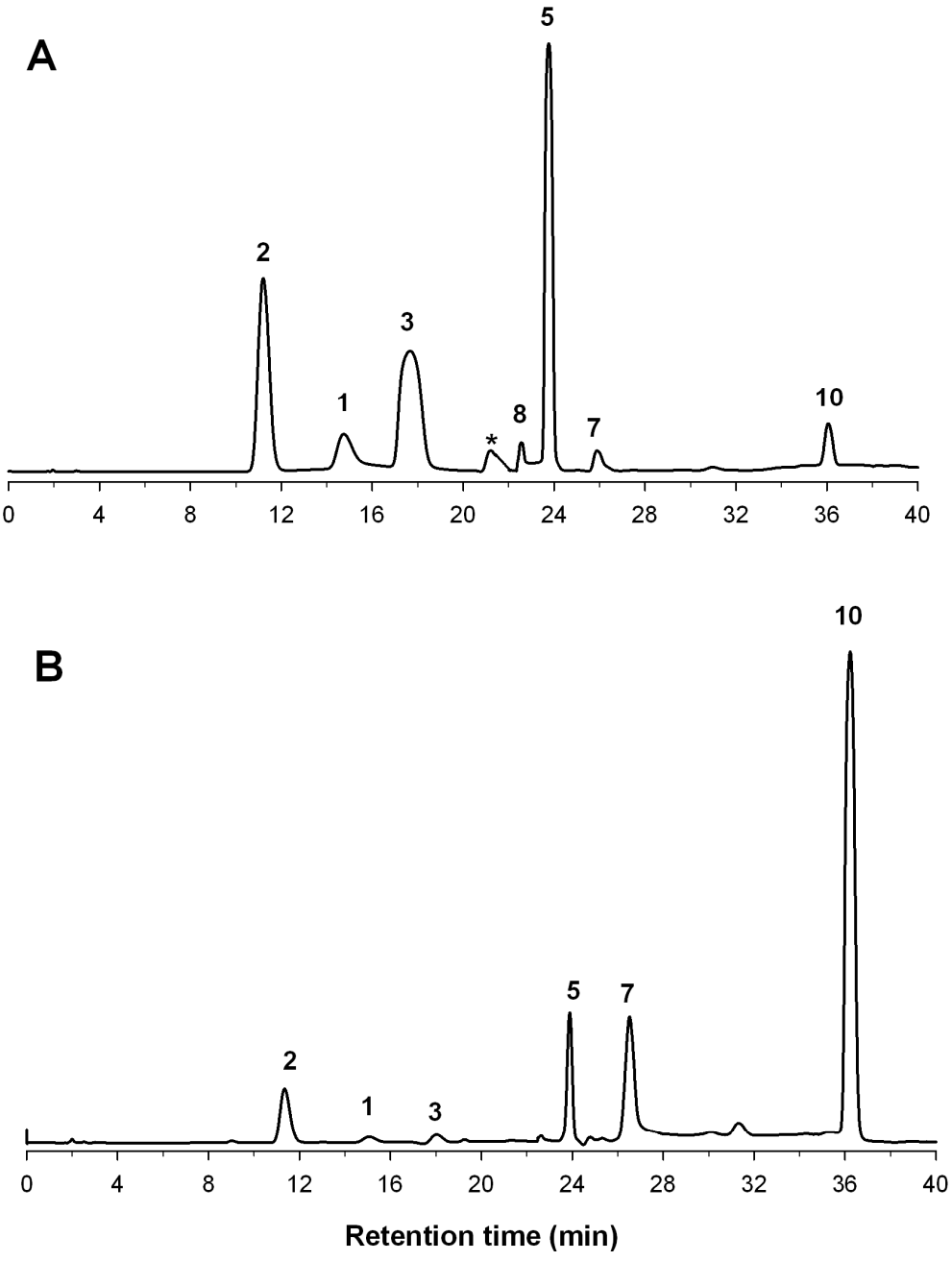

Fig. 4

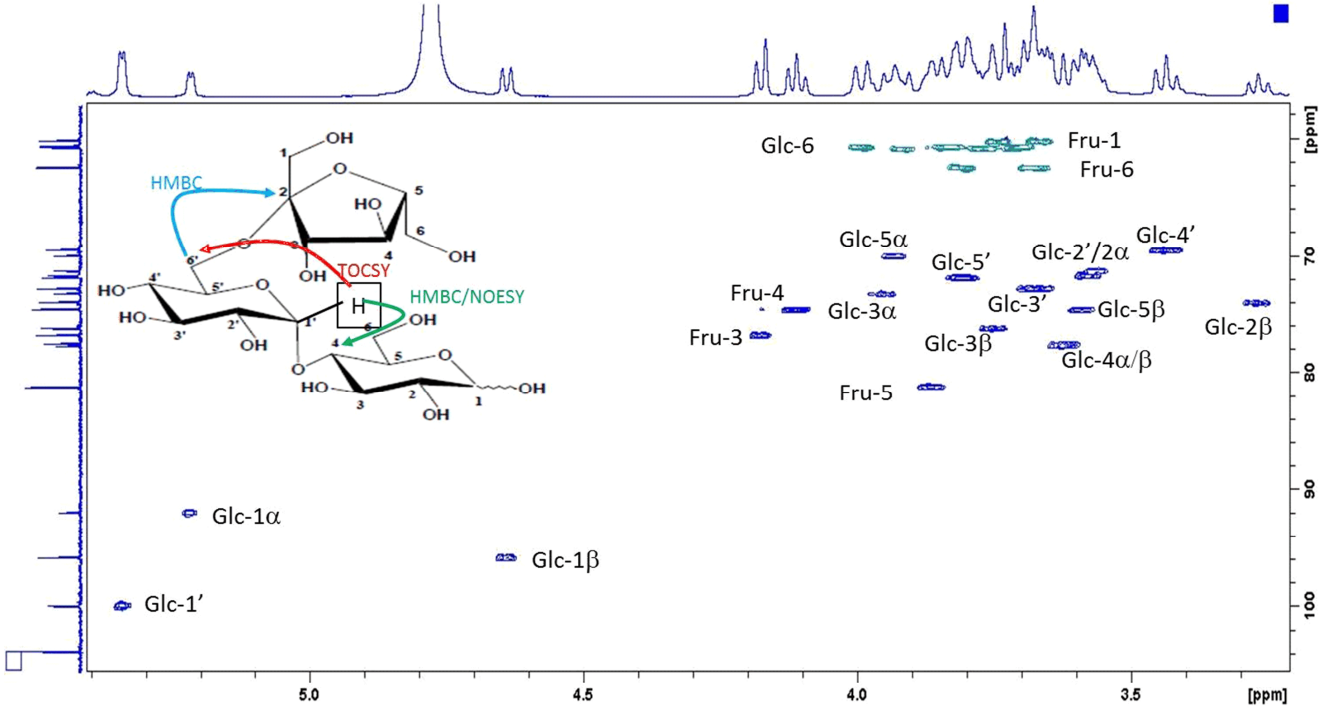

Fig. 5

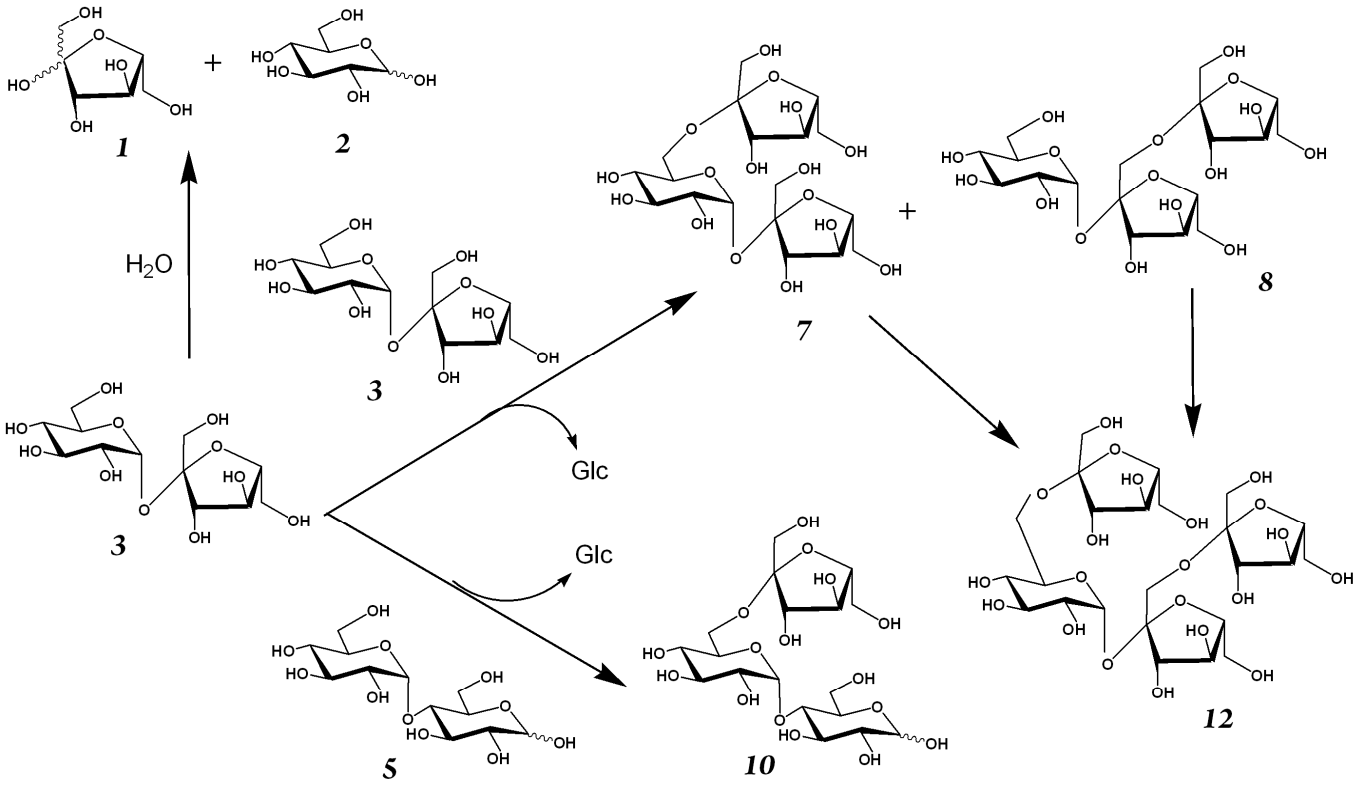

Fig. 6