A study of influential factors on oligosaccharide formation by fructosyltransferase activity during stachyose hydrolysis by Pectinex Ultra SP L

A. Montilla[1], A. Olano[1], C. Martínez-Villaluenga[2], N. Corzo[1*]

[1]Instituto de Investigación en Ciencias de la Alimentación, CIAL, (CSIC-UAM),
Nicolás Cabrera 9, 28049, Madrid, (Spain)

[2]Instituto de Ciencia y Tecnología de Alimentos y Nutrición, ICTAN, (CSIC),
Juan de la Cierva, 3, 28006 Madrid, (Spain)

*Author to whom correspondence should be addressed

Telephone number + 34 91 0017 954
Fax number + 34 91 0017 905
E-mail: nieves.corzo@csic.es

ABSTRACT

Enzyme-catalysed transglycosylation has gained increased interest as an approach to obtain novel prebiotic oligosaccharides. Influence of reaction conditions for oligosaccharide synthesis from stachyose by a commercial enzymatic extract from *Aspergillus aculeatus* (Pectinex Ultra SP-L) was studied. During this reaction, oligosaccharides with degree of polymerization (DP) ranging from 3 to 8 were detected by gas chromatography with flame ionization detection (GC-FID) and MALDI-TOF-MS. Galactosyl-melibiose (DP_3) was synthesized as a result of fructosidase activity while fructosyl-stachyose (DP_5) and difructrosyl-stachyose (DP_6) were formed as consequence of fructosyltransferase activity of Pectinex. The optimal reaction conditions for the synthesis of penta- and hexasaccharides were 60 °C, pH 5.5, 600 mg/mL stachyose and 34 U/mL of enzyme. Composition on oligosaccharides of synthesis mixtures was noticeable affected by reactions conditions. Reaction time played an important role on oligosaccharide mixtures composition constituted by 20.05% pentasaccharides, 0.72% hexasaccharides, 54.82% stachyose, 20.75% galactosyl-melibiose and 1.03% monosaccharides after 1 h and 16.27% pentasaccharides, 3.83% hexasaccharides, 27.26% stachyose, 43.61% galactosyl-melibiose and 2.35% monosaccharides after 3 h. In conclusion, stachyose could be used as a substrate for the enzymatic synthesis of new oligosaccharides that may open new opportunities in the development of future prebiotics.

Keywords: α-galactosides, stachyose, Pectinex Ultra SP-L, transfructosylation, oligosaccharide synthesis

44 **1. Introduction**

45 The consumption of prebiotic carbohydrates has gained increased interest due to
46 their recognition as agents inducing beneficial physiological effects in the colon and in
47 extra-intestinal compartments or reduces the risk of associated intestinal and systemic
48 pathologies (Roberfroid et al., 2010). Prebiotic carbohydrates may be fermented in
49 different parts of digestive tract. Rapidly fermented prebiotics stimulate growth and
50 activity of beneficial bacteria in the proximal colon whereas slowly fermented
51 prebiotics may reach the distal colon where most of chronic diseases (e.g. colorectal
52 cancer and ulcerative colitis) take place (Sanz, Cote, Gibson, & Rastall, 2006; Sanz,
53 Gibson, & Rastall, 2005). Consequently, there is greater interest in finding prebiotics
54 that can persist to more distal regions of the colon. (Hughes et al., 2007; Martinez-
55 Villaluenga et al., 2008; Cardelle-Cobas, Martínez-Villaluenga, Villamiel, Olano &
56 Corzo 2008).

57 In addition, monosaccharide moieties and linkage type may influence the
58 oligosaccharide fermentation rate by colonic microbiota (Sanz et al., 2006). Currently,
59 the development of future prebiotic targets the modulation of microbial fermentation
60 along the gastrointestinal tract (Ouwehand, Derrien, de Vos, Tiihonen, & Rautonen,
61 2005). Future prebiotics could be mixtures of oligosaccharides that have different
62 effects in different parts of the gastrointestinal tract. Besides colon, oral cavity and the
63 small intestine are also considered to be potential prebiotic targets (Ouwehand et al.,
64 2005).

65 α-Galactosides (raffinose, stachyose, verbascose and ajugose) are sucrose-based
66 oligosaccharides found more abundantly in grain legumes (Martinez-Villaluenga, Frias,
67 & Vidal-Valverde, 2008a). These carbohydrates derived from sucrose containing 1-4
68 units of galactose linked by α-(1,6) linkages to the glucose moiety. These

69 oligosaccharides are resistant to digestion promoting the growth of beneficial bacteria in
70 the colon (Van den Broek, Hinz, Beldman, Doeswijk-Voragen, Vincken, & Voragen,
71 2005).

72 *In vitro* assays have shown the ability of *Bifidobacterium* and *Lactobacillus*
73 strains to grow using α-galactosides as carbon source, due to the production of α-
74 galactosidase (Trojanova, Vlkova, Rada, & Marcunek, 2006; Farnworth, Mainville,
75 Desjardins, Gardner, Fliss, & Champagne, 2007; Martinez-Villaluenga, & Gomez,
76 2007). Today, α-galactosides are obtained on industrial scale as a by-product from the
77 production of soy protein isolates and soy protein concentrates (Martinez-Villaluenga,
78 et al., 2008a). The recovery of α-galactosides from soy by-products could be an
79 interesting alternative for their utilization as raw material in the manufacture of future
80 prebiotics.

81 Transglycosylation reactions catalysed by enzymes are being used for the
82 production of oligosaccharides mixtures with a specific composition. Production of
83 galactooligosaccharides mixtures from lactose or lactulose using galactosyltransferases
84 from different sources have been carried out (Chockchaisawasdee, Athanasopoulos,
85 Niranjan, & Rastall, 2005; Cardelle-Cobas, Villamiel, Olano, & Corzo, 2008; Martínez-
86 Villaluenga, Cardelle-Cobas, Corzo, Olano, & Villamiel, 2008b). In addition,
87 fructooligosaccharides mixtures were produced from sucrose by fructosyltransferases
88 from bacteria or moulds (Hang & Woodams, 1995; Yun, 1996; Sangeetha, Ramesh, &
89 Prapulla, 2005; Ghazi, Fernandez-Arrojo, Gomez de Segura, Alcalde, Plou, &
90 Ballesteros, 2006). Since α-galactosides contain a fructosyl end, fructosyltransferases
91 may transfer the fructosyl moiety to a suitable acceptor to give rise new
92 oligosaccharides. Purified fructosyltransferase from *Aspergillus niger* has been used to
93 catalyse the synthesis of new oligosaccharides from raffinose (Uhm, Baek, Jhon, Kim,

94 Hwang, & Ryu, 1999). The relatively inexpensive commercial enzyme preparation
95 Pectinex Ultra SP-L, produced by *Aspergillus aculeatus,* was shown to contain
96 fructosyltransferase activity (Hang & Woodams, 1995; Tanriseven & Aslan, 2005;
97 Ghazi et al., 2006; Montilla, Corzo, Olano, & Jimeno, 2009); therefore, it could be used
98 as catalyst in large-scale production of new oligosaccharides. Therefore, the objective of
99 this work has been to investigate in more detail the fructosyltransferase activity from
100 *Aspergillus aculeatus* of commercial enzymatic preparation Pectinex Ultra SP-L over
101 stachyose hydrolysis.

102

103 **2. Material and methods**

104

105 *2.1. Chemicals*

106

107 D-galactose, D-glucose, D-fructose, melibiose, raffinose, stachyose and phenyl-
108 β-glucoside were purchased from Sigma (St. Louis, MO, USA). The commercial
109 enzyme preparation from *Aspergillus aculeatus*, Pectinex Ultra SP-L, containing
110 fructosyltransferase activity was a generous gift from Novozymes (Dittingen,
111 Switzerland).

112

113 *2.2. Determination of enzymatic activity*

114

115 Fructosyltransferase activity of Pectinex was measured using sucrose as
116 substrate. The activity was assayed at 60 ℃ using sucrose at 300g/L in 0.1 M sodium
117 acetate buffer (pH 5.5). Aliquots (0.1 mL) were withdrawn at different times. The
118 reaction was stopped by adding 10 μL of acetic acid 0.1 N. The amount of fructose and

119 glucose released was determined by gas chromatography (GC-FID) using the method
120 described below. Fructosyltransferase activity was calculated as the difference between
121 the amount of free glucose and fructose which indicated the amount of fructose
122 involved in the transfructosylation reaction. The fructosyltransferase activity was 400
123 U, where 1 unit (U) is defined as the amount of enzyme transferring 1 μmol of fructose
124 per minute per mL under assayed conditions. Enzyme activity measurements were
125 performed in triplicate and the experimental error (RSD relative standard deviation) was
126 lower than 5%.

127 The soluble protein concentration in the enzymatic preparation was determined
128 using the bicinchoninic acid (BCA) assay (Smith et al., 1985). Bovine serum albumin
129 (BSA) was used as standard. Specific activity was defined as U/mg protein. The soluble
130 protein concentration in the enzyme preparation was 82 mg/mL. Therefore, the enzyme
131 expressed a fructosyltransferase specific activity of 4.9 U/mg soluble protein.

132

133 *2.3. Enzymatic synthesis of oligosaccharides*

134

135 Enzymatic synthesis of oligosaccharides from stachyose using Pectinex was
136 carried out under different reaction conditions such as temperature (50, 60 and 70 ºC),
137 pH (3.5; 4.5; 5.5; 6.5 and 7.5), stachyose concentration (100, 300 and 600 g/L),
138 enzyme concentration (17, 34 and 78 U/mL), and time (0.5, 1, 3, 6 and 24 h). Reactions
139 were performed at a final volume of 1.5 mL in microtubes incubated in an orbital shaker
140 at 300 rpm. Aliquots (120 μL) were withdrawn from the reaction mixture at the
141 different times and immediately immersed in boiling water for 5 min to inactivate the
142 enzyme. Samples were stored at -18º C for subsequent analysis. Experiments were
143 carried out in duplicate.

144 The amount of remaining stachyose and the yield of oligosaccharides in the
145 reaction mixtures were expressed as weight percentage of total carbohydrate content.
146
147 *2.4. Purification and characterization of reaction mixtures*
148 Purification was performed following the method described by Morales, Sanz,
149 Olano, & Corzo, (2006). Briefly, a total of 2.5 mL of reaction mixture, containing 1.5 g
150 of carbohydrates was dissolved in 300 mL of water and stirred for 30 min with 9 g of
151 activated charcoal Darco G60, 100 mesh (Sigma, St. Louis, MO) to remove mono and
152 disaccharides. This mixture was vacuum-filtered through Whatman No. 1 filter paper,
153 and activated charcoal was washed with 50 mL of water. The oligosaccharides adsorbed
154 onto the activated charcoal were extracted by stirring for 30 min with 300 mL
155 ethanol/water solution (1:1, v:v) and then vacuum-filtered. The ethanol /water solution
156 was evaporated under vacuum at 30 ºC. Sample was dissolved in 5 mL of deionized
157 water and filtered through 0.22 µm filters (Millipore Corp., Bedford, MA) for further
158 characterization by mass-spectrometry.
159 The enriched fraction containing the main synthesis products was characterized
160 by Matrix-Assisted Laser Desorption/Ionisation Time-of-Flight Mass Spectrometry
161 (MALDI-TOF-MS) on a Voyager DE-PRO mass spectrometer (Applied Biosystems,
162 Foster City, CA) equipped with a pulsed nitrogen laser (λ = 337 nm, 3 ns pulse width,
163 and 3 Hz frequency) and a delayed extraction ion source. Ions generated by laser
164 desorption were introduced into a time of flight analyser (1.3 m flight path) with an
165 acceleration voltage of 20 kV, 74% grid voltage, 0.001% ion guide wire voltage, and a
166 delay time of 300 ns in the reflector positive ion mode. Mass spectra were obtained over
167 the *m/z* range 100-1500. External mass calibration was applied using the monoisotopic
168 $[M + H]^+$ values of des-Arg^1 bradykinin and angiotensin I of the Calibration Mixture 1,

169 Sequazyme Peptide Mass Standards Kit; Applied Biosystems. 2,5-dihydroxybenzoic
170 acid (>98%, Fluka) at 10 mg/mL in water was used as matrix. Sample was diluted 100
171 times in water, and mixed with the matrix at a ratio of 1:4 (v:v). One µL of this solution
172 was spotted onto a flat stainless-steel sample plate and dried in air before analysis.
173
174 *2.5. Carbohydrate analysis by gas chromatography (GC-FID)*
175
176 Trimethyl silylated oximes (TMS-oxime) of mono- di and oligosaccharides were
177 analysed by GC following the method of Montilla, van de Lagemaat, Olano, & del
178 Castillo (2006). GC analysis was performed with a gas chromatograph (3800GC,
179 Varian, California, USA) equipped with a flame ionization detector (FID). The
180 trimethylsilyl oximes were separated using a 8 m x 0.25 mm x 0.25 µm film fused silica
181 capillary column coated with CP-SIL 5CB (methyl silicone from Chrompack,
182 Middelburg, The Netherlands), giving rise to two peaks, corresponding to the syn (E)
183 and anti (Z) isomers. The carrier gas (nitrogen) flow rate was 1.1 mL/min. Injector
184 temperature was 280 ºC, detector temperature was 340 ºC and the oven temperature was
185 programmed from 150 ºC to 165 ºC at 3 ºC/min, then at 5 ºC/min to 340 ºC and keeping
186 this temperature for 10 min. Injections were made in the split mode (1:10). Data
187 acquisition and integration was done using HP ChemStations software (Hewlett-
188 Packard, Wilmington, USA).
189 The oximes were formed following the method of Brobst & Lott (1966). A
190 volume of 7 to 40 µl of sample constituted of approximately 4 mg of sugars, was added
191 to 0.4 mL of internal standard (IS) solution containing 0.5 mg/ml phenyl-β-glucoside.
192 The mixture was dried at 38-40 °C in a rotary evaporator (Büchi Labortechnik AG,
193 Flawil, Switzerland). Sugar oximes were formed by adding 200 µL hydroxylamine

194 chloride (2.5%) in pyridine and heating the mixture at 70 °C for 30 min. Subsequently,
195 the oximes obtained in this step were silylated with hexamethyldisilazane (200 µL) and
196 trifluoroacetic acid (20 µL) and kept at 50 ºC for 30 min. Reaction mixtures were
197 centrifuged at 7000*g* for 5 min at 5 °C. Supernatants were injected in the GC or stored
198 at 4 ºC prior to analysis.

199 Quantitative analysis was carried out by the internal standard method. Response
200 factors were calculated using a triplicate analysis of 6 standard solutions (galactose,
201 glucose, fructose, sucrose, melibiose, raffinose and stachyose) at a concentration range
202 from 0.06 to 2 mg/ml and 0.06 to 4 mg/ml for stachyose.

203

204 **3. Results and discussion**

205

206 The fructosidase, galactosidase and fructosyltransferase activities of Pectinex
207 resulted in the production of melibiose (α-D-Galp-(1→6)-α-D-Glcp), raffinose (α-D-
208 Galp-(1→6)-α-D-Glcp-(1→2)-β-D-Fruf), 6' galactosyl melibiose (α-D-Galp-(1→6)-α-
209 D-Galp-(1→6)-α-D-Glcp), fructosyl stachyose (DP5) (1^F fructofuranosyl stachyose, α-
210 D-Galp-(1→6)-α-D-Galp-(1→6)-α-D-Glcp-(1→2)-β-D-Fruf-(1→2)-β-D-Fruf) and
211 difructosyl stachyose (DP6) (1^F $(fructofuranosyl)_2$ stachyose (α-D-Galp-(1→6)-α-D-
212 Galp-(1→6)-α-D-Glcp-(1→2)-β-D-Fruf-(1→2)-β-D-Fruf-(1→2)-β-D-Fruf) as it has
213 been previously reported (Montilla et al., 2009). Besides, MALTI-TOF-MS analysis of
214 oligosaccharide-enriched fractions allowed the detection of oligosaccharides with DP7
215 (*m/z* 1175.4 and 1191.3) and DP8 (*m/z* 1338.5) (Fig. 1) whereas previous studies have
216 only reported the formation of oligosaccharides DP3 and DP5 from stachyose by
217 fructosyltransferase from *Aspergillus niger* (Uhm et al., 1999)

218

219 Several reports have demonstrated that glycosidic linkages and degree of
220 polymerization of oligosaccharides contribute toward the selectivity of fermentation by
221 beneficial bacteria (Rowland, & Tanaka, 1993; Sanz et al., 2005; 2006). In addition, a
222 wide molecular weight range could give rise to modulation of microbial fermentation.
223 Higher molecular oligosaccharides may be slowly fermented exhibiting higher colonic
224 persistence than low molecular weight carbohydrates, reaching the most distal regions
225 where most of intestinal disorders are encountered (Sanz et al., 2006). On the basis of
226 this information, the production of α-galactosides mixtures from stachyose composed of
227 carbohydrates with DP from 3 to 8 may represent an opportunity in the development of
228 future prebiotics.
229
230 *3.1. Study of the formation of oligosaccharides from hydrolysis of stachyose*
231
232 A series of experiments were performed in which temperature, pH and sustrate
233 and enzyme concentrations were varied to select the optimum conditions for the tri- and
234 oligosaccharides syntesis.
235
236 *3.1.1. Effect of temperature*
237 To determine the influence of temperature on oligosaccharide synthesis, reaction
238 was performed in a temperature range of 50-70 ºC, 300 mg/mL of stachyose, 34 U/mL
239 of enzyme and pH 5.5, for 24 h (Fig. 2). The loss of stachyose (Fig. 2a) ncreased with
240 temperature from 50° to 60°C, but declined with further increase of temperature to 70°C
241 This fact could be attributed to a thermal denaturation of enzyme at 70 ºC.
242 During the first six hours of the reaction fructose release (Fig. 2b) was favoured
243 at 60 ºC, however, after twenty four hours, when most of the stachyose was consumed,

244 the amount of fructose reached at 50ºC was similar to that observed at 60ºC Galactosyl-
245 melibiose production (Fig. 2c) reached maximum yields after 24 h either at 50 ºC or 60
246 ºC (58.9% and 62.9%, respectively).

247 Transferase/hydrolase ratio of the enzyme was optimal at 60 ºC, being observed
248 the highest DP5 oligosaccharide yield (15.3%) after 3 h (Fig. 2d). It is noteworthy a
249 sharply decrease of this pentasaccharide at 50 and 60 ºC after reaching maximum yield
250 while larger amounts of this α-galactoside remained after 24 h at 70 ºC. DP6
251 oligosaccharide production was only possible at 60 ºC, reaching the highest
252 concentration (1.8%) after 6 h. Our results are in agreement with previous works which
253 reported that the optimal temperature for fructooligosaccharides production from
254 sucrose by fructosyltransferase activity of Pectinex was around 60 ºC (Tanriseven &
255 Aslan, 2005; Ghazi, Fernandez-Arrojo, Garcia-Arellano, Ferrer, Ballesteros, & Plou,
256 2007).
257

258 *3.1.2. Effect of pH*

259 Different experiments were carried out at pH values from 3.5 to 7.5 at 60 ºC
260 using reactions mixtures of stachyose (300 mg/mL) and 34 U/mL of enzyme. In assays
261 carried out at pH 3.5 and 7.5 hardly any stachyose hydrolysis was observed (data not
262 shown). The highest rate of stachyose decrease (Fig. 3a), fructose release (Fig. 3b) and
263 galactosyl-melibiose formation (Fig. 3c) were attained at pH 5.5. At pH 4.5 the lowest
264 fructosidase activity was observed.

265 The maximum formation of DP5 (Fig. 3d) was observed at pHs 5.5 and 6.5 after
266 three hours (~ 16%) and then, at pH 6.5 slowly decreased down to (~ 12%) after twenty
267 four hours. However, a markedly decrease to (~ 2%) was observed at pH 5.5 probably
268 due to a higher fructosidase activity of the enzyme under these conditions. **,**

The highest amount of DP6- formed was 1.8%, at pH 5.5 after 6 h of reaction whereas yields at pH 4.5 and 6.5 were negligible. These results are consistent with previous studies of Ghazi et al. (2007) showing that the optimal activity/stability of purified fructosyltransferase from Pectinex was in the pH range 5.0-6.0; similar ~~ly~~ to other fructosyltransferases derived from fungi (Hang, & Woodams, 1996; Rezende, & Felix, 1997).

3.1.3. Effect of enzyme concentration

The effect of enzyme concentration (17, 34 and 68 U/mL) was studied using reaction mixtures containing 300 mg/mL stachyose at pH 5.5 and at 60 ºC (Fig. 4). Loss of stachyose and formation of galactosyl-melibiose noticeably increased when enzyme concentration was raised up to 34 U/mL while a further increase of the enzyme concentration did not result in a significant improve of stachyose conversion and trisaccharide yields (Fig. 4a and 4c). Regarding DP5 formation, enzyme concentration did not exert any influence on maximum yields attained, although formation rate of this pentasaccharide increased with enzyme concentration (Fig. 4d). DP6 yields decreased with enzyme concentration, the highest yield (2.1%) was observed at enzyme concentration of 17 U/mL after 24 h (Fig. 4d). At the same with stachyose, the most important effect observed was an increase of hydrolysis rate with enzyme concentration. Also, hydrolysis rate of the DP5 and DP6 formed during the first three to six hours increased with the enzyme concentration (Fig. 4d). This was consistent with the monosaccharide concentrations observed (Fig. 4b). The highest enzyme concentration tested (68 U/mL) gave rise to the highest fructose (13.3%) and galactose+glucose (4.8%) concentrations after 24 h of reaction. However, in all conditions it was observed that galactosyl-melibiose was stable to hydrolysis; the highest melibiose formation was only 3.4% after 24h with 68 U/mL of enzyme concentration.

294

295 *3.1.4. Effect of initial stachyose concentration*

296 To investigate the influence of substrate concentration on oligosaccharide
297 synthesis, reaction was performed using a range of stachyose concentration from 100 to
298 600 mg/mL (Fig. 5). Reactions were performed at 60 ºC, pH 5.5 and 34 U/mL of
299 enzyme for 24 h. Fig. 5a shows that at the higher initial substrate concentration the
300 higher rate of stachyose conversion was reached. The fructosidase activity of Pectinex
301 was favoured with low stachyose concentrations (100 mg/mL) and longer reaction time,
302 thus increasing amounts of free fructose was released reaching up to 13.8% after 24 h of
303 reaction (Fig. 5b).

304 Formation of galactosyl-melibiose increased with higher initial stachyose
305 concentration up to 6 h (Fig. 5c), and the highest galactosyl-melibiose yield (67.5%)
306 was found with 100 mg/mL of initial stachyose concentration after 24 h of reaction. The
307 galactosyl-melibiose yield obtained by Pectinex using stachyose as substrate was much
308 higher than that obtained by Van Laere et al., (1999) who achieved a 33 % using α-
309 galactosidase from *Bifidobacterium adolescentis* and 300 mM melibiose. Tzortzis, Jay,
310 Baillon, Gibson, & Rastall, (2003) and Goulas, Goulas, Tzortzis, & Gibson, 2009
311 obtained a 26% and 21% of galactosyl-melibiose using α-galactosidase from
312 *Lactobacillus reuteri* and 670 mM melibiose and α-galactosidase from *Bifidobacterium*
313 *bifidum* and 1170 mM melibiose, respectively. Galactosyl-melibiose is an interesting
314 compound with prebiotic and symbiotic properties with *Lactobacillus acidophilus* and
315 *L. reuteri*. Prebiotics properties has been found in synthesised galactosyl melibiose
316 mixtures which produced a higher increase in bifidobacteria and lactobacilli population
317 and decrease of clostridia and *E. coli* population than produced by FOS, melibiose and

318 raffinose used as reference carbohydrates (Tzortzis, Goulas, Baillon, Gibson, & Rastall,
319 2004).
320 Transfructosylation reaction–mainly occurred at reaction times less than three
321 hours and increased with initial substrate concentrations up to 600 mg/mL (Fig. 5d).
322 The highest yields of transfructosylation products DP5 (20.1%) and DP6 (3.8%), were
323 found at the highest initial stachyose concentration (600 mg/mL) after 1 h and 3 h,
324 respectively. Our research group used previously this concentration to obtain and
325 charaterize DP5 and DP6 compounds (Montilla et al., 2009). These results are
326 consistent with previous studies showing that high substrate concentration exerts a
327 noticeable influence on the formation of stachyose-derived oligosaccharides by
328 fructosyltransferases due to an increase in transferase/hydrolysis ratio (Mahoney, 1998;
329 Cardelle-Cobas, et al. 2008). Ghazi et al. (2007) reported that the fructosyltransferase
330 activity in a purified enzyme from *A. aculeatus* is approximately 20-fold higher than
331 hydrolysis activity at sucrose concentrations greater than 342 g/L.
332
333 **4. Conclusions**
334 These results shown the noticeable effect of reaction conditions on the
335 oligosaccharide formation during the enzymatic treatment of stachyose with Pectinex
336 Ultra SP-L. The optimal reaction conditions for the synthesis of penta- and
337 hexasaccharides were 60 ºC, pH 5.5, 600 mg/mL stachyose and 34 U/mL of enzyme.
338 Under these conditions the time of reaction may noticeably influence the composition
339 of α-galactoside mixtures, thus, 0.72% hexasaccharides, 20.05% pentasaccharides,
340 54.82% stachyose, 20.75% galactosyl-melibiose and 1.03% monosaccharides was found
341 after 1 h of reaction; and 3.83% hexasaccharides, 16.27% pentasaccharides, 27.26%
342 stachyose, 43.61% galactosyl-melibiose and 2.35% monosaccharides after 3 h of time

343 course reaction. However, to obtain the maximum yield of galactosyl-melibiose
344 (67.5%), the assays should be carried out at 60 ºC and pH 5.5, using 100 mg/mL
345 stachyose and 34 U/mL of enzyme during 24h.

346 α-Galactosides which are industrially available in large amounts as a by-product
347 from the production of soy protein isolate seems to be a promising raw material for the
348 production of new oligosaccharides through hydrolysis and transglycosylation using a
349 GRAS commercial enzyme preparation. Further research is warranted to evaluate the
350 prebiotic properties of these new α-galactosides mixtures that may be used to influence
351 the microbial composition in the distal colon, where most of gut disorders occur.

352

353 **Acknowledgements**

354

355 We are grateful to Ramiro Martinez (Novozymes A/S, Spain) for providing us
356 with Pectinex Ultra SP-L and Plácido Galindo for his help in acquiring the MALDI-
357 TOF-MS spectral data. This work has been financed under a R+D program of the
358 Spanish Ministry of Science and Innovation Science, Projects AGL-2008-00941/ALI
359 and Consolider Ingenio 2010 (FUN-C-FOOD) CSD 2007-00063; R + D program of the
360 Comunidad de Madrid, Project ALIBIRD P2009/AGR-1469 and as R + D program of
361 the Comunidad de Castilla-La Mancha POII10-0178-4685.

362

363 **References**

364

365 Brobst, K. M., & Lott, C. E. (1966). Determination of some components in corn syrup
366 by gas-liquid chromatography of trimethylsilyl derivatives. *Cereal Chemistry 43,* 35-
367 43.

368 Cardelle-Cobas, A., Villamiel, M., Olano, A., & Corzo, N. (2008). Study of
369 galactooligosaccharides formation from lactose using Pectinex Ultra SP-L. *Journal
370 of the Science of Food and Agriculture*. *88*, (6) 954-961.
371 Cardelle-Cobas, A., Martínez Villaluenga, C., Villamiel, M., Olano, A., Corzo, N.,
372 2008b. Synthesis of oligosaccharides derived from lactulose and Pectinex Ultra SP-
373 L. *Journal of Agricultural and Food Chemistry*, *56*, 3328-3333.
374 Chockchaisawasdee, S., Athanasopoulos, V. I., Niranjan, K., & Rastall, R. A. (2005).
375 Synthesis of galacto-oligosaccharide from lactose using β-galactosidase from
376 *Kluyveromyces lactis*: Studies on batch and continuous UF membrane-fitted
377 bioreactors. *Biotechnology and Bioengineering*. *89*, 434-443.
378 Farnworth, E. R., Mainville, I., Desjardins, M. P., Gardner, N., Fliss, I., & Champagne,
379 C. (2007). Grow of probiotic bacteria and bifidobacteria in a soy yogurt formulation.
380 *International Journal of Food Microbiology, 116*, 174-181.
381 Ghazi, I., Fernandez-Arrojo, L., Gomez de Segura, A., Alcalde, M., Plou, F. J., &
382 Ballesteros, A. (2006). Beet sugar syrup and molasses as low-cost feedstock for the
383 enzymatic production of fructo-oligosaccharides. *Journal of Agricultural and Food
384 Chemistry*. *54*, 2964-2968.
385 Ghazi, I., Fernandez-Arrojo, L., Garcia-Arellano, H., Ferrer, M., Ballesteros, A., &
386 Plou, F. J. (2007). Purification and kinetic characterization of a fructosyltransferase
387 from *Aspergillus aculeatus*. *Journal Biotechnology, 128*, 204-211.
388 Goulas, T., Goulas, A., Tzortzis, G., & Gibson, G. R. (2009). A novel α-galactosidase
389 from *Bifidobacterium bifidum* with transgalactosylating properties: gene molecular
390 cloning and heterologous expression. *Applied Microbiology and Biotechnology, 82,*
391 471–477.

392 Hang, Y. D., & Woodams, E. E. (1995). Fructosylransferase activity of commercial
393 enzyme preparations used in fruit processing. *Biotechnology Letters, 17*, 741-744.
394 Hang Y. D., & Woodams E. E. (1996). Optimization of enzyme production of fructo-
395 oligosaccharides from sucrose. *Lebensmittel Wissenschaft und Technologie, 29,* 578-
396 580.
397 Hughes, S. A., Shewry, P. R., Li, L. Gibson, G.R , Sanz, M. L., Rastall, R. A. (2007). In
398 vitro fermentation by human fecal microflora of wheat arabinoxylans . *Journal of*
399 *Agricultural and Food Chemistry, 55,* 4589-*4595.*
400 Mahoney, R. R. (1998). Galactosyl-oligosaccharide formation during lactose
401 hydrolysis: a review. *Food Chemistry, 6*, 147-154.
402 Martinez-Villaluenga, C. & Gomez, R. (2007). Characterization of bifidobacteria as
403 starters in fermented milk containing raffinose family of oligosaccharides from lupin
404 as prebiotic. *International Dairy Journal. 17*, 116-122.
405 Martinez-Villaluenga, C., Frias, J., & Vidal-Valverde, C. (2008a). Alpha-galactosides:
406 antinutritional factors or functional ingredients? *Critical Review of Food Science and*
407 *Nutrition*, *48*, 301-316.
408 Martínez-Villaluenga, C., Cardelle-Cobas, A., Corzo, N., Olano, A., & Villamiel, M.
409 (2008b). Optimization of conditions for galactooligosaccharide synthesis during
410 lactose hydrolysis by a β-galactosidase from *Kluyveromyces lactis* (Lactozym 3000 L
411 HP G). *Food Chemistry, 107*, 258-264.
412 Martínez-Villaluenga, C., Cardelle-Cobas, A., Olano, A., Corzo, N., Villamiel, M., &
413 Jimeno, M.L., 2008c. Enzymatic synthesis and identification of two trisaccharides
414 produced from lactulose by transgalactosylation. *Journal of Agricultural and Food*
415 *Chemistry, 56*, 557–563.

416 Montilla, A., van de Lagemaat, J., Olano, A., & del Castillo, M. D. (2006).
417 Determination of oligosaccharides by conventional High-Resolution Gas
418 Chromatography. *Chromatographia, 63*, 453-458.
419 Montilla, A., Corzo, N., Olano, A., & Jimeno, M. L. (2009). Identification of
420 oligosaccharides formed during stachyose hydrolysis by Pectinex Ultra SP-L.
421 *Journal of Agricultural and Food Chemistry, 57,* 5007-5013.
422 Morales, V., Sanz, M. L., Olano, A., & Corzo, N. (2006). Rapid separation on activated
423 charcoal of high oligosaccharides in honey. *Chromatographia, 64*, 233-238.
424 Ouwehand, A. C., Derrien, M., de Vos, W., Tiihonen, K., & Rautonen, N. (2005).
425 Prebiotics and other microbial substrates for gut functionality. *Current Opinion in*
426 *Biotechnology*, *16*, 212-217.
427 Rezende, S. T., & Felix, C. R. (1997). Raffinose-hydrolyzing activity of *Aspergillus*
428 *fumigatus. Biotechnology Letters, 19*, 217–220.
429 Roberfroid, M., Gibson, G. R., Hoyles, L., McCartney, A. L., Rastall, R., Rowland, I.,
430 et al. (2010). Prebiotic effects: metabolic and health benefits. *British Journal of*
431 *Nutrition, 104*, S1-S63.
432 Rowland, I. R., & Tanaka, R. (1993). The effects of transgalactosylated
433 oligosaccharides on gut flora metabolism in rats associated with a human fecal
434 microflora. *Journal of Applied Bacteriology, 74*, 667-674.
435 Sangeetha, P. T., Ramesh, M. N., & Prapulla, S. G. (2005). Recent trends in the
436 microbial production, analysis and application of Fructooligosaccharides. *Trends in*
437 *Food Science and Technology, 16*, 442-457
438 Sanz, M. L., Gibson, G. R., & Rastall, R. A. (2005). Influence of disaccharide structure
439 on prebiotic selectivity in vitro. *Journal of Agricultural and Food Chemistry, 53*,
440 5192-5199.

441 Sanz, M. L., Cote, G. L., Gibson, G. R., & Rastall, R. A. (2006). Influence of glycosidic
442 linkages and molecular weight on the fermentation of maltose-bases oligosaccharides
443 by human gut bacteria. *Journal of Agricultural and Food Chemistry, 54*, 9779-9784.
444 Smith, P. K. , Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H.,
445 Provenzano, M. D., et al. (1985). Measurement of protein using bicinchoninic acid.
446 *Analytical Biochemistry,150*, 76-85.
447 Tanriseven, A., & Aslan, Y. (2005). Immobilization of Pectinex Ultra SP-L to produce
448 fructooligosaccharides. *Enzyme and Microbial Technology*. *36*, 550-554.
449 Trojanova, I., Vlkova, E., Rada, V., & Marcunek, M. (2006). Different utilization of
450 glucose and raffinose in *Bifidobacterium breve* and *Bifidobacterium animalis*. *Folia*
451 *Microbiologica. 51*, 320-324.
452 Tzortzis, G., Jay, A. J., Baillon, M. L. A., Gibson, G. R., & Rastall, R. A. (2003).
453 Synthesis of α-galactooligosaccharides with α-galactosidase from *Lactobacillus*
454 *reuteri* of canine origin. *Applied Microbiology and Biotechnology, 63*, 286-292.
455 Tzortzis, G., Goulas, A. K., Baillon, M. L. A., Gibson, G. R., & Rastall, R. A. (2004). In
456 vitro evaluation of the fermentation properties of galactooligosaccharides synthesised
457 by alpha-galactosidase from *Lactobacillus reuteri*. *Applied Microbiology and*
458 *Biotechnology, 64,* 106-111.
459 Uhm, T. B., Baek, N. I., Jhon, D. Y., Kim, D. M., Hwang, K. T. & Ryu, E. J. (1999).
460 Synthesis the new oligosaccharides from raffinose by *Aspergillus niger*
461 fructosyltransferase. *Biotechnology Techniques, 13*, 169-171.
462 Van den Broek, L. A. M., Hinz, S. W. A., Beldman, G., Doeswijk-Voragen, C. H. L.,
463 Vincken, J. P., & Voragen, A. G. J., (2005) Glycosyl hydrolases from
464 *Bifidobacterium adolescentis* DSM20083. An overview. *Lait. 85*, 125-133.

465 Van Laere, K. M. J., Hartemink, R., Beldman, G., Pitson, S., Dijkema, C., Schols, H.
466 A., & Voragen, A. G. J. (1999). Transglycosidase activity of *Bifidobacterium*
467 *adolescentis* DSM 20083 α-galactosidase. *Applied Microbiology and Biotechnology*,
468 52, 681-688.
469 Yun, J. W. (1996). Fructooligosaccharides – Occurrence, preparation, and application.
470 *Enzyme and Microbial Technology,19*, 107-117.
471

472 **FIGURE CAPTIONS**

473 **Figure 1.** MALDI-TOF-MS profile of the purified fraction of oligosaccharides formed
474 during enzymatic hydrolysis of stachyose with Pectinex Ultra SP-L (DP, degree of
475 polymerisation).

476 **Figure 2.** Effect of temperature on stachyose hydrolysis and oligosaccharide production
477 during the enzymatic treatment of stachyose (300 mg/mL) with Pectinex Ultra SP-L
478 (34U/m/L) in 0.1 M sodium acetate buffer pH 5.5 at (♦) 50 ºC; (■) 60 ºC; (▲) 70ºC.

479 **Figure 3**. Effect of pH on stachyose hydrolysis and oligosaccharide production during
480 the enzymatic treatment of stachyose (300 mg/mL) with Pectinex Ultra SP-L (34
481 units/mL) at 60ºC in 0.1 M sodium acetate buffer (♦) pH 4.5; (■) pH 5.5; (▲) pH 6.5.

482 **Figure 4.** Effect of enzyme concentration on stachyose hydrolysis and oligosaccharide
483 production during the enzymatic treatment of stachyose (300 mg/mL) at 60ºC and pH
484 5.5 with Pectinex Ultra SP-L (♦) 17 U/mL; (■) 34 U/mL; (▲) 68 U/mL.

485 **Figure 5.** Effect of stachyose concentration on hydrolysis and oligosaccharide
486 production from reaction mixtures of stachyose (♦) 100 mg/mL; (■) 300 mg/mL; (▲)
487 600 mg/mL and Pectinex Ultra SP-L (34 U/mL), at pH 5.5 and 60 ºC.

488 (*): Data from Montilla et al., (2009).

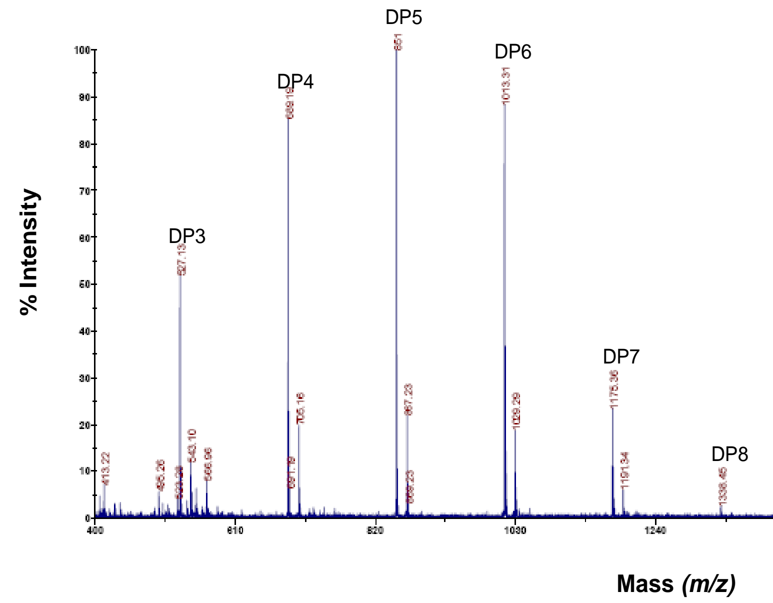

Figure 1. Montilla et al., 2011

489

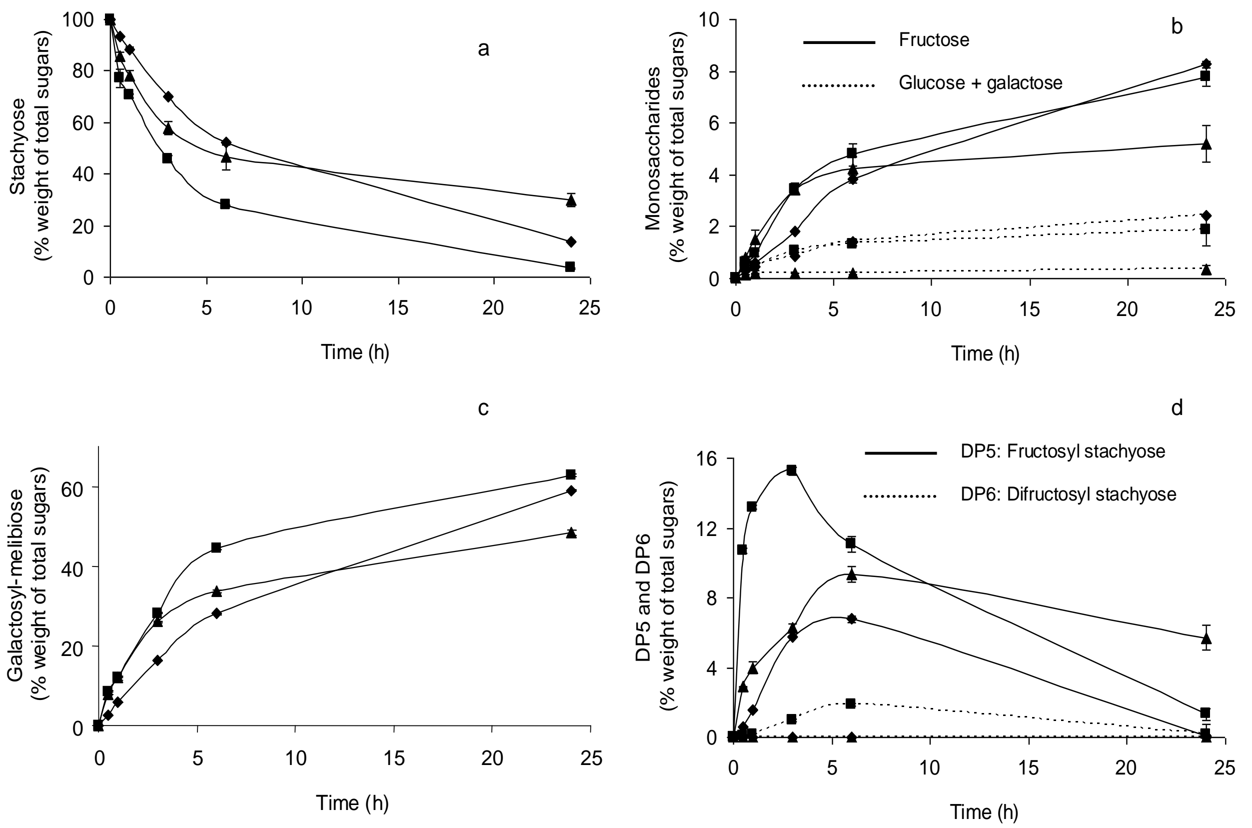

Figure 2.- Montilla et al., 2011

490
491
492
493

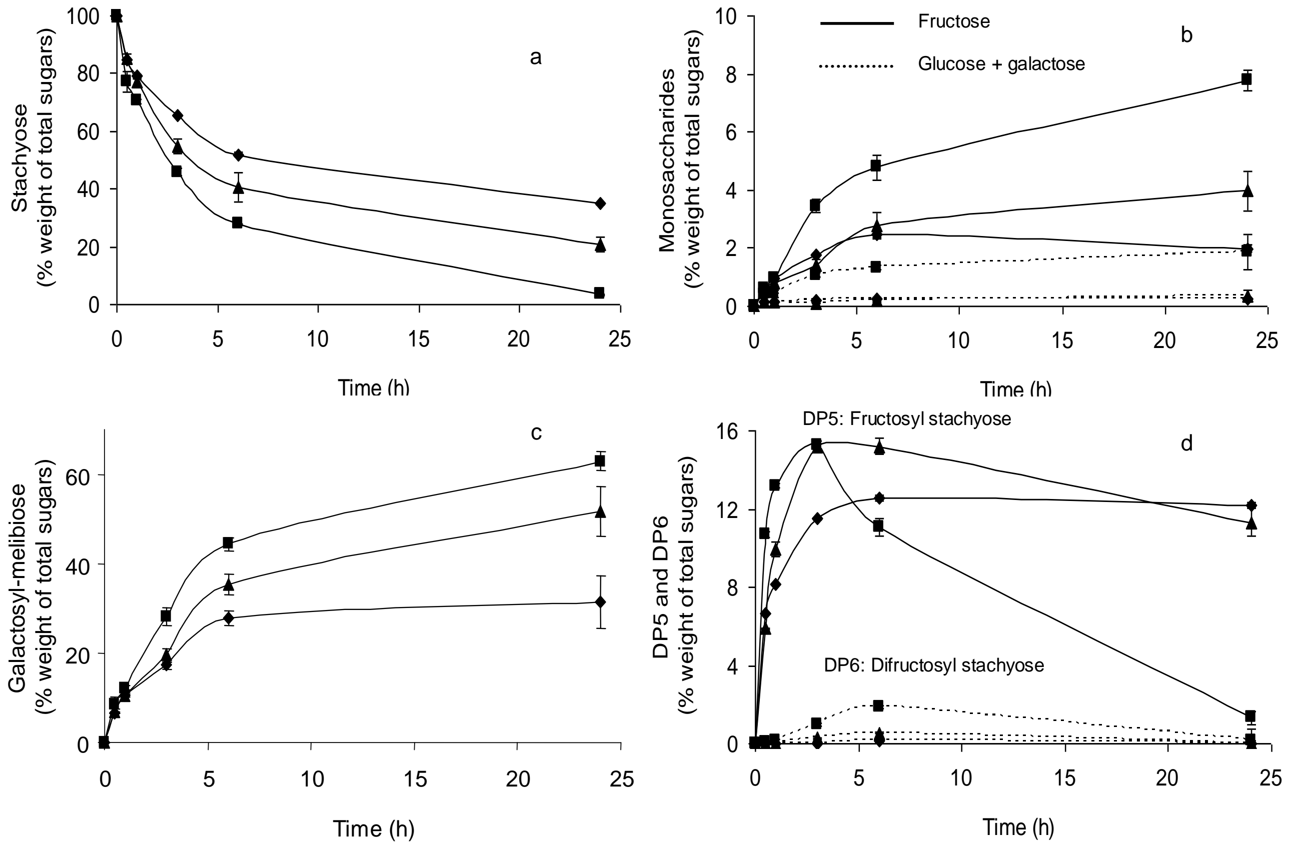

Figure 3.- Montilla et al., 2011

494

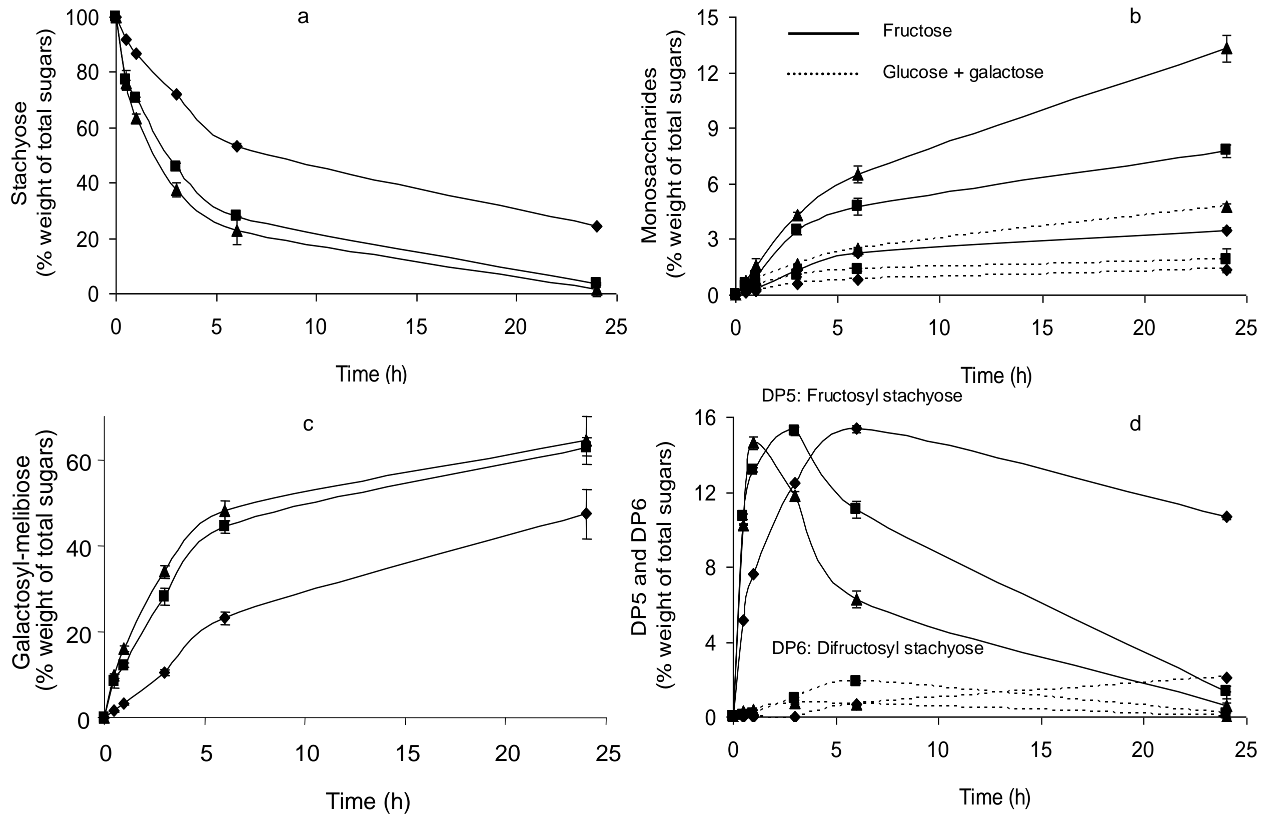

Figure 4.- Montilla et al., 2011

495

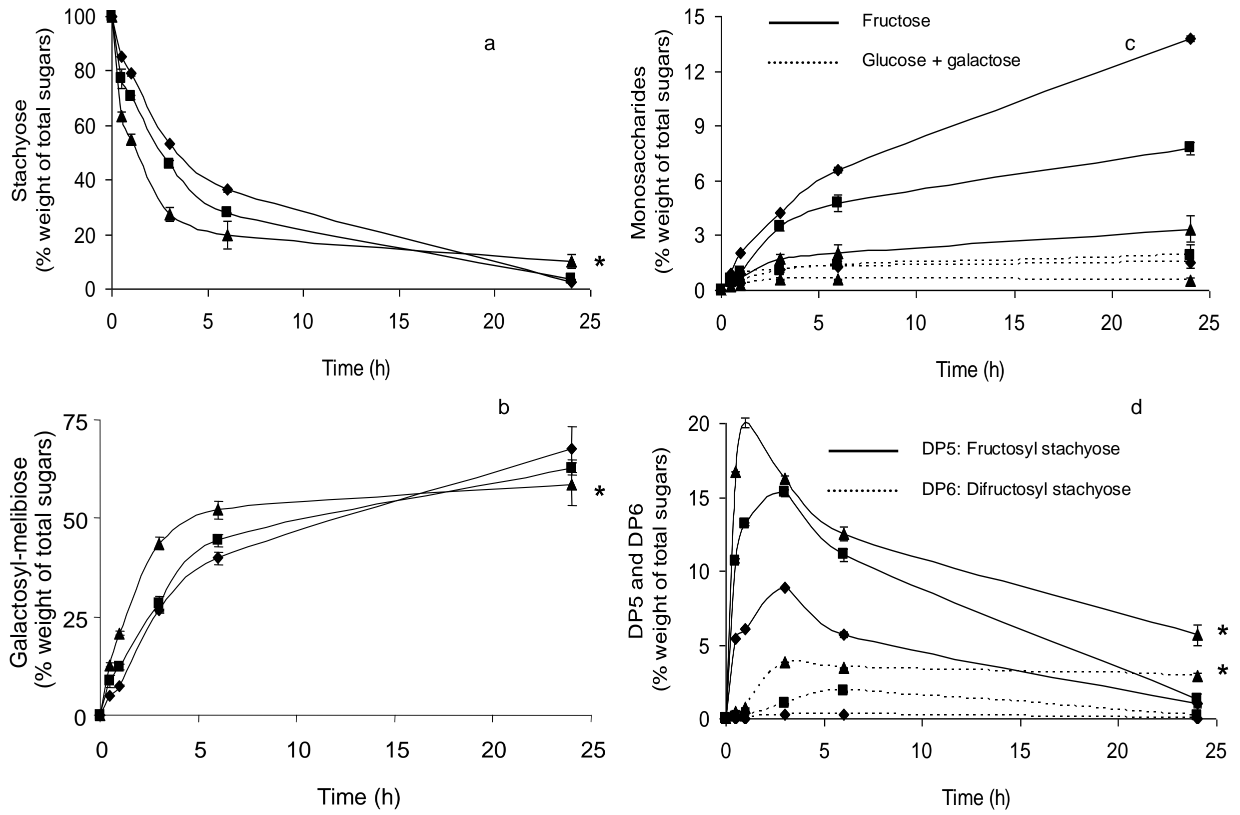

Figure 5.- Montilla et al.,

496
497
498