Synthesis and structural characterization of raffinosyl-oligofructosides upon transfructosylation by *Lactobacillus gasseri* DSM 20604 inulosucrase

Marina Díez-Municio[a], Miguel Herrero[a], Blanca de las Rivas[b], Rosario Muñoz[b], M. Luisa Jimeno[c], and F. Javier Moreno[a*]

(a) Instituto de Investigación en Ciencias de la Alimentación, CIAL (CSIC-UAM), CEI (UAM+CSIC), C/ Nicolás Cabrera 9, 28049 Madrid, Spain.

(b) Instituto de Ciencia y Tecnología de Alimentos y Nutrición, ICTAN (CSIC), C/ Juan de la Cierva 3, 28006 Madrid, Spain.

(c) Centro Química Orgánica "Lora-Tamayo" (CSIC), C/ Juan de la Cierva 3, 28006 Madrid, Spain.

* Corresponding author: Tel.: +34 91 0017948; E-mail address: javier.moreno@csic.es

16 ***Abstract***

17 A new process based on enzymatic synthesis of a series of raffinose-derived
18 oligosaccharides or raffinosyl-oligofructosides (RFOS) with degree of polymerization
19 (DP) from 4 to 8 was developed in the presence of raffinose. This process involves a
20 transfructosylation reaction catalyzed by an inulosucrase from *Lactobacillus gasseri*
21 DSM 20604 (IS). The main synthesized RFOS were structurally characterized by
22 Nuclear Magnetic Resonance (NMR). According to the elucidated structures, RFOS
23 consist of β-2,1-linked fructose unit(s) to raffinose: α-D-galactopyranosyl-(1→6)-α-D-
24 glucopyranosyl-(1↔2)-β-D-fructofuranosyl-((1←2)-β-D-fructofuranoside)n (where *n*
25 refers to the number of transferred fructose moieties). The maximum yield of RFOS
26 was 33.4% (in weight respect to the initial amount of raffinose) and was obtained at the
27 time interval of 8-24 h of transfructosylation reaction initiated with 50% (*w/v*) of
28 raffinose. Results revealed the high acceptor and donor affinity of IS towards raffinose,
29 being fairly comparable to that of sucrose for the production of fructooligosaccharides
30 (FOS), including when both carbohydrates coexisted (sucrose:raffinose mixture, 250 g
31 L^{-1} each). The production of RFOS was also attempted in the presence of
32 sucrose:melibiose mixtures; in this case, the predominant acceptor-product formed was
33 raffinose followed by a minor production of a series of oligosaccharides with varying
34 DP. The easiness of RFOS synthesis and the structural similarities with both raffinose
35 and fructan series of oligosaccharides warrant the further study of the potential bioactive
36 properties of these unexplored oligosaccharides.

37

38 ***Keywords:*** raffinose, transfructosylation reaction, α-galactosides, inulosucrase,
39 bioactive oligosaccharides.

40 ***Introduction***

41 Oligosaccharides belonging to raffinose family (also known as α-galactosides)
42 and β-fructans are the two most widespread water-soluble carbohydrates in the plant
43 kingdom (Martínez-Villaluenga et al. 2008; Van den Ende 2013). They can be
44 enzymatically biosynthesized by transferring successive galactosyl- or fructosyl-
45 residues, respectively, from donor to acceptor sucrose (Martínez-Villaluenga and Frias,
46 2014). During the last decades, these types of oligosaccharides have attracted
47 considerable interest due to their health-promoting effects on gastrointestinal and
48 immune systems, as well as on mineral absorption, lipid metabolism, oxidative stress or
49 glucose homeostasis, among others (Di Bartolomeo et al. 2013; Zhang et al. 2013;
50 Martínez-Villaluenga and Frias, 2014). Based on the link between the molecular
51 structure and the physiological effects exerted by oligosaccharides, increasing attention
52 is being paid to longer and branched fructan and raffinose series, since they might
53 provide healthier effects throughout the whole colon due to their prebiotic properties
54 (Van den Ende 2013). In this context, the development of novel and/or tailor-made
55 oligosaccharides through enzymatic processes is of great interest because enzyme
56 substrate-, regio- and stereospecificity may provide structurally controlled
57 oligosaccharides with high yields (Díez-Municio et al. 2014; Ortíz-Soto and Seibel,
58 2014).

59 Several previous works have described the synthesis of novel raffinose-derived
60 oligosaccharides through the action of, mainly, transglycosidases by using raffinose (α-
61 D-galactopyranosyl-(1→6)-α-D-glucopyranosyl-(1↔2)-β-D-fructofuranoside) as
62 acceptor and sucrose as donor. Côté et al. (2009) made use of the advantages of an
63 alternansucrase [EC 2.4.1.140] from *Leuconostoc mesenteroides* NRRL B-21297 to
64 transglucosylate raffinose which led to a series of glucosylated-raffinose

65 oligosaccharides with degrees of polymerization (DP) from 4 to 10. Later on, these
66 raffinose-derived oligosaccharides were shown to exert *in vitro* prebiotic properties
67 (Hernández-Hernández et al. 2011). In contrast, raffinose was shown to be a poor
68 acceptor for microbial dextransucrase [EC 2.4.1.5] (Côté et al. 2009) or β-
69 fructofuranosidase (Gimeno-Pérez et al. 2014) given the low yield production of the
70 corresponding glucosylated or fructosylated tetrasaccharides. Uhm et al. (1999) reported
71 the limited production of a fructosylated tetrasaccharide (12.9 mol %) and an
72 unidentified pentasaccharide (1.6 mol %) from raffinose using a fructosyltransferase
73 from *Aspergillus niger.* Furthermore, different microbial levansucrases [EC 2.4.1.10]
74 have been also employed to use raffinose both as donor and acceptor of fructosyl
75 moieties to synthesize mostly polymers of the levan type, with the absence or with a
76 minor amount of oligosaccharides (Hestrin et al. 1956; Park et al. 2003; Andersone et
77 al. 2004; van Hijum et al. 2004). In contrast, Visnapuu et al. (2009; 2011) carried out
78 the synthesis of oligosaccharides derived from raffinose with DP up to 6 by using
79 levansucrases from *Pseudomonas syringae* and *Pseudomonas chlororaphis* subsp.
80 *aurantiaca*, although they were not quantified and the type of glycosidic linkage was
81 not elucidated. The tetrasaccharide stachyose (galactosyl-raffinose) has been also used
82 as an efficient precursor to form fructosylated oligosaccharides up to DP 8 using a
83 commercial enzymatic preparation from *Aspergillus aculeatus* (Montilla et al. 2009;
84 2011).

85 More recently, we have described the ability of a recombinant inulosucrase [EC
86 2.4.1.9] from *Lactobacillus gasseri* DSM 20604 to efficiently synthesize novel
87 oligosaccharides, termed maltosylfructosides, by the transfer of the fructosyl moiety
88 from sucrose toward maltose (Díez-Municio et al. 2013). The acceptor promiscuity of
89 this recombinant enzyme is reinforced by its capacity to not only produce

90 fructooligosaccharides (FOS) from sucrose, but also to convert raffinose into a range of
91 oligosaccharides as previously shown by Anwar et al. (2010). Nevertheless, these
92 raffinose-derived oligosaccharides were neither quantified nor structurally characterized
93 and the synthesis was carried out at a single concentration of raffinose.

94 In the present work, the optimization of a novel enzymatic synthesis process of
95 raffinosyl-oligofructosides (RFOS) by transfructosylation reaction using the
96 recombinant inulosucrase from *Lactobacillus gasseri* DSM 20604 (IS) is addressed for
97 the first time. The comprehensive structural characterization of the main different
98 products obtained has been performed by nuclear magnetic resonance (NMR).
99 According to the elucidated structures, the produced raffinose-derived oligosaccharides
100 could possess potential bioactive properties. Moreover, the optimized synthesis of the
101 well-known prebiotic FOS is also described for comparison.

102

103 ***Materials and methods***

104 *Carbohydrates and chemicals*

105 Fructose, glucose, sucrose, melibiose and raffinose were purchased from Sigma-
106 Aldrich (Steinheim, Germany) and 1-kestose, nystose and 1^F-fructofuranosylnystose
107 from Wako Pure Chemical Industries (Osaka, Japan). Acetonitrile (HPLC grade) was
108 obtained from VWR (Barcelona, Spain). All other reagents were of analytical grade and
109 commercially available.

110

111 *Production, purification and characterization of recombinant inulosucrase (IS)*

112 A fragment of the recombinant IS protein lacking the cell-anchoring-motif from
113 *L. gasseri* DSM 20604 (Anwar et al. 2010) was overproduced in *Escherichia coli* and
114 purified as previously described by Díez-Municio et al. (2013).

115 The protein concentration of the purified IS was 16.2 mg mL^{-1} according to the
116 bicinchoninic acid (BCA) assay using as standard a dextransucrase of *Leuconostoc*
117 *mesenteroides* B-512F purchased from CRITT Bio-Industries (Toulouse, France).

118 The enzyme activities of IS were measured as a function of the amounts of
119 glucose and fructose released from a solution of sucrose (100 g L^{-1}) as described by
120 Anwar et al. (2010). The total activity of IS was expressed as the amount of free glucose
121 while the amount of formed fructose was measured for the determination of the
122 hydrolytic activity. The transfructosylation activity (transferred fructose) was defined as
123 the difference between the amount of released glucose and fructose. In consequence, the
124 IS expressed a total activity of 17.4 units per milligram (U mg^{-1}), where 1 unit is
125 defined as the amount of enzyme releasing 1 μmol of glucose per minute under the
126 assayed conditions. The hydrolytic activity was 6.9 U mg^{-1}, where 1 unit is defined as
127 the amount of enzyme releasing 1 μmol of fructose per minute under the assayed
128 conditions. Finally, the transfructosylation activity was 10.5 U mg^{-1}, where 1 unit is
129 defined as the amount of enzyme required to transfer 1 μmol of fructose per minute at
130 other molecules under the assayed conditions. Enzyme activity measurements were
131 repeated three times, and the experimental error was $< 5\%$.
132

133 *Enzymatic synthesis of raffinosyl-oligofructosides (RFOS)*

134 The production of RFOS and fructooligosaccharides (FOS) through
135 transfructosylation reactions catalyzed by IS was carried out using raffinose and sucrose
136 as substrates, respectively. The reaction conditions were previously established for the
137 synthesis of maltosyl-fructosides (Díez-Municio et al. 2013) using an enzyme
138 concentration of 1.6 U mL^{-1}, at pH 5.2 (25 mM sodium acetate buffer, supplemented
139 with 1 mM $CaCl_2$) and 55ºC as reaction temperature. Three different concentrations of

140 starting sucrose or raffinose (both donor and acceptor of fructose moieties) were
141 studied: 25, 50 and 65 g in 100 mL. Moreover, the production of FOS and RFOS was
142 studied using reaction mixtures consisting in sucrose and raffinose (25:25, expressed in
143 g 100 mL^{-1}) or sucrose and melibiose (25:25, expressed in g 100 mL^{-1}). To facilitate
144 the complete solubilization of the starting substrates, all assayed carbohydrate solutions
145 were preheated up to 65-70 ºC before addition of the enzyme.

146 Samples were incubated in individual tubes of 1.5 mL in an orbital shaker at
147 1,000 rpm. The enzymatic reactions were allowed to proceed up to 48 h. Aliquots were
148 taken from the reaction mixture at suitable time intervals (1, 3, 8, 24, 32 and 48 h). The
149 enzyme was inactivated by heating at 100 ºC for 5 min and inactivated samples were
150 then diluted with acetonitrile:water (40:60, *v/v*), filtered using a 0.45 µm syringe filter
151 (Symta, Madrid, Spain), and analyzed by LC-RID. Results are shown as mean ± sd of
152 triplicate assays.

153

154 *Chromatographic determination of carbohydrates by Liquid Chromatography with*
155 *Refractive Index Detector (LC-RID).*

156 Enzymatic reactions were monitored by LC-RID on an Agilent Technologies
157 1220 Infinity LC System – 1260 RID (Boeblingen, Germany). The separation of the
158 synthesized oligosaccharides was carried out on a Kromasil (100-NH_2) column (250 x
159 4.6 mm, 5 µm particle size) (Akzo Nobel, Brewster, NY, USA) using acetonitrile:water
160 (70:30, *v/v*) as the mobile phase and eluted in isocratic mode at a flow rate of 1.0 mL
161 min^{-1} for 80 min. Injection volume was 50 µL (1 mg of total carbohydrates). Data
162 acquisition and processing were performed using Agilent ChemStation software
163 (Agilent Technologies, Boeblingen, Germany).

164 Main carbohydrates in the reaction mixtures were initially identified by
165 comparing the retention times (t_R) with those of commercially available standards.
166 Quantitative analysis was performed by the external standard method, using calibration
167 curves in the range 0.01 - 10 mg for glucose (quantification of monosaccharides),
168 sucrose and melibiose (quantification of disaccharides), raffinose (quantification of
169 trisaccharides), nystose (quantification of tetrasaccharides) and 1^F-
170 fructofuranosylnystose (quantification of pentasaccharides and acceptor products of
171 polymerization degree above 5). All analyses were carried out in triplicate.
172 Determination coefficients obtained from these calibration curves, which were linear
173 over the range studied, were always $R^2 > 0.999$. Reproducibility of the method was
174 estimated on the basis of the intra-day and inter-day precision, calculated as the relative
175 standard deviation (RSD) of concentrations of oligosaccharide standards obtained in $n \geq$
176 5 independent measurements, obtaining RSD values below 10% in all cases.
177

178 *Purification and structural characterization of the raffinosyl-oligofructosides (RFOS)*
179 *by nuclear magnetic resonance (NMR)*

180 Given the lack of commercially available standards for RFOS, the main
181 synthesized oligosaccharides (DP 4-7), obtained after 24 h of transfructosylation
182 reaction from raffinose at 500 g L^{-1} under the optimized conditions, were isolated and
183 purified by preparative LC-RID as previously described (Díez-Municio et al. 2014) for
184 its subsequent characterization.

185 Structure elucidation of the purified oligosaccharides was accomplished by
186 nuclear magnetic resonance spectroscopy (NMR). NMR spectra were recorded at 298
187 K, using D_2O as solvent, on a Varian SYSTEM 500 NMR spectrometer (^{1}H 500 MHz,
188 ^{13}C 125 MHz) equipped with a 5-mm HCN cold probe. Chemical shifts of ^{1}H (δ_H) and

189 ^{13}C (δ_C) in parts per million (ppm) were determined relative to an internal standard of
190 sodium [2,2,3,3-$^{2}H_4$]-3-(trimethylsilyl)-propanoate in D_2O (δ_H 0.00) and 1,4-dioxane
191 (δ_C 67.40) in D_2O, respectively. One-dimensional (1D) NMR experiments (^{1}H, and ^{13}C)
192 were performed using standard Varian pulse sequences. Two-dimensional (2D) [^{1}H–^{1}H]
193 NMR experiments (gradient correlation spectroscopy, gCOSY; total correlation
194 spectroscopy, TOCSY; and rotating-frame Overhauser effect spectroscopy, ROESY)
195 were carried out with the following parameters: delay time of 1 s, spectral width of
196 1179.2 Hz in both dimensions, 4096 complex points in t2, 4 transients (16 for ROESY)
197 for each of 128 time increments, and a linear prediction to 256. The data were zero-
198 filled to 4096 $\times$ 4096 real points. 2D [^{1}H–^{13}C] NMR experiments [gradient
199 heteronuclear single-quantum coherence (gHSQC) and gradient heteronuclear multiple-
200 bond correlation (gHMBC)] used the same ^{1}H spectral window, a ^{13}C spectral window
201 of 30165 Hz, 1 s of relaxation delay, 1024 data points, and 128 time increments, with a
202 linear prediction to 256. The data were zero-filled to 4096 $\times$ 4096 real points. Typical
203 numbers of transients per increment were 4 and 16, respectively.
204
205 ***Results***
206 *Synthesis of RFOS by the recombinant inulosucrase from L. gasseri DSM 20604 using*
207 *raffinose as starting substrate.*
208 In addition to the reaction conditions, i.e. enzyme concentration, pH and
209 temperature, which were previously optimized to increase the
210 transfructosylation/hydrolysis ratio of IS (Díez-Municio et al. 2013), the use of high
211 substrate concentrations is another factor that influences the transferase activity of
212 transglycosidase enzymes (Canedo et al. 1999; Robyt 1995). Consequently, up to three
213 different initial concentrations of raffinose, that is 25%, 50% and 65% (*w/v*), were

214 studied for the synthesis of RFOS. However, reliable results could not be obtained at the
215 highest assayed concentration due to lack of solubility of raffinose (data not shown).

216 **Figure 1** shows the LC-RID profiles corresponding to transfructosylation
217 reaction after 24 h using 25% (*w/v*) of raffinose as starting substrate. The detection of
218 fructose (peak 1) and melibiose (α-D-galactopyranosyl-(1→6)-D-glucose, peak 3)
219 indicated that raffinose (peak 4) was efficiently cleaved by IS at the bond between
220 glucose and fructose. In addition, the detection of fructose in low levels could also
221 indicate its transfer to other raffinose molecules to give a series of oligosaccharides with
222 DP ranging from 4 to 8 (peaks 5-9 in **Figure 1**) and whose abundance decreased as the
223 oligosaccharide chain increased. This behavior is indicative of the capacity of raffinose
224 to act both as donor and acceptor in the transfructosylation reaction catalyzed by IS.
225 Finally, the detection of a minor peak (named 2) identified as inulobiose (β-D-
226 fructofuranosyl-(2→1)-D-fructose) according to data reported by Díez-Municio et al.
227 (2013), revealed the capacity of fructose to also act as a minor acceptor in the
228 transfructosylation reaction. Interestingly, when 50% (*w/v*) of raffinose was tested as
229 substrate, the chromatographic profile obtained after 24 h was essentially the same to
230 that shown in **Figure 1**, although with different yields.

231 **Tables 1** and **2** summarize the quantitative data of the carbohydrates present in
232 the reaction mixture throughout the transfructosylation process initiated with 25% (*w/v*)
233 and 50% (*w/v*) of raffinose, respectively. By using a 25% (*w/v*) concentration of
234 raffinose, the synthesis of total RFOS was increased during the first 8 h of reaction and
235 then, achieved a plateau from 8 to 24 h, followed by a decrease until the end of the
236 reaction (48 h) (**Table 1**). Under these conditions, the maximum production of RFOS
237 was 70.7 g L^{-1} found at 24 h, which is equivalent to a yield of 29.6%, in weight with
238 respect to the initial amount of quantified raffinose (**Table 3**). Likewise, only 10% of

239 raffinose remained in the reaction mixture after 24 h of reaction. Notwithstanding, it
240 should be noted that the production of RFOS found at 8 h was 69.6 g L^{-1}. Thus, from an
241 economic point of view, it is not feasible to perform the enzymatic reaction for another
242 16 h in order to obtain just 1.1 g/L more of oligosaccharide production. However, the
243 composition of the final product in terms of DP differs as a function of the reaction
244 time. While at 8 h of reaction the major product is the RFOS with DP 4 (representing
245 58.2% of the composition), at 24 h of reaction RFOS with DP 5-7 reach their maximum
246 production (**Table 1**). This change in the composition of the DP fractions and the
247 decrease in the total content of RFOS from 32 h to the end of the reaction could be
248 explained by the fact that once raffinose is largely hydrolyzed and also used as acceptor
249 by IS, the RFOS could serve, in turn, as substrates for the enzyme. When 50% *(w/v)*
250 was set as initial concentration of raffinose, the maximum production of RFOS,
251 achieved at 24 h of reaction, was 2.5-fold higher than that obtained with 25% *(w/v)* of
252 raffinose, reaching 172.6 g L^{-1} (**Table 2**). This value is equivalent to a yield of 33.4% in
253 weight with respect to the initial amount of quantified raffinose (**Table 3**). In this case,
254 RFOS with DP 5-7 reached their maximum production also after 24 h of reaction, while
255 the maximum production of RFOS with DP 4 was obtained after 8 h of reaction (**Table**
256 **2**). As it could be expected, the higher the raffinose concentration, the higher the
257 production yield of synthesized RFOS. Despite the high level of initial concentration of
258 raffinose used for the RFOS synthesis, 82% of raffinose was hydrolyzed or converted
259 into RFOS after 24 h of reaction and only 13.6% of raffinose remained at the end of the
260 enzymatic process. Notable levels of the disaccharide melibiose (α-gal-(1→6)-α-glu)
261 were also obtained as a result from the production of oligosaccharides derived from
262 raffinose (α-gal-(1→6)-α-glu-(1↔2)-β-fru) by the transfructosidase activity of IS.
263 Although there is no extensive toxicological data available for melibiose, it is supposed

264 to be safe for oral consumption because it can be found in a wide variety of foods
265 (Lakio et al. 2013), as well as naturally in plants such as cocoa beans and processed
266 soybeans (Tomita et al. 2007). Melibiose is a disaccharide consisting of the same two
267 monosaccharides as lactose, glucose and galactose, but linked by a different glycosidic
268 bond. It has been described to be resistant to the gastrointestinal digestion (Mineo et al.
269 2002) and metabolized by the gut microbiota (Van Laere et al. 1999). Therefore, as any
270 non-digestible carbohydrate, melibiose can be considered as a low calorie ingredient.
271 Melibiose has been also described to be released when dietary raffinose is metabolized
272 by gut bacteria, suggesting that various physiological functions of raffinose might make
273 their contribution in the form of melibiose (Tomita et al. 2007). Nevertheless, to
274 increase the purity of the synthetized oligosaccharides unreacted substrates and mono-
275 /disaccharides present after enzymatic oligosaccharide formation could be removed by
276 physicochemical purification or using different fractionation processes.
277
278 *Synthesis of FOS derived from sucrose by the recombinant inulosucrase from L. gasseri*
279 *DSM 20604. A comparison with the RFOS synthesized from raffinose.*
280 Considering that sucrose is the ordinary substrate for transfructosidase enzymes,
281 we addressed the synthesis of FOS catalyzed by IS under the same reaction conditions
282 than those used for the synthesis of RFOS, in order to compare the ability of raffinose
283 and sucrose to act as substrates for this enzyme. Therefore, initial concentrations of 25%
284 and 50% (*w/v*) of sucrose were employed for the synthesis of FOS. In this case,
285 considering that sucrose is more soluble than raffinose in aqueous solutions, an
286 additional set of samples with 65% (*w/v*) of starting sucrose could be also tested.
287 Overall, FOS from DP 3 (1-kestose) to DP 9, as well as minor amounts of neo-kestose
288 and inulobiose could be detected by LC-RID (chromatograms not shown). **Figure 2**

289 illustrates the concentration of sucrose, glucose, fructose and the total FOS synthesized
290 during the transfructosylation process from the three assayed concentrations of sucrose.
291 Similarly to raffinose, the production of FOS increased with the concentration of
292 sucrose and, consequently, the maximum production of FOS was of 283.45 g L^{-1} after
293 32 h of transfructosylation reaction starting from 65% of sucrose (**Figure 2C**).
294 However, similar values in oligosaccharides production and yields were found for both
295 carbohydrates when FOS were synthesized from equivalent concentrations of sucrose to
296 those obtained for the synthesis of RFOS (i.e., 25% and 50% of sucrose). Concretely,
297 67.3 g L^{-1} and 168.8 g L^{-1} of total FOS with DP from 3 to 9 were produced from 25%
298 *(w/v)* and 50% *(w/v)* of sucrose, respectively, after 3 and 32 h of transfructosylation
299 reaction (**Figures 2A** and **2B** and **Table 3**). Therefore, these results highlight the
300 suitability of raffinose to act as substrate for the synthesis of oligosaccharides catalyzed
301 by IS, being its ability to produce acceptor products comparable to that of sucrose.

302 Nevertheless, the productivity and specific productivity values (determined after
303 the first hour of reaction) corresponding to the synthesis of FOS from 25% and 50%
304 *(w/v)* of sucrose were 1.25 and 1.56-fold higher, respectively, than those values
305 determined for RFOS synthetized from equivalent concentrations of raffinose (**Table 3**).
306 Thus, a higher initial velocity of the incorporation of fructose moieties into sucrose than
307 into raffinose is suggested, which could be attributed to the fact that sucrose has a lower
308 Michaelis-Menten constant (Km), since it is the predominant donor substrate for
309 glycansucrases.

310

311 *Synthesis of RFOS and FOS derived from sucrose:raffinose mixtures by the*
312 *recombinant inulosucrase from L. gasseri DSM 20604.*

313 Taking into account the appropriateness of both series of oligosaccharides, the
314 production of FOS and RFOS was also explored in the presence of a sucrose:raffinose
315 mixture (25:25, expressed in g 100 mL^{-1}). As it is shown in **Figure 3**, a decrease of
316 sucrose and raffinose with a concomitant synthesis of a mixture of FOS (DP from 3 to
317 8) and RFOS (DP from 4 to 8) was observed from the first hour of reaction, suggesting
318 the ability of the enzyme to interchangeably use both substrates as acceptor and donor.
319 Likewise, the levels of fructose were substantially lower than those of melibiose and
320 glucose, indicating the predominance of the transfructosylation reaction. The maximum
321 level of production of combined transfer products (considering the sum of FOS and
322 RFOS) was 180.6 g L^{-1} obtained after 24 hours of reaction, equivalent to a yield of
323 33.8% (**Table 3**). These values were fairly similar or slightly higher than those found
324 for single synthesis of FOS or RFOS from 50% *(w/v)* of sucrose or raffinose. In
325 addition, by comparing the quantitative data with those obtained with 25% *(w/v)* of
326 either raffinose or sucrose separately (**Table 1** and **Figure 2A**), the highest levels of
327 RFOS synthesized with the starting reaction mixture of sucrose and raffinose were
328 fairly similar (74.4 g L^{-1}) whereas FOS were produced in an even higher yield (106.2 g
329 L^{-1}) (**Figure 3**). Tian and Karboune (2012) also observed a higher production of FOS by
330 a levansucrase from *Bacillus amyloliquefaciens* in the presence of raffinose and sucrose
331 as compared to the use of sucrose alone.
332 Interestingly, productivity and specific productivity values calculated from the
333 starting reaction mixture of sucrose and raffinose (25% of each, *w/v*) were below the
334 values obtained after the single synthesis of FOS from sucrose at 50% *(w/v)*. However,
335 these values were above those produced with the individual synthesis of RFOS from
336 raffinose at 50% *(w/v)* (**Table 3**), confirming the previous finding about the velocity of
337 the incorporation of fructose moieties into sucrose and raffinose.

338

339 *Synthesis of raffinose, RFOS and FOS derived from sucrose:melibiose mixtures by the*

340 *recombinant inulosucrase from L. gasseri DSM 20604.*

341 The ability of IS to produce raffinose and, specially, RFOS from mixtures of

342 sucrose (donor) and melibiose (acceptor) (25:25, expressed in g 100 mL^{-1}) was also

343 tested. This study was based on previous findings about the capacity of this enzyme to

344 specifically transfer fructose moieties of sucrose to either C-1 of the reducing end or C-

345 6 of the nonreducing end of maltose, to mainly produce the trisaccharide erlose [α-D-

346 glucopyranosyl-(1→4)-α-D-glucopyranosyl-(1↔2)-β-D-fructofuranoside] followed by

347 neo-erlose [β-D-fructofuranosyl-(2→6)-α-D-glucopyranosyl-(1→4)-α-D-

348 glucopyranose] and oligosaccharides of higher DP by elongation of the saccharide chain

349 from both glucose units with successive fructosyl units (Díez-Municio et al. 2013). In

350 our case, melibiose was a relatively good acceptor-substrate since 59% of the starting

351 amount was used as acceptor after 8 h of reaction, although the main acceptor-product

352 was raffinose, whose maximum production was 167.3 g L^{-1} after 8 h. In contrast, the

353 total RFOS yield (DP from 4 to 7) was low (42.6 g L^{-1} were obtained after 48 h of

354 reaction). In addition to RFOS, 69.6 g L^{-1} of total FOS (DP from 3 to 8) were also

355 produced due to the presence of sucrose.

356

357 *Structural elucidation of raffinosyl-oligofructosides by nuclear magnetic resonance*

358 *(NMR).*

359 The four main unknown chromatographic peaks (5-8, **Figure 1**) were purified by

360 preparative LC-RID and successfully characterized by NMR (structures **A - D**,

361 respectively, **Figure 4**) by the combined use of 1D and 2D [^{1}H–^{1}H] and [^{1}H–^{13}C] NMR

362 experiments (gCOSY, TOCSY, multiplicity-edited gHSQC and gHMBC). Determined

363 ^{1}H and ^{13}C NMR chemical shifts are summarized in **Table 4**. The full set of spectra is
364 available in the Supporting Information (Figures S1-S17).
365 The main synthesized RFOS (peak 5, **Figure 1**) was the structure **A**. The 1D ^{1}H
366 NMR spectrum of **A** showed two resonances in the anomeric region (δ5.30, and δ4.85),
367 and 1D ^{13}C NMR spectrum showed signals corresponding to 24 carbons including four
368 anomeric carbons (δ106.59 , δ106.20, δ101.31 and δ95.25), indicative of the presence of
369 a tetrasaccharide with four hexose sugars in the structure. A multiplicity-edited gHSQC
370 spectrum was used to link the carbon signals to the corresponding proton resonances.
371 Thus, the anomeric carbon at δ101.31 correlated with an alpha anomeric proton at δ4.85
372 (J(H1,H2) = 3.7 Hz) and the anomeric carbon at δ95.25 correlated with an alpha
373 anomeric proton at δ5.30 (J(H1,H2) = 3.9 Hz). The anomeric carbons at δ106.59 and
374 δ106.20 were quaternary carbons. In addition, six methylene carbons at δ68.65, δ65.20,
375 δ65.14, δ63.97, δ63.80 and δ63.27 were identified. The ^{1}H–^{1}H COSY and ^{1}H–^{1}H
376 TOCSY experiments revealed the ^{1}H signals of galactopyranose, glucopyranose and
377 fructofuranose residues (**Figure 4**). The ^{1}H–^{1}H ROESY experiment showed correlations
378 between the H2 and H1 methylene protons for the two fructose units. From these data it
379 could be concluded that the tetrasaccharide consisted of a unit of α-galactopyranose, a
380 unit of α-glucopyranose, and two units of β-fructofuranose.
381 The position of glycosidic linkages was analyzed as follows: gHMBC showed
382 correlations between the α-Gal-C1 anomeric carbon (101.31 ppm) and α-Glu-H6
383 methylene protons (3.90, 3.54 ppm), between the α-Glu-H1 anomeric proton (5.30 ppm)
384 and one of the β-Fru anomeric carbons (106.20 ppm), and between the β-Fru-H1
385 methylene protons (δ3.67 and δ3.62) and the other β-Fru anomeric carbon (106.59ppm).
386 Consequently, the main synthesized RFOS (peak 5, **Figure 1**) was identified as the
387 tetrasaccharide α-D-galactopyranosyl-(1→6)-α-D-glucopyranosyl-(1↔2)-β-D-

fructofuranosyl-(1←2)-β-D-fructofuranoside which can be named as fructosyl-raffinose (**Figure 4A**). The remaining structures (peaks 6-8, **Figure 1**) were identified, following the same procedure, as fructosylated-raffinose oligosaccharides with DP 5, 6, and 7, respectively. The same relevant NMR correlations were found for these compounds, in consequence, the structure of peak 6 (**Figure 1**) was elucidated as α-D-galactopyranosyl-(1→6)-α-D-glucopyranosyl-(1↔2)-β-D-fructofuranosyl-(1←2)-β-D-fructofuranosyl-(1←2)-β-D-fructofuranoside (**Figure 4B**). Structures of peaks 7 and 8 (**Figure 1**) were elucidated as α-D-galactopyranosyl-(1→6)-α-D-glucopyranosyl-(1↔2)-β-D-fructofuranosyl-(1←2)-β-D-fructofuranosyl-(1←2)-β-D-fructofuranosyl-(1←2)-β-D-fructofuranoside (**Figure 4C**) and α-D-galactopyranosyl-(1→6)-α-D-glucopyranosyl-(1↔2)-β-D-fructofuranosyl-(1←2)-β-D-fructofuranosyl-(1←2)-β-D-fructofuranosyl-(1←2)-β-D-fructofuranosyl-(1←2)-β-D-fructofuranoside (**Figure 4D**).

Taking into account the mechanism of action described for the synthesis of compounds **A** - **D**, these results led us to tentatively determine that peak 9 (**Figure 1**) could correspond to the octasaccharide α-D-galactopyranosyl-(1→6)-α-D-glucopyranosyl-(1↔2)-β-D-fructofuranosyl-(1←2)-β-D-fructofuranosyl-(1←2)-β-D-fructofuranosyl-(1←2)-β-D-fructofuranosyl-(1←2)-β-D-fructofuranosyl-(1←2)-β-D-fructofuranoside.

Discussion

This work describes a new and feasible synthesis process of a series of raffinose-derived oligosaccharides, termed raffinosyl-oligofructosides (RFOS) with DP ranging from 4 to 8. This procedure is based on the efficient transfructoyslation of raffinose catalyzed by a recombinant inulosucrase from *L. gasseri* DSM 20604 (IS). Regardless the starting concentration of raffinose, the predominant RFOS present throughout the

413 transfructosylation reaction was that of DP 4; however, as the reaction proceeded, the
414 presence of DP 4 was proportionately lower (**Tables 1** and **2**), which could be indicative
415 of the capacity of RFOS of low DP to act in turn as acceptors for further
416 transfructosylation to yield oligosaccharides of a higher molecular weight.

417 Concerning the highest yields obtained for these novel compounds, i.e. 29.6 and
418 33.4% in weight respect to the respective initial amount of starting raffinose (25 or
419 50%, respectively, **Table 3**), they can be considered high compared to other studies that
420 used raffinose as precursor and/or addressed the synthesis of new fructosylated
421 oligosaccharides (Uhm et al. 1999; Yamamori et al. 2002; Gimeno-Pérez et al. 2014).
422 Remarkably, the yields obtained for the synthesis of RFOS in the current work were
423 higher than those obtained for the synthesis of FOS under the same reaction conditions
424 but using sucrose instead of raffinose as starting substrate (**Table 3**). Considering that
425 sucrose is the preferable substrate for transfructosidase enzymes, this finding highlights
426 the efficiency of the synthesis of RFOS. Nonetheless, the higher solubility of sucrose as
427 compared to raffinose allowed that the synthesis of FOS could be carried out from 65%
428 of sucrose, increasing, thus, the FOS yield up to 43.5%.

429 As it was elucidated by NMR, the synthesis of RFOS is produced by the
430 elongation of the raffinose chain from the fructose moiety by adding successive
431 fructosyl units through β-2,1-linkages to give oligosaccharides with the general
432 structure α-D-galactopyranosyl-(1→6)-α-D-glucopyranosyl-(1↔2)-β-D-
433 fructofuranosyl-((1←2)-β-D-fructofuranoside)$_n$. This study has also revealed the high
434 acceptor and donor affinity of IS towards raffinose, being fairly comparable to that of
435 sucrose. This observation remained when both carbohydrates coexisted, as it could be
436 deduced from the concentration and yields obtained of FOS and/or RFOS. However, the
437 productivity values of FOS were higher (i.e., 1.25 and 1.56-fold) than those obtained for

438 the synthesis of RFOS (**Table 3**). This fact can be attributed to a higher transfer rate of
439 fructose moieties into sucrose than into raffinose, supported by a lower Michaelis-
440 Menten constant (Km) of the former.

441 Raffinose has shown a different behavior for the synthesis of RFOS compared to
442 the synthesis of maltosyl-fructosides (Díez-Municio et al. 2013) or lactosyl-
443 oligofructosides (Díez-Municio et al. 2015) also catalyzed by IS from sucrose:maltose
444 or sucrose:lactosucrose reaction mixtures, respectively. In this sense, despite the fact
445 that lactosucrose (β-D-galactopyranosyl-(1→4)-α-D-glucopyranosyl-(1↔2)-β-D-
446 fructofuranoside) and raffinose present only a slight structural difference, which is the
447 type of glycosidic linkage between the galactosyl and glucosyl moieties (i.e. β-1,4 or α-
448 1,6), lactosucrose did not have the capacity to act as a donor, requiring the presence of
449 sucrose as a donor to produce fructosyl-derivatives of lactosucrose. On the contrary,
450 raffinose is able to act also as donor. This finding stresses the different flexibility of IS
451 for the donor and acceptor substrate-binding subsites. In this regard, Ozimek et al.
452 (2006) described the mode of action of bacterial fructosyltransferases, indicating that
453 the donor substrate, usually sucrose, enters the active site and occupies the -1 and +1
454 subsites (following the nomenclature proposed by Davies et al. 1997), the glycosidic
455 bond is cleaved and a covalent fructosyl-enzyme intermediate is formed at -1 whereas
456 glucose is released. Then, an acceptor substrate may enter the active site, binds to the +1
457 and +2 subsites and react with the fructosyl-enzyme intermediate at -1, resulting in the
458 oligosaccharide formation. Therefore, our data indicate the feasibility of raffinose to
459 occupy the -1 and +1 subsites to act as a donor in contrast to lactosucrose, suggesting
460 the importance of the type of glycosidic linkage that bonds the galactose unit to the
461 sucrose moiety to enter the IS donor-substrate subsite. Nevertheless, IS seems to be
462 more versatile on the acceptor specificity.

463 Concerning the glycosidic linkage specificity of this enzyme, our results
464 demonstrate that IS unambiguously transfers fructose units to melibiose and raffinose
465 with a β-2,1-bond to form raffinose (and minor amounts of RFOS) and RFOS from DP
466 4 to 8, respectively, in a similar way to lactosucrose (Díez-Municio et al. 2015).
467 However, when maltose is used as acceptor, IS was capable of transferring fructose
468 moieties through either β-2,6-linkages to the non-reducing glucose residue or β-2,1-
469 linkages to the reducing glucose unit of maltose to produce two types of maltosyl-
470 fructosides (Díez-Municio et al. 2013). Therefore, chemoselectivity and reaction
471 specificity of the IS could be determined by the type of acceptor, in good agreement
472 with previous findings observed for other microbial transglycosidases (Ortíz-Soto and
473 Seibel, 2014).
474 In conclusion, the RFOS synthesized and characterized in this work are
475 galactosylated derivatives of FOS. Therefore, they could be considered as hetero-
476 fructooligosaccharides. This group of oligosaccharides has been described to have
477 potential applications as bioactive components in the food, pharmaceutical and/or
478 cosmetic industries (Gimeno-Pérez et al. 2014). In this particular case, the easiness of
479 RFOS synthesis by using only raffinose as starting substrate, as well as the structural
480 similarities with both raffinose and fructan series of oligosaccharides, whose health-
481 beneficial effects have been largely discussed and demonstrated, makes of great interest
482 the further study of the potential bioactive properties of RFOS. Moreover, to the best of
483 our knowledge, the combined production of FOS and RFOS in the presence of a
484 sucrose:raffinose mixture has been explored for the first time in this study.
485
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490 ***Compliance with Ethical Standards***

491

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494

495 Conflict of Interest: All authors declare that they have no conflict of interest.

496

497 Ethical approval:

498 This article does not contain any studies with human participants or animals performed

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617 ***Figure captions***

618

619 **Figure 1**. LC-RID profile after 24 h of transfructosylation reaction catalyzed by the
620 recombinant inulosucrase from *Lactobacillus gasseri* DSM 20604 (IS) (1.6 U mL^{-1}) at
621 55 °C, in 25 mM sodium acetate buffer supplemented with 1 mM $CaCl_2$ (pH 5.2) using
622 250 g L^{-1} of raffinose as starting substrate. Peak identification: 1, fructose; 2,
623 inulobiose; 3, melibiose; 4, raffinose; 5-9, raffinosyl-oligofructosides (RFOS) with
624 increasing DP (from 4 to 8).

625

626 **Figure 2.** Concentrations of sucrose, glucose, fructose and total fructooligosaccharides
627 (FOS) upon transfructosylation reaction catalyzed by the recombinant inulosucrase from
628 *Lactobacillus gasseri* DSM 20604 (IS) (1.6 U mL^{-1}) at 55 °C, in 25 mM sodium acetate
629 buffer supplemented with 1 mM $CaCl_2$ (pH 5.2) using A) 250 g L^{-1}, B) 500 g L^{-1} and C)
630 650 g L^{-1} of sucrose as starting substrate. Vertical bars represent standard deviations (*n*
631 = 3).

632

633 **Figure 3.** Concentrations of sucrose, raffinose, glucose, fructose, melibiose, total
634 fructooligosaccharides (FOS) and total raffinosyl-oligofructosides (RFOS) upon
635 transfructosylation reaction catalyzed by the recombinant inulosucrase from
636 *Lactobacillus gasseri* DSM 20604 (IS) (1.6 U mL^{-1}) at 55 °C, in 25 mM sodium acetate
637 buffer supplemented with 1 mM $CaCl_2$ (pH 5.2) using 250 g L^{-1} of sucrose and 250 g L^{-}
638 ^{1} of raffinose as starting substrates. Vertical bars represent standard deviations ($n = 3$).

639

640 **Figure 4.** Structures and ^{13}C-NMR spectra of the synthesized raffinosyl-

641 oligofructosides (RFOS) by inulosucrase from *Lactobacillus gasseri* DSM 20604 (IS)

642 upon transfructosylation reaction of the raffinose.

643 A) RFOS DP4: α-D-Gal-(1→6)-α-D-Glc-(1→2)-β-D-Fru-(1→2)-β-D-Fru; B) RFOS

644 DP5: α-D-Gal-(1→6)-α-D-Glc-(1→2)-β-D-Fru-(1→2)-β-D-Fru-(1→2)-β-D-Fru; C)

645 RFOS DP6: α-D-Gal-(1→6)-α-D-Glc-(1→2)-β-D-Fru-(1→2)-β-D-Fru-(1→2)-β-D-Fru-

646 (1→2)-β-D-Fru; D) RFOS DP7: α-D-Gal-(1→6)-α-D-Glc-(1→2)-β-D-Fru-(1→2)-β-D-

647 Fru-(1→2)-β-D-Fru-(1→2)-β-D-Fru-(1→2)-β-D-Fru.

648

Table 1. Carbohydrate composition (g L^{-1}) determined by LC-RID and produced upon the transfructosylation reaction catalyzed by the recombinant inulosucrase from *Lactobacillus gasseri* DSM 20604 using 250 g L^{-1} raffinose as starting substrate. Values shown as mean ± sd (n =3).

					Raffinosyl-oligofructosides (RFOS)					
Time (h)	**Fructose**	**Melibiose**	**Raffinose**	**Inulobiose**	**DP 4**	**DP 5**	**DP 6**	**DP 7**	**DP 8**	**Total**
0	0.0	0.0	238.5±0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
1	11.8±1.2	43.9±2.7	150.5±6.2	1.0±0.2	29.9±0.7	5.4±0.6	1.4±0.1	0.2±0.0	0.0	36.9
3	18.8±1.3	64.4±4.1	103.7±0.7	1.4±0.1	38.8±1.4	10.7±0.7	4.5±0.4	1.1±0.1	0.0	55.1
8	25.3±1.5	90.8±4.5	60.8±0.2	1.6±0.2	40.5±1.2	15.8±0.9	9.8±0.7	3.3±0.2	0.2±0.0	69.6
24	32.2±0.6	110.6±3.0	23.3±1.5	2.6±0.2	33.6±1.7	19.9±0.8	11.2±0.9	4.9±0.9	1.1±0.1	70.7
32	27.5±3.7	96.2±12.5	21.9±2.4	2.4±0.4	22.6±1.8	13.6±2.3	8.4±1.5	3.7±0.9	0.9±0.0	49.2
48	34.0±4.0	111.2±13.9	22.6±2.0	3.0±0.4	26.2±6.3	16.9±4.4	8.6±1.9	4.2±0.7	1.3±0.2	57.2

Table 2. Carbohydrate composition (g L^{-1}) determined by LC-RID and produced upon the transfructosylation reaction catalyzed by the recombinant inulosucrase from *Lactobacillus gasseri* DSM 20604 using 500 g L^{-1} raffinose as starting substrate. Values shown as mean ± sd (n =3).

					Raffinosyl-oligofructosides (RFOS)					
Time (h)	**Fructose**	**Melibiose**	**Raffinose**	**Inulobiose**	**DP 4**	**DP 5**	**DP 6**	**DP 7**	**DP 8**	**Total**
0	0.0	0.0	517.3±0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
1	9.1±0.3	59.8±1.6	388.0±4.4	1.0±0.0	58.7±0.9	8.8±0.0	1.6±0.1	0.1±0.0	0.0	69.2
3	16.9±0.2	88.2±0.7	290.6±0.8	2.0±0.3	78.2±4.0	19.5±0.2	6.8±0.0	1.3±0.2	0.0	105.8
8	18.2±0.8	120.6±5.5	208.3±5.2	2.3±0.0	91.6±1.9	30.7±1.5	17.3±1.0	5.2±0.3	0.4±0.0	145.2
24	30.9±0.2	170.3±1.5	94.9±1.8	3.1±0.0	85.0±0.5	44.0±0.2	28.8±0.5	12.7±0.2	2.1±0.2	172.6
32	22.7±0.1	153.4±0.2	78.9±1.7	2.8±0.1	76.1±1.9	40.2±0.3	24.6±0.1	12.0±0.4	2.2±0.2	155.1
48	24.2±0.4	156.8±1.9	70.5±0.7	2.7±0.0	66.7±1.6	38.3±0.2	25.3±0.2	13.4±0.2	3.3±0.1	147.0

Table 3. Yield, productivity and specific productivity of transfer products formed upon the transfructosylation reaction catalyzed by the recombinant inulosucrase from *Lactobacillus gasseri* DSM 20604 using sucrose, raffinose or sucrose:raffinose mixture as starting substrates at different concentrations (250, 500 or 650 g L^{-1}). Maximum concentration ranges (g L^{-1}) of transfer products and the time (h) intervals to which were reached are also shown.

Starting substrate	Concentration (g L^{-1}) of starting substrate	Maximum concentration ranges (g L^{-1}) of transfer products [a]	Time (h) intervals at the maximum concentration of transfer products	Yield [b]	Productivity [c]	Specific Productivity [d]
Sucrose	**250**	66.6 - 67.3	3 - 24	24.9	46.3	0.070
	500	143.8 - 168.8	8 - 32	31.2	108.1	0.165
	650	232.0 - 283.5	8 - 32	43.5	123.1	0.187
Raffinose	**250**	69.6 - 70.7	8 - 24	29.6	36.9	0.056
	500	145.0 - 172.6	8 - 24	33.4	69.1	0.105
Sucrose:Raffinose	**250:250**	164.2 – 180.6	8 - 24	33.8	86.2	0.131

[a] **Transfer products** refers to FOS, RFOS or a mixture of FOS and RFOS depending on whether the starting substrate is sucrose, raffinose or a mixture of sucrose and raffinose, respectively.

[b] **Yield** (g transfer products / 100 g starting substrate) represents the maximum mass of transfer products obtained during the synthesis per unit mass of initial substrate. Experimental values for the starting substrate concentration were precisely determined by LC-RID analysis.

[c] **Productivity** (g transfer products $L^{-1}\cdot h^{-1}$) represents the concentration of transfer products formed per unit of reaction time (determined after the first hour of reaction).

[d] **Specific productivity** (g transfer products mg enzyme$^{-1}\cdot h^{-1}$) represents the mass of transfer products produced per unit mass of inulosucrase from *L.gasseri* DSM 20604 added and per unit of reaction time.

Table 4. ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR spectral data for oligosaccharides **A-E**[a].

Structure	Position	Gal		Glu		Fru-1		Fru-int		Fru-term	
		δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
A (n=0) α-D-galactopyranosyl-(1→6)-α-D-glucopyranosyl-(1→2)-β-D-fructofuranosyl-(1→2)-β-D-fructofuranoside	1	4.85 (3.7)	101.31	5.30 (3.9)	95.25	3.60 3.56	63.80			3.58 3.54	63.27
	2	3.69	71.35	3.42	73.86	--	106.20			--	106.59
	3	3.75	72.07	3.90	74.29	4.14	79.37			4.04	79.46
	4	3.85	72.22	3.41	72.30	3.91	76.70			3.94	77.32
	5	3.81	73.89	3.60	75.54	3.74	84.07			3.72	83.98
	6	3.60	63.97	3.90 3.54	68.65	3.67 3.62	65.14			3.68 3.64	65.20
B α-D-galactopyranosyl-(1→6)-α-D-glucopyranosyl-(1→2)-β-D-fructofuranosyl-(1→2)-β-D-fructofuranosyl-(1→2)-β-D-fructofuranoside	1	4.85 (3.8)	101.31	5.30 (3.9)	95.24	3.68 3.56	63.93	3.71 3.57	63.71	3.61 3.54	63.21
	2	3.68	71.34	3.41	73.88	--	106.15	--	105.89	--	106.51
	3	3.75	72.03	3.90	74.30	4.14	79.50	4.08	80.31	4.04	79.56
	4	3.85	72.20	3.41	72.29	3.91	76.70	3.93	77.29	3.96	77.16
	5	3.81	73.88	3.61	75.54	3.73	84.07	3.72	83.91	3.72	83.91
	6	3.60	63.97	3.90 3.53	68.64	3.68 3.62	65.14	3.68 3.62	65.10	3.69 3.61	65.07
C α-D-galactopyranosyl-(1→6)-α-D-glucopyranosyl-(1→2)-β-D-fructofuranosyl-(1→2)-β-D-fructofuranosyl-(1→2)-β-D-fructofuranosyl-(1→2)-β-D-fructofuranoside	1	4.85 (3.8)	101.31	5.30 (3.9)	95.19	3.68 3.56	63.89	3.71 3.57	63.74, 63.51	3.61 3.54	63.26
	2	3.68	71.34	3.41	73.88	--	106.15	--	105.90, 105.88	--	106.52
	3	3.75	72.06	3.90	74.30	4.14	79.39	4.08	80.29, 80.21	4.04	79.57
	4	3.85	72.21	3.41	72.29	3.91	76.67	3.93	77.28, 77.15	3.96	77.16
	5	3.81	73.88	3.61	75.53	3.73	84.10	3.72	83.92, 83.90	3.72	83.92
	6	3.60	63.97	3.90 3.53	68.66	3.68 3.62	65.14	3.68 3.62	65.12, 65.04	3.69 3.61	65.05
D α-D-galactopyranosyl-(1→6)-α-D-glucopyranosyl-(1→2)-β-D-fructofuranosyl-(1→2)-β-D-fructofuranosyl-(1→2)-β-D-fructofuranosyl-(1→2)-β-D-fructofuranosyl-(1→2)-β-D-fructofuranoside	1	4.85 (3.8)	101.31	5.30 (3.9)	95.17	3.60 3.56	63.87	3.71 3.57	63.70, 63.57, 63.56	3.61 3.54	63.31
	2	3.68	71.34	3.42	73.87	--	106.14	--	105.90, 105.90, 105.90	--	106.52
	3	3.75	72.05	3.90	74.30	4.14	79.36	4.08	80.21, 80.19, 80.15	4.04	79.58
	4	3.85	72.20	3.41	72.28	3.91	76.66	3.93	77.27, 77.19,77.13	3.96	77.17
	5	3.81	73.87	3.60	75.54	3.74	84.11	3.72	83.93, 83.91, 83.89	3.72	83.92
	6	3.60	63.96	3.90 3.54	68.66	3.67 3.62	65.14	3.68 3.62	65.12, 65.05, 64.98	3.69 3.61	65.05
E α-D-galactopyranosyl-(1→6)-α-D-glucopyranosyl-(1→2)-β-D-fructofuranosyl-(1→2)-β-D-fructofuranosyl-(1→2)-β-D-fructofuranosyl-(1→2)-β-D-fructofuranosyl-(1→2)-β-D-fructofuranosyl-(1→2)-β-D-fructofuranoside	1	4.85 (3.8)	101.31	5.30 (3.9)	95.17	3.68 3.56	63.94	3.71 3.57	63.71, 63.67, 63.62, 63.55	3.61 3.54	63.28
	2	3.68	71.33	3.41	73.87	--	106.13	--	105.94, 105.94, 105.93,105.92	--	106.52
	3	3.75	72.05	3.90	74.30	4.14	79.36	4.08	80.31, 80.18, 80.11, 80.08	4.04	79.58
	4	3.85	72.20	3.41	72.28	3.91	76.67	3.93, 3.95	77.29, 77.24, 77.13, 77.10	3.96	77.19
	5	3.81	73.87	3.61	75.52	3.75	84.07	3.72	83.92, 83.92, 83.91, 83.89	3.72	83.92
	6	3.60	63.95	3.90 3.53	68.66	3.68 3.64	65.14	3.68 3.62	65.11, 65.07, 64.97, 64.96	3.69 3.61	65.04

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

Figure 1. *Díez-Municio et al.*

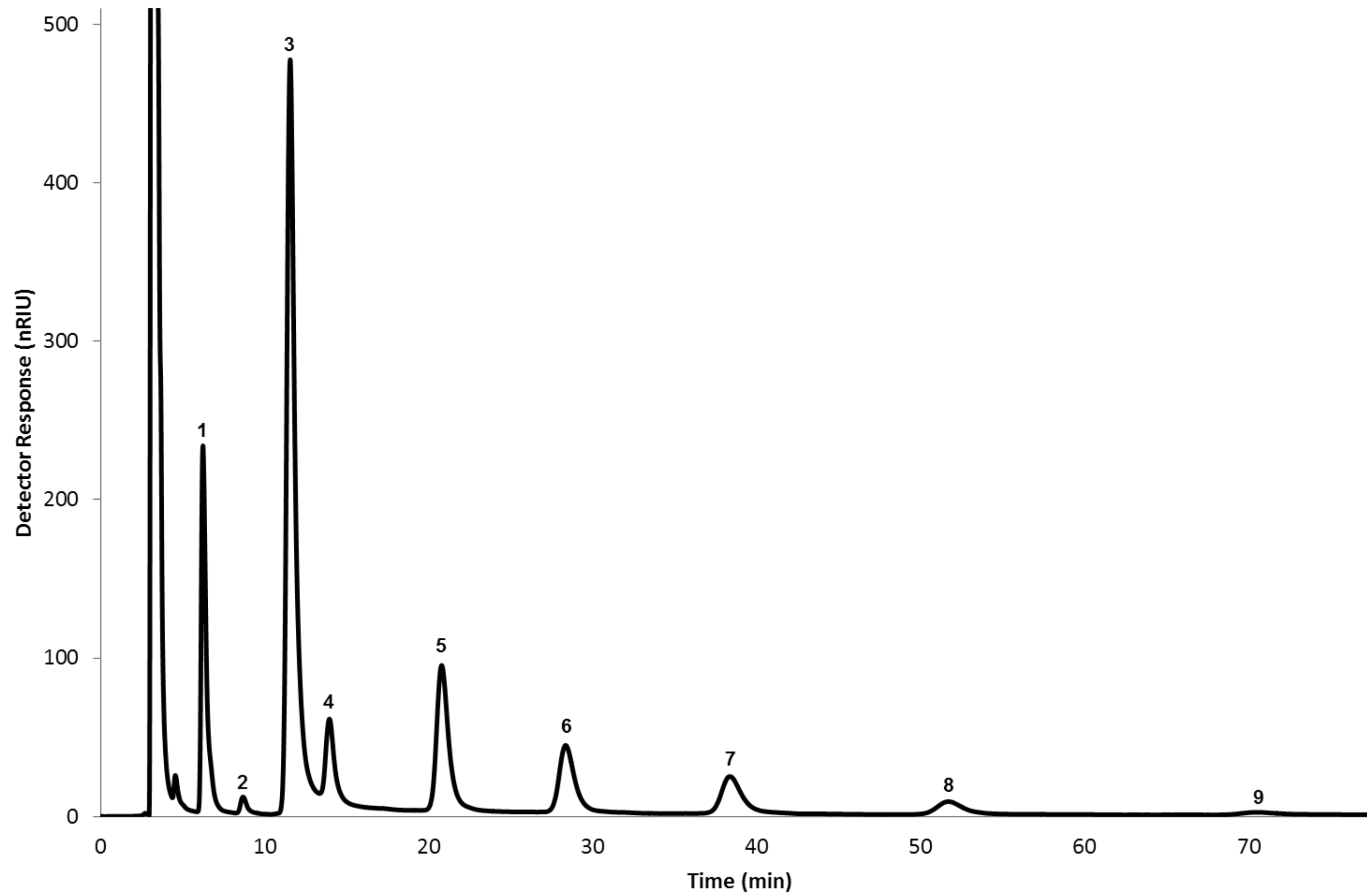

Figure 2. *Díez-Municio et al.*

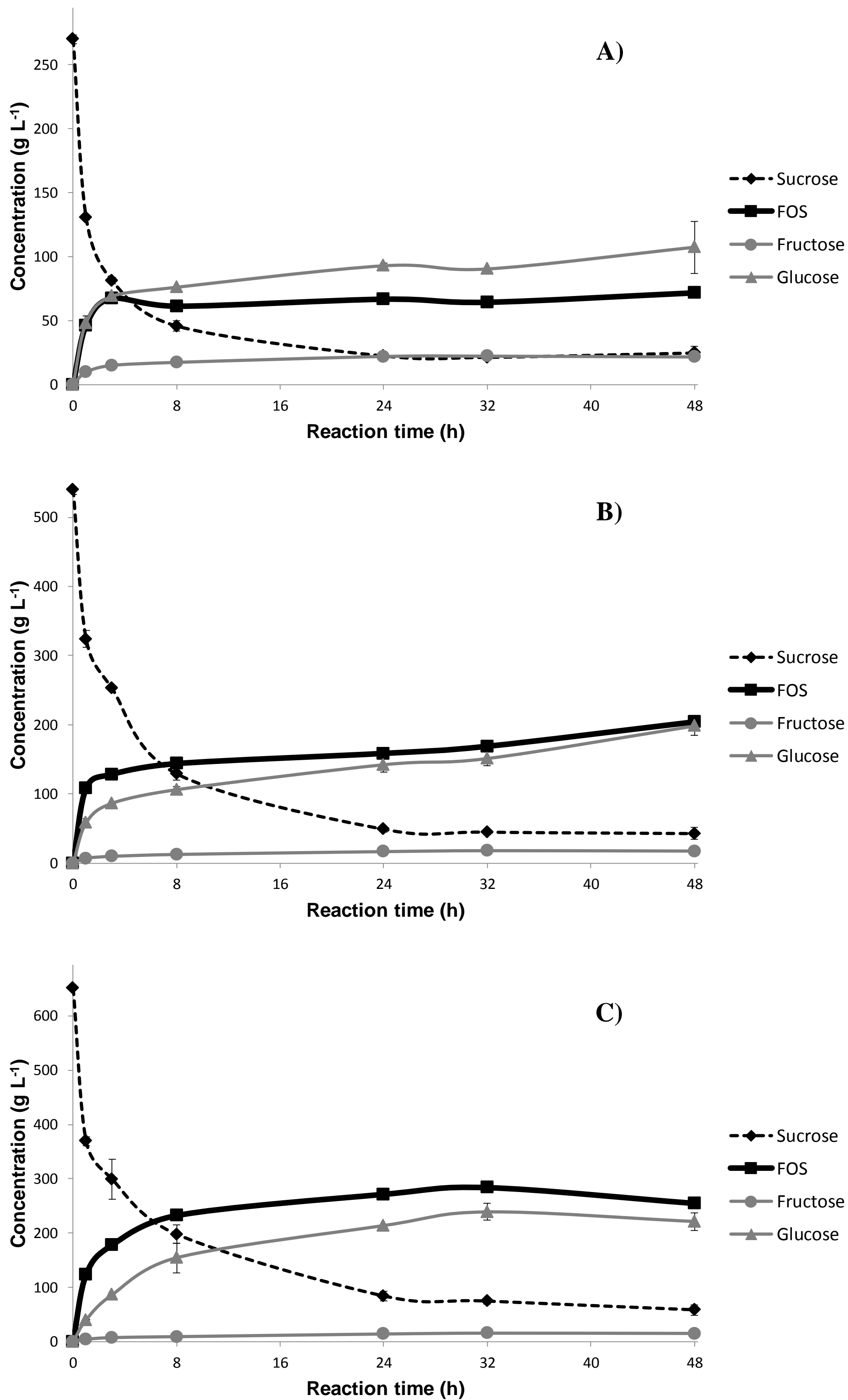

Figure 3. *Díez-Municio et al.*

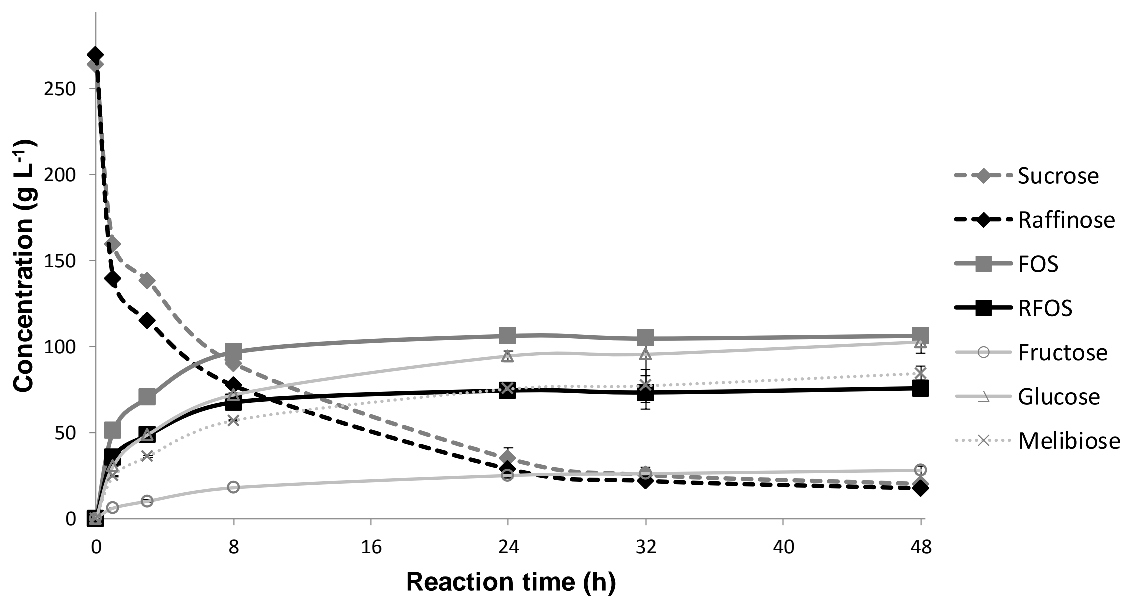

Figure 4. *Díez-Municio et al.*