Effect of acceptor carbohydrates on oligosaccharide and polysaccharide synthesis by dextransucrase DsrM from *Weissella cibaria*

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Abstract

The digestibility of isomalto-oligosaccharides (IMO) as well as their metabolism by gut microbiota depends on the degree of polymerization and the ratio of α-(1→4) to α-(1→6) linkages. Both parameters are influenced by the method of production. Commercial IMO are produced by transglycosylation of starch hydrolysates, or by transglycosylation with dextransucrase and sucrose as glucosyl-donor and maltose as glucosyl-acceptor. This study aimed to quantitatively and qualitatively assess the acceptor reaction with dextransucrase. α-Glucans were selected by systematic variation of degree of polymerization and linkage type; the dextransucrase DsrM from *Weissella cibaria* 10M was used as biocatalyst. The efficiency of α-glucans as acceptor carbohydrates decreased in the order DP2 > DP3 > DP1; among disaccharides, the efficiency decreased in the order α-(1→6) > α-(1→4) > α-(1→3); the α-(1→2) linked kojibiose did not support oligosaccharide formation. Equimolar addition of efficient acceptor molecules and sucrose shifted the dextransucrase reaction to oligosaccharides as virtually exclusive product. DsrM readily extended a commercial IMO preparation by adding α-(1→6)-linked glucose moieties. Conversion of commercial IMO by dextransucrase reduced their *in vitro* digestibility as analysed by two different protocols. This study facilitates the synthesis of oligosaccharides produced in the acceptor reaction with dextransucrase with controlled yields and degree of polymerization, and hence with optimal functional properties in food applications.

Keywords. Dextransucrase, dextran, isomalto-oligosaccharides, panose, isomaltose, prebiotic, *in vitro* digestibility.

Highlights

- α-Glucans are efficient acceptor carbohydrates for dextransucrase DrsM.
- The efficiency of acceptors for DsrM decreased in the order DP2 > DP3 > DP1
- Among disaccharides, the efficiency decreased α-(1→6) > α-(1→4) > α-(1→3)
- Equimolar addition of isomaltose and sucrose yielded only oligosaccharides.
- Conversion of commercial IMO by dextransucrase reduced their *in vitro* digestibility

1. Introduction

Non-digestible oligosaccharides are functional food ingredients that confer health benefits through metabolism by the gastrointestinal microbiota (Bindels, Delzenne, Cani, & Walter, 2015). Commercial oligosaccharides including fructo-oligosaccharides, galacto-oligosaccharides, xylo-oligosaccharides and isomalto-oligosaccharides (IMO) are extracted from natural sources or synthesized by enzymatic methods (Courtois, 2009). IMO consisting of α-(1→6) linked glucose moieties are the most significant contributor to the global oligosaccharide market (Nakakuki, 2002); commercial IMO preparations additionally contain oligosaccharides with mixed α-(1→4) and α-(1→6) linkages (Hu, Ketabi, Buchko, & Gänzle, 2013; Goffin et al, 2011; Madsen, Stanley, Swann, & Oswald, 2017).

The digestibility of IMO as well as the metabolism by the gut microbiota depend on the degree of polymerization (DP) and the linkage type (Ryan, Fitzgerald, & van Sinderen, 2006; Ketabi, Dieleman & Gänzle, 2011; Gänzle & Follador, 2012; Iwaya et al., 2012, Hu et al., 2013). The method of production determines DP and the linkage type in IMO, and hence the digestibility. Commercial IMO are produced by enzymatic hydrolysis of starch to resistant maltodextrins (van der Maarel, 2002), by enzymatic transglycosylation of starch hydrolysates with α-glucosidase (Pan & Lee, 2005), or by enzymatic transglycosylation with dextransucrase using maltose as glycosyl acceptor (Chen & Gänzle, 2016; Goulas, Cooper, Grandison & Rastall, 2004). In addition to non-digestible oligosaccharides, commercial IMO contain the digestible isomaltose and trisaccharides including panose and isomaltotriose for which the digestibility is poorly documented (McCleary, Sloane, Draga, & Lazewska, 2013). Prebiotic properties of commercial IMO, however, were consistently demonstrated in animal and human studies (Ketabi, Dieleman & Gänzle, 2011; Goffin, et al, 2011; Likotrafiti, Tuohy, Gibson & Rastall, 2014; Wang, 2009). IMO are hydrolysed by

brush border isomaltase and digestion depends on oligosaccharide transport across membranes (Hooton, Lentle, Monro, Wickham, & Simpson, 2015). *In vitro* digestibility assays, however, employ amyloglucosidase (McCleary, 2014), which hydrolyses oligosaccharides with α-(1→4) and α-(1→6) linkages (Pazur & Ando, 1960).

Transglycosylation of starch hydrolysates allows control of the DP by controlling the extent of starch hydrolysis; however, this also results in an increased ratio of α-(1→4) linkages. In transglycosylation reactions with dextransucrase as biocatalyst and sucrose and maltose as substrates, the DP is controlled by the choice of the biocatalyst and by the ratio of glycosyl-acceptor and glycosyl-donors (Robyt & Eklund, 1983; Shi et al., 2016). Suitable acceptor carbohydrates compete with water and dextran for transfer of the glucose moiety in a concentration-dependent manner (van Hijum, Kralj, Ozimek, Dijkhuizen, & van Geel-Schutten, 2006). Reactions with dextransucrase as biocatalyst, sucrose as donor and maltose as acceptor predominantly produce α-(1→6) linked panose-series oligosaccharides (Shi et al., 2016). In addition to maltose, isomaltose is a suitable acceptor carbohydrate for oligosaccharide synthesis by dextransucrases (Robyt & Eklund, 1983; Shi et al., 2016). Few studies, however, quantified the oligosaccharide yield and acceptor preference of dextransucrase; moreover, oligosaccharide synthesis by combination of starch-based and sucrose-based oligosaccharide synthesis has not been reported. This study therefore aimed to quantitatively and qualitatively assess the acceptor reaction with the dextransucrase DsrM from *W. cibaria*, which was characterized previously with respect to catalytic properties, dextran formation, and technological functionality of the dextran (Chen et al., 2016; Chen & Gänzle, 2016). α-Glucans were selected by systematic variation of DP and linkage type; moreover, commercial IMO were included in the analysis. Products were analysed by ion exchange chromatography and size exclusion chromatography to characterize

products of the reaction. The molecular weight (M_W) of polysaccharides was measured by asymmetric field-flow fractionation (AF4) coupled to multi-angle laser light scattering and the digestibility of oligosaccharides was determined *in vitro* by two methods.

2. Materials and Methods

2.1 Materials

Mono- di,- and trisaccharides and dextran standards were purchased from Sigma Aldrich (Oakville, ON, Canada). *Escherichia coli* BL21 (DE3) was purchased from Invitrogen Co (Carlsbad, US). The commercial isomaltooligosaccharide (IMO) preparation Vitafiber® was provided by BioNeutra Inc. (Edmonton, Canada).

2.2 Expression and purification of the recombinant his-tagged DsrM from W. cibaria

E. coli BL21 Star (DE3) (Invitrogen) harboring pET28a$^+$-*dsrM* from *W. cibaria* 10M (Chen et al., 2016, Chen & Gänzle, 2016) was cultivated aerobically at 37°C in Luria-Bertani (LB) broth containing 50 mg L^{-1} kanamycin (Invitrogen). Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a concentration of 0.2 mM when cultures grew to an OD_{600nm} of 0.6. Cultivation was continued for another 20 h at 20°C and 200 rpm. Cells were harvested by centrifugation, resuspended in phosphate buffered saline (PBS, pH 7.4), and disrupted by ultrasonication. Cell debris was removed by centrifugation at 12,000 ×g for 20 min and the supernatant was loaded to a Ni-NTA Spin column (Qiagen) equilibrated with 10mM imidazole in 100 mM PBS and 300 mM NaCl (pH 8.0). The column was washed with 100 mM imidazole in 100 mM PBS and 300 mM NaCl (pH 8.0); DsrM was eluted with elution buffer containing 500 mM imidazole in 100 mM PBS and 300 mM NaCl (pH 8.0). Protein identity was verified by SDS-PAGE analysis of the crude cellular extract and purified DsrM (Figure S1 of the online supplementary material). The protein content was measured by Bradford method using bovine serum albumin as standard.

2.3 Dextransucrase enzyme activity assay and optimization of temperature and pH for the acceptor reaction of sucrose and maltose

DsrM activity was determined as follows: Purified enzyme was incubated in 25 mM sodium acetate buffer (pH 5.2) containing 1 mM $CaCl_2$ and 100 mM sucrose. Unless stated otherwise, 1 µM enzyme was added to reactions. Samples were taken at 5 min intervals and the reaction was stopped by heating to 90°C for 10 min. The concentration of glucose and fructose was determined enzymatically by glucose assay reagent and the Fructose Assay Kit (Sigma-Aldrich). The amount of free glucose represents hydrolysis activity and the amount of fructose represents the total activity of the enzyme. One unit (U) of hydrolysis or total activity corresponds to the release of 1 µmol glucose or fructose from 100 mM sucrose in 25 mM sodium acetate (pH 5.2) and 1 mM $CaCl_2$ at 37 °C, respectively. Transferase activity was calculated as difference between total and hydrolysis activity.

To determine the effect of temperature on the transfer reaction of DsrM with sucrose and maltose as acceptor carbohydrate, enzymatic activities were assayed at pH 5.2 and temperatures ranging from 6 to 42^{o}C. To determine the effect of pH on the transfer reaction of DsrM, the reaction was performed at 30^{o}C in sodium acetate buffers adjusted to pH ranging from 4.0 to 5.2, or in buffer containing 50 mM citric acid and 100 mM Na_2HPO_4 with the pH adjusted to 5.6 to 8.4.

2.4 Oligosaccharides synthesis by DsrM with different acceptor carbohydrates

DsrM was incubated with sucrose, melibiose, arabinose, raffinose, galactose, lactose, or the glucose-series acceptors glucose, maltose, isomaltose, nigerose, kojibiose, maltotriose, isomaltotriose as acceptor carbohydrates at 30°C in 25 mM sodium acetate buffer (pH 5.2) containing 1 mM $CaCl_2$ for 24 h. The enzyme was inactivated by heating at 90^{o}C for 10 min. All enzymatic syntheses and analyses were carried out in duplicate or triplicate biological repeats.

2.5 Analysis of oligosaccharides

Qualitative analysis of oligosaccharides was performed with high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD). Samples were diluted 100-fold with water and separated on a Carbopac PA20 column coupled to an ED40 chemical detector (Dionex, Oakville, Canada). Water (A), 0.2M NaOH (B) and 1*M* NaAc (C) were used as solvents with the following gradient: 0 min, 68.3% A, 30.4%B and 1.3%C; 25 min, 54.6% A, 30.4% B and 15.0% C; 28min, 50% A and 50% C; 31min, 10% A, 73% B and 17%C; followed by re-equilibration. Galactose, glucose, fructose, melibiose, sucrose, isomaltose, lactose, kojibiose, nigerose, maltose, raffinose, isomaltotriose, panose, and maltotriose were used as external standards (Table S1 of the online supplementary material). Consistent with IUPAC nomenclature, all oligosaccharides that could be assigned a precise DP by HPAEC-PAD, i.e. oligosaccharides with a DP up to 30, were termed oligosaccharides to differentiate these from polymeric dextran.

Quantification of oligosaccharides was achieved by an Agilent 1200 series LC system (Agilent Technologies, Palo Alto, CA) equipped with a Supelcosil LC-NH_2 column (250mm×4.6mm, 5µm, Sigma Aldrich) and coupled to a refractive index (RI) detector. Samples were diluted with acetonitrile / water (50:50, v/v) and eluted with acetonitrile/water 70: 30 (v/v) at a flow rate of 0.8 mL min^{-1} and 30°C. The reaction products were quantified by using isomaltotriose as external standard. The oligosaccharide yield was calculated as:

$$Yield\ (\%) = 100 \times \frac{mol\ glucose\ transferred\ to\ oligosaccharides}{mol\ glucose\ in\ the\ initial\ substrate\ sucrose}$$

2.6 Size distribution of oligosaccharides and polysaccharides produced by DsrM

The distribution of oligosaccharides and polysaccharides was analyzed by size exclusion chromatography (SEC) using a Superdex peptide column (GE Healthcare Life Sciences,

Mississauga, ON, Canada) that was eluted with water and coupled to a RI detector. The column was calibrated with mono, di, and trisaccharides, and with dextran preparations (Sigma-Aldrich) with a relative M_W of 2800 and 8000. Molar mass determination of dextran was performed with AF4 coupled to multi-angle light scattering (MALS) and RI detectors (Postnova, Salt Lake City, UT, USA). The cellulose membrane (Postnova) of the accumulation wall had a M_W cut off of 10 kDa. Poly-styrolsulphonate standard and BSA were used for calibration of detectors. Samples were diluted with 10mM NaCl and injected at a flow rate of 0.2 mL min^{-1} and a cross flow of 1 mL min^{-1} for 6 min. After injection of 50 µl, the cross flow rate remained constant for 2 min, linearly decreased to 0.1 mL min^{-1} over 10 min, and was maintained at 0.1 mL min^{-1} for 10 min. The molar mass was determined from the laser scattering signals and RI signal by AF 2000 software (Postnova). The refractive index increment (dn/dc) value of 0.146 ml g^{-1} was employed (Vilaplana & Gilbert, 2010).

2.7 Production of oligosaccharides by DsrM with commercial IMO as acceptor

Purified DsrM was incubated with 250 mM sucrose and 3.75%, 7.5%, 15% or 30% (w/v) of a commercial IMO preparation mixture in 25 mM sodium acetate buffer (pH 5.2) containing 1 mM $CaCl_2$ for 24 h. The enzyme was inactivated and the formation of oligosaccharides was analyzed by HPAEC-PAD. Size distribution of oligosaccharides and dextran was performed by SEC and the molar mass of dextran was measured by AF4 as described above.

2.8 In vitro *digestibility of oligosaccharides*

Oligosaccharides were produced with maltose or a commercial IMO as acceptor and dextransucrase was inactivated by heating to 90°C for 10 min. Sucrose, maltose, glucose, and fructose were removed by addition of 10% (v/v) alginate-immobilized commercial baker's yeast (*Saccharomyces cerevisiae*), representing 1% dry yeast biomass, followed by incubation for 24 h

182 at 30°C. Alginate encapsulated yeasts were employed to facilitate removal of yeast by
183 centrifugation at 7000 x g; the supernatant containing oligosaccharides was collected and freeze-
184 dried. Removal of monosaccharides, sucrose, and maltose was verified by HPAEC-PAD. The *in*
185 *vitro* digestibility was determined with two methods. The first method employs pancreatic amylase
186 and amyloglucosidase and was validated to quantify starch digestibility in swine (van Kempen,
187 Regmi, Matte & Zijlstra, 2010). The second method employs brush border enzymes present in the
188 rat intestinal mucosa and was previously used to determine digestibility of IMO in rats (Tsunehiro,
189 Okamoto, Furuyama, Yatake & Kaneko, 1999).

190 *2.8.1 Digestion with an enzyme mixture of pancreatin, invertase and amyloglucosidase*

191 Freeze dried oligosaccharides (1.000 g) were transferred to a 50-mL tube containing 10 mL pepsin
192 solution, containing 50 mg pepsin (250U/mg), and 50 mg guar gum in 0.05 M HCl (Englyst et al.
193 1999; van Kempen et al, 2010); 5-10 glass beads with 5 mm diameter were also added to the tube.
194 The first digestion step, mimicking the gastric digestion, lasted 30 min at 37°C with agitation at
195 200 rpm. Then 10 mL of 0.25 M sodium acetate solution and 5 mL of enzyme mixture containing
196 0.7 g pancreatin from porcine pancreas (Sigma-Aldrich) (45 U/mg lipase, 42 U/mg amylase and
197 3.0 U/mg protease), 3 mg invertase (Sigma-Aldrich) and 50 µL amyloglucosidase from *Aspergillus*
198 *niger* (~300U/mL) (Sigma-Aldrich) were added. The solution was further incubated for 4 h
199 (Englyst et al. 1996; van Kempen et al. 2010). After 4 h, 500 µL of sample was removed, reactions
200 were stopped by addition of 0.5 mL absolute ethanol, and the glucose concentration was measured
201 with a glucose oxidase kit (Megazyme, Bray, Ireland).

202 *2.8.2. Digestion with rat intestinal extract*

203 This digestion method employs an acetone extract of the rat intestinal mucosa (Sigma-Aldrich).
204 The reaction mixture containing 1 mL sample dissolved in water to 10 g/L, 1 mL of 50 mM sodium

maleate buffer (pH 6.0) with 1 % rat enzyme mixture (Tadashi et al, 2005), and 3-7 glass beads with 5 mm diameter was incubated at 37 °C for 4 h with agitation at 200 rpm. The reaction was stopped by heating to 90 °C for 5 min. The samples were cooled on ice and subsequently centrifuged at 7000 x g for 3 min. The glucose concentration was measured with a glucose oxidase kit (Megazyme).

2.9 Statistical analysis

Oligosaccharide synthesis and analysis was carried out in duplicate or triplicate independent experiments; results are expressed as means ± standard deviation, or shown as representative chromatograms. The *in vitro* digestibility was determined in at least 6 independent experiments; results are expressed as means ± standard error of the mean. Significant differences of the oligosaccharide yield with different acceptor carbohydrates were evaluated by one way analysis of variance (ANOVA) and the Holm-Sidak post hoc analysis and assessed at a 5% probability of error ($P<0.05$). Significant differences of the *in vitro* digestibility of oligosaccharides were determined by two way ANOVA and the Holm-Sidak post hoc analysis ($P<0.05$).

3. Results

3.1 Effect of pH and temperature on the acceptor reaction of maltose and sucrose by DsrM

To confirm that the optimum pH and temperature for oligosaccharide synthesis correspond to the optimum pH and temperature values for overall enzyme activity (Chen et al., 2016; Chen & Gänzle, 2016), oligosaccharide formation by DsrM was evaluated with sucrose and maltose as glucosyl-acceptor under different incubation conditions. The concentrations of the sucrose and maltose remaining after 24 h of reaction was lowest after incubation at pH ranging from 4.4 to 5.2. This pH range corresponded to the highest concentration of panose-series oligosaccharides (Fig. S2 of the online supplementary material). Variation of the incubation temperature from 10 to 40°C had

no major influence on the yield of panose-series oligosaccharides (Fig. S2B). Sodium acetate buffer at pH of 5.2 and 30°C were used in all subsequent reactions to match the optimum of DsrM activity (Chen et al., 2016; Chen & Gänzle, 2016).

3.2 Determination of the efficacy of glucosyl acceptor carbohydrates

To determine the preferred glucosyl-acceptor for DsrM, acceptor reactions were performed with 500 mmol L^{-1} sucrose and glucose, isomaltose, isomaltotriose, maltose, maltotriose, kojibiose, and nigerose as acceptors. Qualitative analysis to obtain information on DP and linkage type of acceptor products was achieved with HPAEC-PAD (Fig. 1 and 2); quantification of the oligosaccharide yields was achieved by HPLC-RI (Table 1). Sucrose consumption was dependent on the acceptor carbohydrate; unreacted sucrose remained in reactions with glucose or kojibiose as acceptors (Fig. 1). Homologous oligosaccharide series were obtained with all acceptor carbohydrates except kojibiose; high oligosaccharide yields were obtained with isomaltose and maltose whereas the lowest oligosaccharides yields were obtained with glucose (Fig. 1 and Table 1). Plotting the log(DP) versus the retention time demonstrated linear relationships for all acceptor carbohydrates (Fig. 2). The retention times (Rt) order of disaccharides with different α-linkage types increases in the order α-(1,6), α-(1,2), α-(1,3), and α-(1,4) (Koizumi et al, 1989) (Table S1). Compared with trisaccharides with different α-linkage types, the Rt difference between α-(1→6)-α-(1→6) and α-(1→6)-α-(1→4) is 3.7 min. The Rt difference between α-(1→6)-α-(1→4) and α-(1→4)-α-(1→4) was 1.9 min. The use of isomaltose, isomaltotriose, and panose as external standards and the log-linear relationship of Rt and DP demonstrated that homologous series of oligosaccharides are obtained by α-(1→6) extension of the acceptor carbohydrate.

The acceptor reaction with glucose showed IMO with DP of 2 – 5 as main products but oligosaccharides with Mw up to DP 30 were also detected (Fig. 1 and 2). With glucose, isomaltose,

and isomaltotriose as acceptors, the di-acceptor products were obtained with the highest yield (Fig. 1 and Table 1). Products of the acceptor reaction were quantified on a Supelcosil-NH_2-column with RI detection (Table 1). The oligosaccharide yield with isomaltose as acceptor was virtually quantitative, indicating that oligosaccharides are the predominant product of catalysis. The oligosaccharide yield with maltose was 95%, maltotriose and isomaltotriose yielded 39 and 49%, respectively. A yield of 25% was obtained with glucose (Table 1). DsrM thus preferred DP2>DP3>DP1 and disaccharides with linkage type α-(1→6) > α-(1→4) > α-(1→3) > α-(1→2) as acceptor carbohydrates.

3.2 Determination of the efficacy of other acceptor carbohydrates

Fructose, galactose, lactose, melibiose, raffinose, xylose, and arabinose were also employed in the acceptor reaction. In all these acceptor reactions, small amounts of leucrose, an isomer of sucrose, D-glu-α-(1,5)-D-fructopyranose eluting at 9.4 min, and of IMO were formed, reflecting the availability of fructose and glucose, respectively, as acceptor carbohydrates (Fig. 1, 2, and Fig. S3 of the online supplementary data). The formation of leucrose increased in reactions with fructose as acceptor (Fig. S3). Using other non-glucan acceptor carbohydrates, monosaccharides (arabinose, galactose, and xylose) were not effective glucosyl acceptors. Small peaks that possibly reflect monoglycosylation were observed with raffinose, galactose, and arabinose as acceptors; small peaks indicating glucosylation of lactose and melibiose were observed at 13.9 and 13.5 min, respectively. Reactions with sucrose (Figure 2), as well as xylose, galactose, lactose, and melibiose (data not shown) also yielded small amounts of IMO with a DP ranging from 7 to > 25, demonstrating that glucose was a predominant acceptor in these reactions.

3.3 Effect of acceptor carbohydrates on yield and size of polysaccharides produced by DsrM.

To investigate the effect of different molar ratios of sucrose to maltose or glucose on the DP of the products, the size distribution of poly- and oligosaccharides was analyzed by SEC and AF4. These two methods provide complementary information. Separation on SEC with the Superdex peptide column separates oligosaccharides with a M_W ranging from 10^2 to 10^4; AF4 separates polysaccharides with a M_W higher than 10^4. DsrM reactions with sucrose as sole substrate produced almost exclusively polysaccharides with a relative M_W higher than 10^4 (Chen et al., 2016 and data not shown). Addition of maltose or glucose increased the ratio of oligosaccharides to polysaccharides in a dose- and acceptor dependent manner (Figure 3). Oligosaccharides with a M_W lower than 2800 were the main products of sucrose conversion when equimolar concentrations of maltose were present (Fig. 2 and Fig. 3A). Products obtained with glucose had a higher M_W, oligosaccharides with a DP of 7 – 17 were the main products when sucrose and glucose were present in equimolar concentrations (Fig. 1 and 3B).

The size distribution of polysaccharides produced in presence or absence of acceptor carbohydrates was analyzed with AF4-MALS. In the absence of acceptor carbohydrates, DsrM produced dextran with a relative M_W of 2×10^8 (Fig. 4). Addition of glucose as acceptor reduced the yield but not the average M_W of dextran (Fig. 4). In contrast, the relative M_W of dextran produced in presence of maltose acceptor ranged from 10^5 to 10^7, demonstrating that the yield as well as the M_W of dextran was reduced.

3.4 Oligosaccharide formation with a commercial IMO preparation as acceptors

Commercial IMO preparations produced from starch consist predominantly of isomaltose-series oligosaccharides with a DP of 2 – 4, and panose series oligosaccharides with a DP of 3 – 5 (Goffin et al., 2011; Hu et al., 2013) and are thus partially digestible. To determine whether the M_W of commercial IMO is modified by DsrM, IMO were incubated with 500 mM sucrose and

296 DsrM, and the resulting pattern and yield of oligosaccharides were determined by HPAEC-PAD
297 and SEC, respectively (Fig. 5 and 6). The ratio of IMO to sucrose ranged from 2.5 % w/v IMO to
298 20 % w/v IMO. All reactions produced predominantly oligosaccharides; polymeric dextran was
299 present only at low concentrations, or below the detection limit (Fig. 6). At a low concentration of
300 IMO (2.5%), the main products obtained were IMO (Fig. 5) with a relative M_W higher than 2800
301 (Fig. 5 and 6). With increasing IMO concentration, the average M_W of products was reduced and
302 panose-series oligosaccharides were more prominent products (Fig. 5 and 6). This pattern of
303 products reflect the preferential glycosylation of α-(1→6) over α-(1→4) linked disaccharides, and
304 of disaccharides over trisaccharides that was observed with pure compounds (Table 1). When 20%
305 commercial IMO were added as acceptor, IMO-series oligosaccharides with DP of 3 – 7 and
306 panose-series oligosaccharides with DP of 4 – 7 were the major components (Fig. 5 and 6).
307 Oligosaccharide analysis by HPAEC-PAD and SEC thus consistently demonstrated that the
308 conversion of commercial IMO with DsrM and sucrose allows the controlled extension of the DP
309 by 2 – 5; the increase of the M_W can be controlled by the ratio of IMO to sucrose.

310 *3.4* In vitro *digestibility of oligosaccharides produced by dextransucrase*

311 Sucrose, maltose, and isomaltose are digestible disaccharides but the digestibility of IMO and
312 panose-series oligosaccharides with a DP of 3 or higher is unknown. The *in vitro* digestibility of
313 acceptor products obtained with maltose and the commercial IMO was compared to the acceptor
314 carbohydrates with two *in vitro* methods (Figure 7). Maltose and resistant maltodextrins were used
315 as digestible and non-digestible controls, respectively (Figure 7). Commercial IMO were partially
316 digestible with both methods. Extension of the commercial IMO with DsrM significantly reduced
317 their digestibility (*P=0.011*). Remarkably, the digestibility of panose-series oligosaccharides was
318 not different from maltose when amyloglucosidase was present but the same oligosaccharides were

only partially digestible when intestinal brush border enzymes α-glucosidases were used to simulate digestion (Figure 7).

4. Discussion

This study qualitatively and qualitatively assessed the acceptor reaction of DsrM from *W. cibaria* through systematic variation of acceptor carbohydrates differing in DP and linkage type. Multiple chromatographic methods identified and quantified oligosaccharides and polysaccharides produced by DsrM, and determined their DP. The digestibility of reaction products was evaluated *in vitro*. Results extend current knowledge on the acceptor reaction of dextransucrases, and improve the toolset for tailored production of oligosaccharides with specific molecular weight distribution and digestibility.

The transfer reaction of dextransucrase yields polysaccharides or oligosaccharides depending on the type and concentration of acceptor carbohydrates. Dextransucrase activity in presence of sucrose only yields predominantly dextran; suitable acceptor carbohydrates shift the reaction to oligosaccharides (Robyt and Eklund, 1983; Demuth et al., 2002; Shi et al., 2016). Accordingly, glucose and α-linked disaccharides composed of glucose were suitable acceptors for DsrM. Quantitation of oligosaccharides after systematic variation of linkage type and DP of acceptor products, and assessment of the molecular weight distribution of oligo- and polysaccharides demonstrated that isomaltose was the most efficient acceptor carbohydrate for oligosaccharide synthesis. This contrasts prior observations with dextransucrases from *Leuconostoc* spp. (Robyt and Eklund, 1983; Demuth et al., 2002; Shi et al., 2016) and may relate to differences between dextransucrases from *W. cibaria* and *L. mesenteroides* (Chen & Gänzle, 2016). The use of trisaccharides as acceptors decreased the transglycosylation efficiency. Conditions that match the optimum of the maltose acceptor reaction rate for *L. mesenteroides* dextransucrase, 0.5 M maltose

and 0.5 M sucrose (Robyt and Eklund, 1983, Heincke, Demuthy, Jördening & Buchholz, 1999; Shi et al, 2016), allowed fast conversion of sucrose; however, a residue of the acceptor carbohydrate remained.

Separation of homologous series of gluco-oligosaccharides by HPAEC-PAD provides a log-linear relationship of degree of polymerization and the retention factor k (the ratio of the adjusted retention time and the void volume) (Koizumi, Kubota, Tanimoto, & Okada, 1989; Demuth et al., 2002). Corresponding analysis demonstrated that DsrM synthesizes homologous series of oligosaccharides with different acceptors with minimal formation of branched oligosaccharides that were observed with the recombinant dextransucrase E392-rDSR from *W. confusa* (Shi et al., 2016).

The efficient acceptors maltose and isomaltose produced oligosaccharides with a DP ranging from 3 – 10 with a high yield while maltotriose, isomaltotriose, glucose, and nigerose generated lower oligosaccharide yields but generated oligosaccharides with a higher DP (Table 1, Fig 1 and Fig. 2). The molecular weight of dextran produced by DsrM was not only related to the type of acceptor but also to its concentration. The use of maltose and sucrose in equimolar concentrations virtually abolished dextran formation and resulted in a strongly reduced molecular weight of the dextran formed; in contrast, the use of glucose and sucrose in equimolar concentrations decreased the yield of dextran formation but not the molecular weight of the polysaccharide (Fig. 3 and Fig. 4). Taken together, our analyses provide an unprecedented control of the yield and DP of poly- and oligosaccharides produced in the acceptor reaction with dextransucrase, and hence their functional properties in food applications.

The use of starch as a relatively cheap substrate in commercial production of IMO leads to products with a substantial proportion of disaccharides (Hu et al., 2013; Madsen et al., 2017) which are

365 excluded from the U.S. definition of dietary fibre and are partially digestible (Hooton et al., 2015;
366 Anonymous, 2016). Moreover, the current AOAC method for quantification of dietary fibre in
367 foods does not account for (commercial) IMO because amyloglucosidase degrades α-(1→4) as
368 well as α-(1→6) linked gluco-oligosaccharides (McCleary et al., 2013; McCleary, 2014). The
369 combination of the conversion of starch and sucrose as substrates for IMO production may be
370 advantageous because it includes starch as a cheap carbohydrate source but additionally allows
371 modification of DP and linkage type through the dextransucrase acceptor reaction. This study
372 provided proof of concept for this approach by employing a commercial IMO preparation as
373 acceptor for DsrM (Hu et al., 2013; Madsen et al., 2017). Because isomaltose and panose are
374 efficient acceptor carbohydrates for dextransucrases (Shi et al., 2016; Table 1 and Fig. 2),
375 commercial IMO were efficient as acceptors for DsrM. Addition of 30% commercial IMO as
376 acceptor minimized dextran synthesis and yielded isomaltose-series oligosaccharides with DP of
377 3 – 7 and panose-series oligosaccharides with DP of 4 – 7 as major components (Fig. 5 and 6).
378 Human studies on the digestibility of commercial IMO demonstrate that these are partially digested
379 and absorbed in the small intestine while other components are fermented by the intestinal
380 microbiota (Kohmoto et al, 1992; Oku & Nakamura, 2003), however, the digestibility of individual
381 components of commercial IMO remains poorly documented. Current methods for determination
382 of starch digestibility *in vitro* employ amyloglucosidase in addition to pancreatic enzymes (Englyst
383 et al., 1996; van Kempen et al., 2010) and may thus not be suitable for IMO. The use of intestinal
384 extracts from pigs or rats in *in vitro* digestion protocols was proposed to more accurately reflect
385 the digestibility of IMO by brush border α-glucosidases (Tsunehiro et al., 1999; McCleary et al.,
386 2013; Tanabe, Nakamura & Oku, 2014). α-Glucosidases from pancreatin are highly specific for α-
387 (1→4) linked glucosides (Champ, Martin, Noah, & Gratas, 1999) while brush border

α-glucosidases also recognize isomaltose as a substrate (Tsunehiro et al., 1999; Tanabe et al., 2014). This study compared the digestibility of commercial IMO and panose-series oligosaccharides by two *in vitro* methods employing pancreatic enzymes and amyloglucosidase, or intestinal α-glucosidases. In keeping with prior observations, maltose and resistant maltodextrins were digestible and indigestible, respectively. Commercial IMO were approximately 50% digestible; when accounting for differences in product composition, this value conforms reasonably well to the digestibility of commercial IMO that was observed in human volunteers (Kohmoto et al, 1992; Oku & Nakamura, 2003). The digestibility of the dextransucrase-extended commercial IMO was significantly reduced in comparison to commercial IMO. The discrepancy between the *in vitro* digestibility of commercial and experimental IMO in protocols employing pancreatic enzymes and amyloglucosidase, or intestinal enzymes, was related to the digestibility of panose-series oligosaccharides. Panose and panose series oligosaccharides are hydrolysed by amyloglucosidase but are digested more slowly than maltose by α-glucosidases from the pig or rat intestine (McCleary et al., 2013; this study). Moreover, both amyloglucosidase and brush border enzymes hydrolyze α-(1→6) linked oligosaccharides, however, brush border isomaltase is confined to membrane vesicles and their activity is limited by the membrane permeability of the substrates (Hooton et al., 2015; Tanabe et al., 2014). The *in vivo* digestibility of oligosaccharides with defined molecular weight, however, remains to be determined.

In conclusion, the efficiency of α-glucans as acceptor carbohydrates for DsrM of *W. cibaria* decreases in the order DP2 > DP3 > DP1; among disaccharides, the efficiency decreases in the order α-(1→6) > α-(1→4) > α-(1→3); the α-(1→2) linked kojibiose does not support oligosaccharide formation in the acceptor reaction of DsrM. Equimolar addition of efficient acceptor molecules and sucrose shifted the dextransucrase reaction to oligosaccharides as virtually

exclusive product. In keeping with the observation that α-(1→6) linked di- and trisaccharides are efficient acceptor carbohydrates, DsrM readily extended commercial IMO by adding one to three α-(1→6)-linked glucose moieties. Conversion of commercial IMO by dextransucrase reduced their *in vitro* digestibility. However, *in vivo* data on the digestibility of tri- and tetrasaccharides with α-(1→6) is insufficient (Kohmoto et al, 1992; Oku & Nakamura, 2003) and *in vitro* protocols to determine digestibility provide conflicting results depending on the enzyme source.

Acknowledgements. The National Science and Engineering Research Council of Canada (NSERC) and BioNeutra Inc. are acknowledged for financial support under the Collaborative Research and Development program.

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Figure legends.

Figure 1. Analysis of gluco-oligosaccharides produced by recombinant dextransucrase DsrM by HPAEC-PAD. Oligosaccharides were produced by incubation of DsrM for 24 h with 500 mM of sucrose and 500 mM of acceptor carbohydrates indicated in the panels. Peaks that were identified by external standards are labeled; arrows indicate oligosaccharides that were obtained by successive α-(1→6)-glucosyl transfer. Minor peaks are amplified fourfold as gray inlets. The chromatograms represent two biological repeats.

Figure 2. HPAEC-PAD analysis of oligosaccharides produced by recombinant dextransucrase DsrM. The log-transformed degree of polymerization is plotted against K. Lines were obtained by linear regression; all correlation coefficients (r^2) were greater than 0.999. Identical graphs were obtained with glucose, isomaltose, or isomaltotriose as acceptors and only one representative graph is shown. The product with the smallest molecular weight represents the mono-acceptor product of the series with DP2, 3, or 4 depending on the acceptor carbohydrate. Acceptor products obtained with sucrose were superimposed with acceptor products obtained with glucose.

Figure 3. Effect of maltose (**Panels A and C)** and glucose **(Panels B and D)** on the size distribution of isomaltooligosaccharides (**Panels A and B) and** α-glucans (**Panels C and D)** produced in enzymatic reactions with DsrM for 24 h. Reactions were conducted with 500 mM sucrose and the acceptor concentration indicated on the x-axis. Enzymatic reactions were analysed by HPSEC-RI with glucose, lactose, raffinose and dextrans as external standards; peaks were integrated as α-glucans with M_W of more than 8000 (■/□), as α-glucans with Mw of 2800 to 8000 (●/○), and as oligosaccharides with M_W between 500 to 2800 (▲) or 300 to 2800 (Δ). Data are shown as means ± standard deviation of two independent experiments analyzed in duplicate.

Figure 4. Cumulative weight distribution of dextran produced by DsrM with 500 mM sucrose and no acceptor sugar (○), 500 mM maltose (▲), or 500 mM glucose (●) for 24 h in the presence of 500 mM sucrose. The molecular weight distribution was analyzed by asymmetric field-flow fractionation coupled with multi angle laser scattering detector.

Figure 5. Effect of the concentration of isomaltooligosaccharides on the formation of oligosaccharides. Enzymatic reactions used DsrM for 24 h in the presence of 500 mM sucrose and different concentrations of a commercial IMO preparation as acceptor carbohydrate. Separation of the IMO preparation is shown in the upper panel as reference. Enzymatic reactions were analyzed by HPAEC-PAD. Peaks that were identified by external standards are indicated. Chromatograms are representative of two independent experiments.

Figure 6. Size distribution of isomaltooligosaccharides **(Panel A)** and α-glucans **(Panel B)** produced in enzymatic reactions with DsrM for 24 h. Reactions were conducted with 500 mM sucrose and a commercial IMO preparation as acceptor at concentration indicated on the x-axis. Enzymatic reactions were analysed by HPSEC-RI with glucose, lactose, raffinose and dextrans as external standards; peaks were integrated as α-glucans with M_W of more than 8000 (■), as α-glucans with Mw of 2800 to 8000 (●), and as oligosaccharides with M_W between 500 to 2800 (▲). Data are shown as means ± standard deviation of two independent experiments analyzed in duplicate.

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for different oligosaccharides that were obtained with the same *in vitro* protocols and do not share a common superscript differ significantly ($P<0.05$). Values for the same oligosaccharides that were obtained with the two *in vitro* protocols differ significantly ($P<0.05$) if marked by an asterisk.

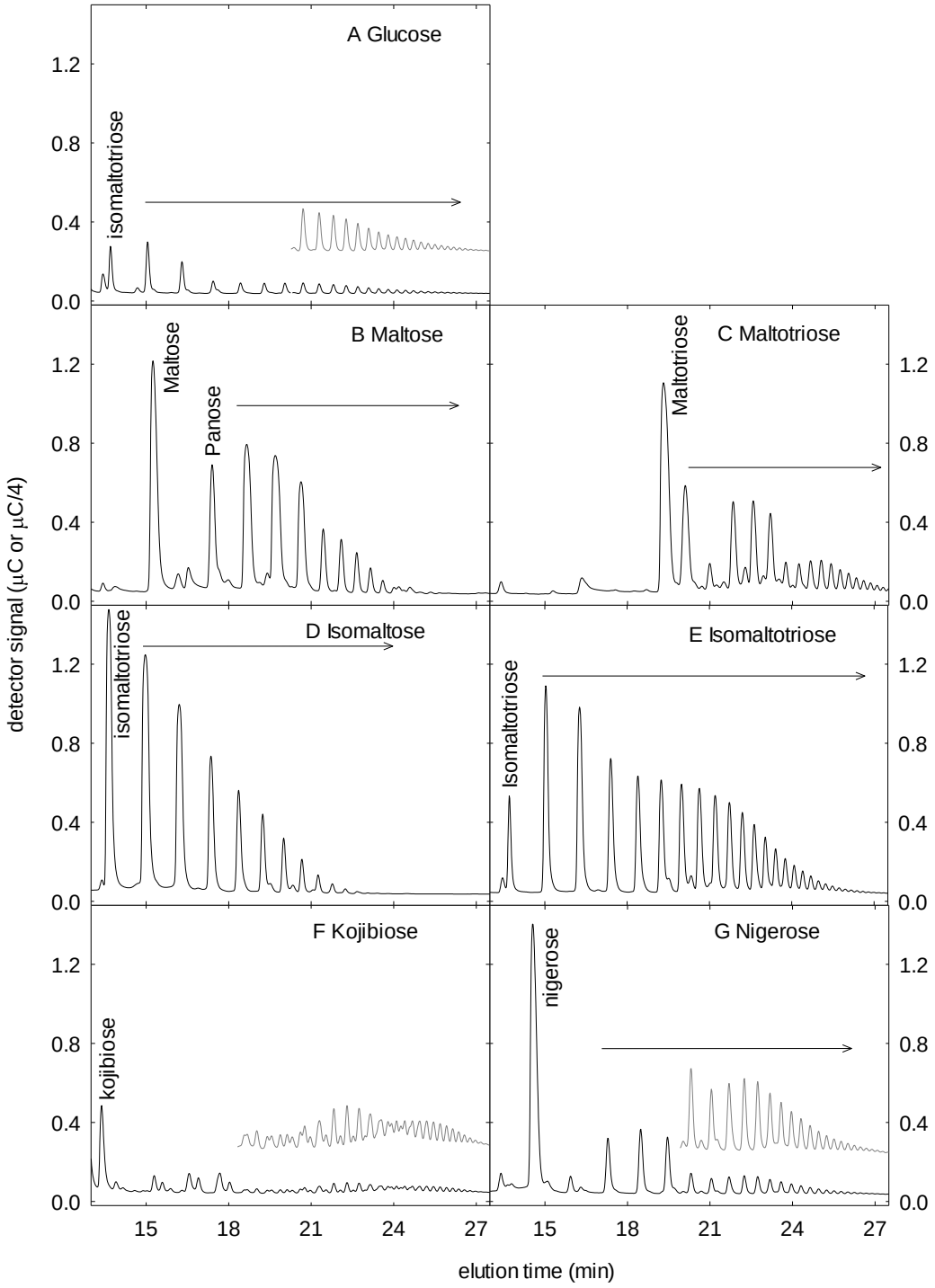
Table 1. Effect of acceptor carbohydrates on the yield of oligosaccharides in the acceptor reaction analyzed by HPLC. Data are shown as means ± standard deviation of two biological repeats. Values in the same row differ significantly ($P<0.05$) if they do not share a common superscript.

(mM) [a]	glucose	maltose	isomaltose	nigerose	maltotriose	isomaltotriose
DP3	7.2 ± 1.3^{d}	31.9 ± 3.8^{b}	46.0 ± 2.3^{a}	18.6 ± 1.0^{c}	n/a	n/a
DP4	6.4 ± 0.5^{e}	29.8 ± 2.6^{b}	50.2 ± 2.5^{a}	28.6 ± 0.3^{b}	23.4 ± 0.3^{c}	12.7 ± 0.5^{d}
DP5	4.8 ± 0.5^{e}	27.4 ± 1.9^{b}	40.5 ± 1.9^{a}	26.6 ± 0.2^{b}	12.0 ± 0.1^{d}	16.5 ± 0.2^{c}
DP6	1.7 ± 0.7^{e}	28.2 ± 0.8^{a}	24.9 ± 1.0^{b}	10.7 ± 0.2^{d}	13.2 ± 0.3^{c}	12.8 ± 0.3^{c}
DP7	1.7 ± 0.6^{e}	19.2 ± 0.1^{a}	16.7 ± 0.5^{b}	6.2 ± 0.1^{d}	10.5 ± 0.4^{c}	11.1 ± 0.0^{c}
DP8	2.7 ± 0.2^{f}	8.5 ± 0.4^{c}	10.4 ± 0.2^{a}	4.5 ± 0.1^{e}	5.5 ± 0.2^{d}	9.3 ± 0.0^{b}
DP9	2.1 ± 0.2^{d}	$4.3\pm0.3^{b,c}$	7.7 ± 0.8^{a}	4.8 ± 0.0^{b}	3.7 ± 0.0^{c}	7.5 ± 0.1^{a}
DP10	2.1 ± 0.4^{b}	2.1 ± 0.1^{b}	2.3 ± 0.5^{b}	0.7 ± 0.0^{c}	$2.8\pm0.1^{a,b}$	3.3 ± 0.1^{a}
Yield of OS (%)[b]	25 %	95%	110 %	59 %	39%	49%

[a] The dn/dc obtained for isomaltotriose was used to calculate the concentration of other α-glucooligosaccharides.

[b]The oligosaccharide yield was calculated as

$$Yield\ (\%) = 100\frac{mol\ glucose\ transferred\ to\ oligosaccharides}{mol\ glucose\ in\ the\ initial\ substrate\ sucrose}$$



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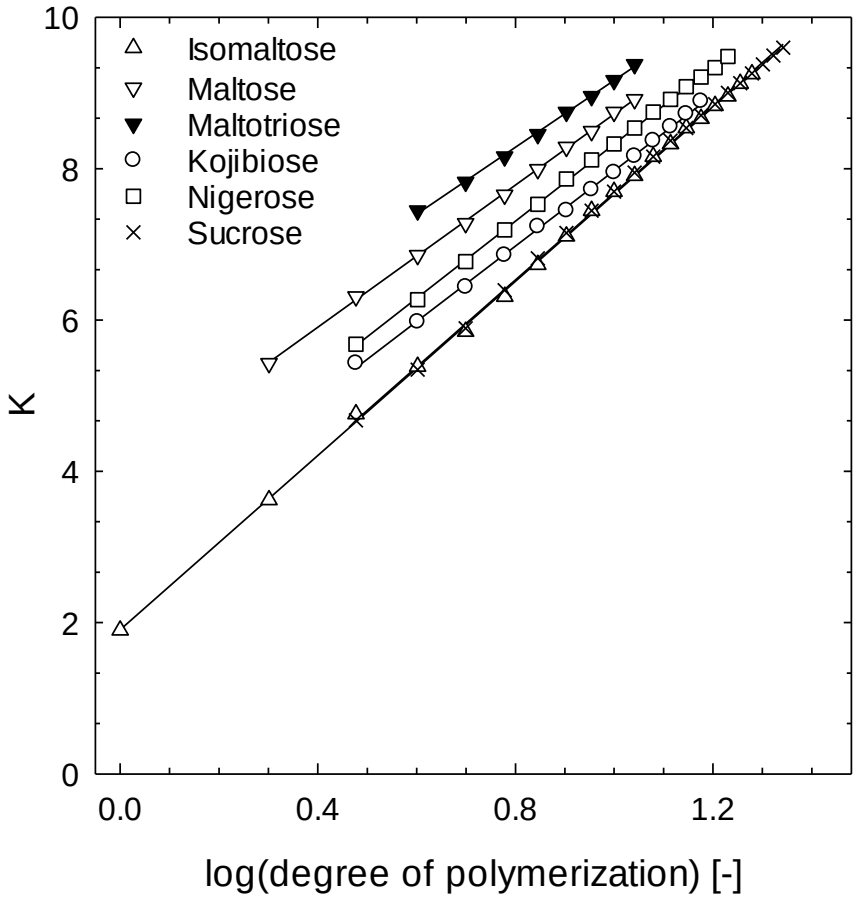

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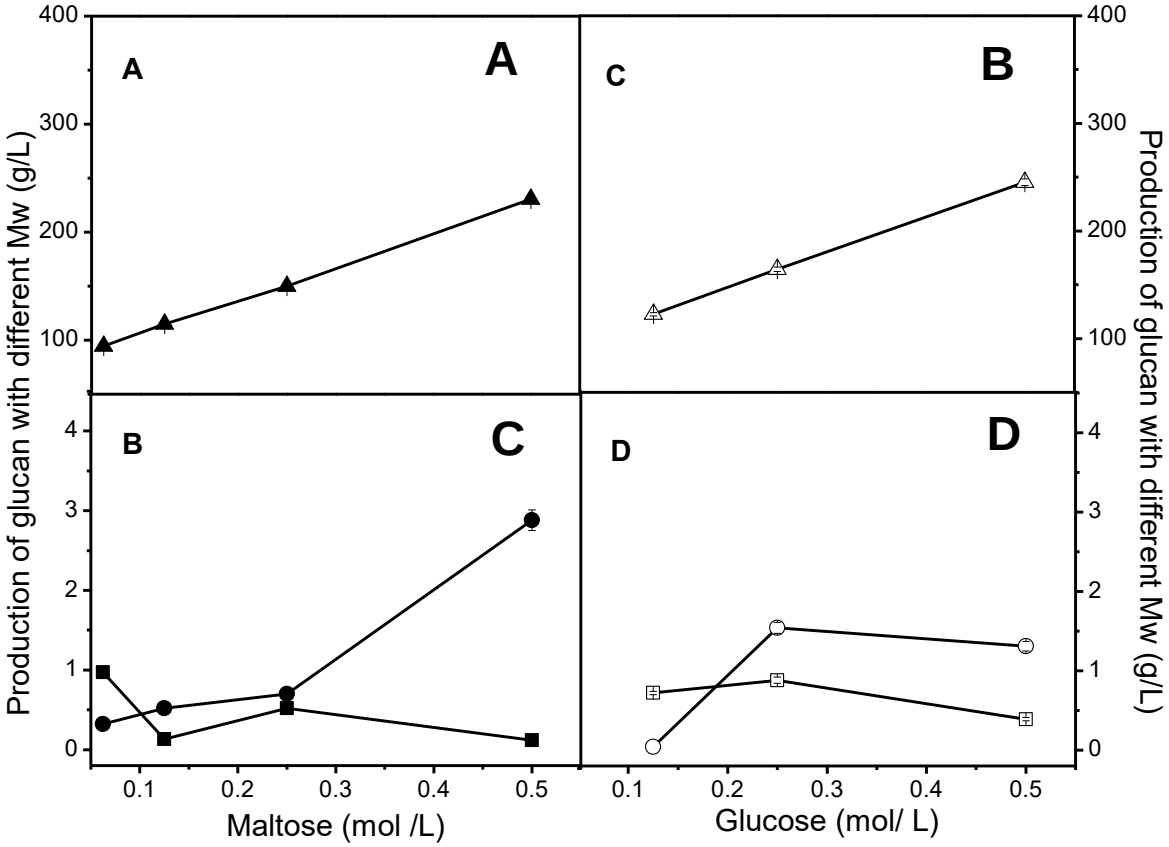

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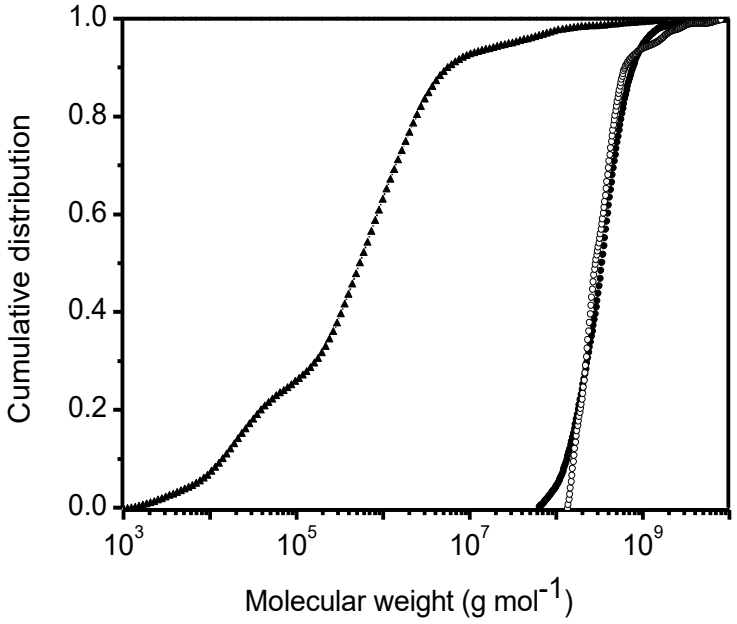

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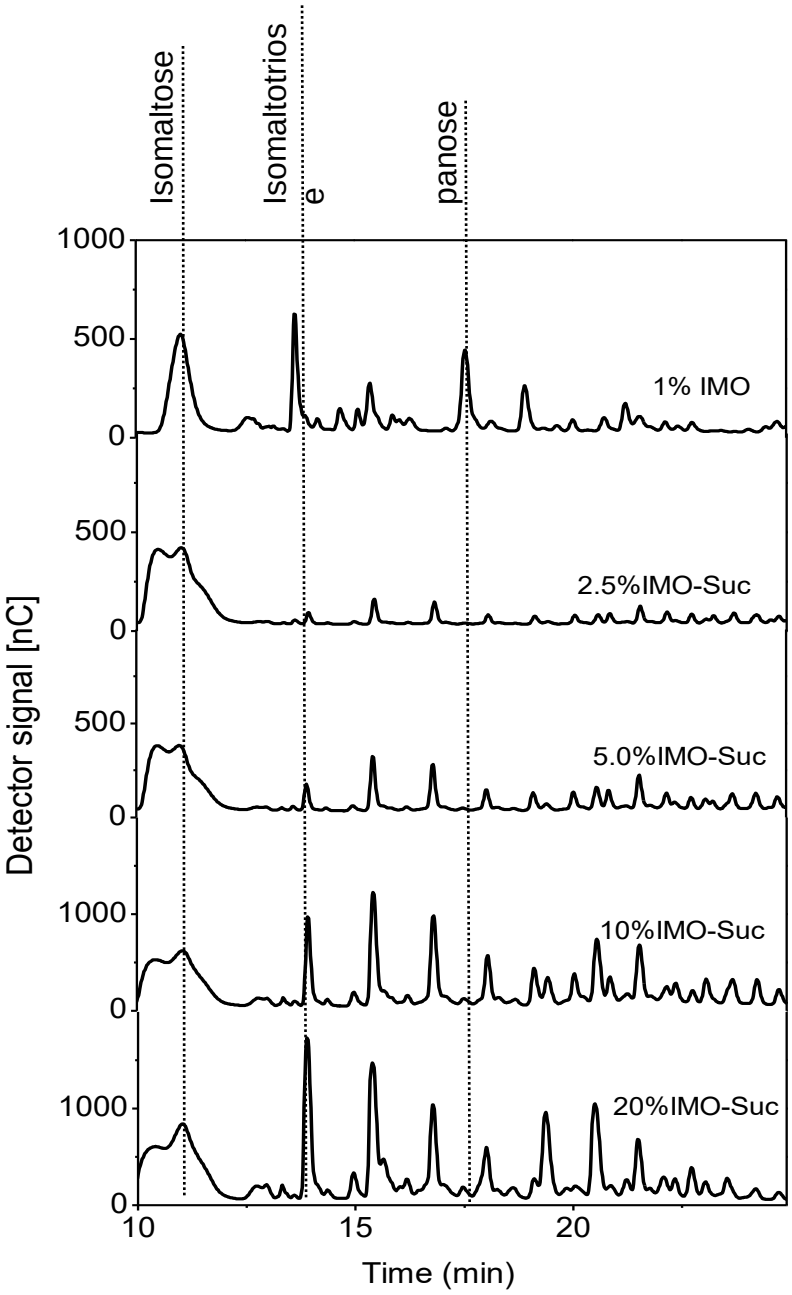

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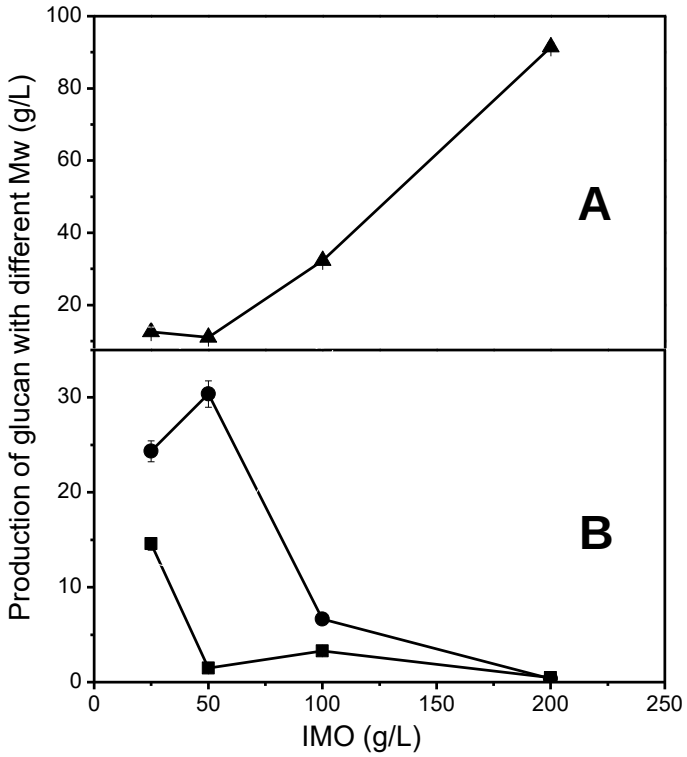

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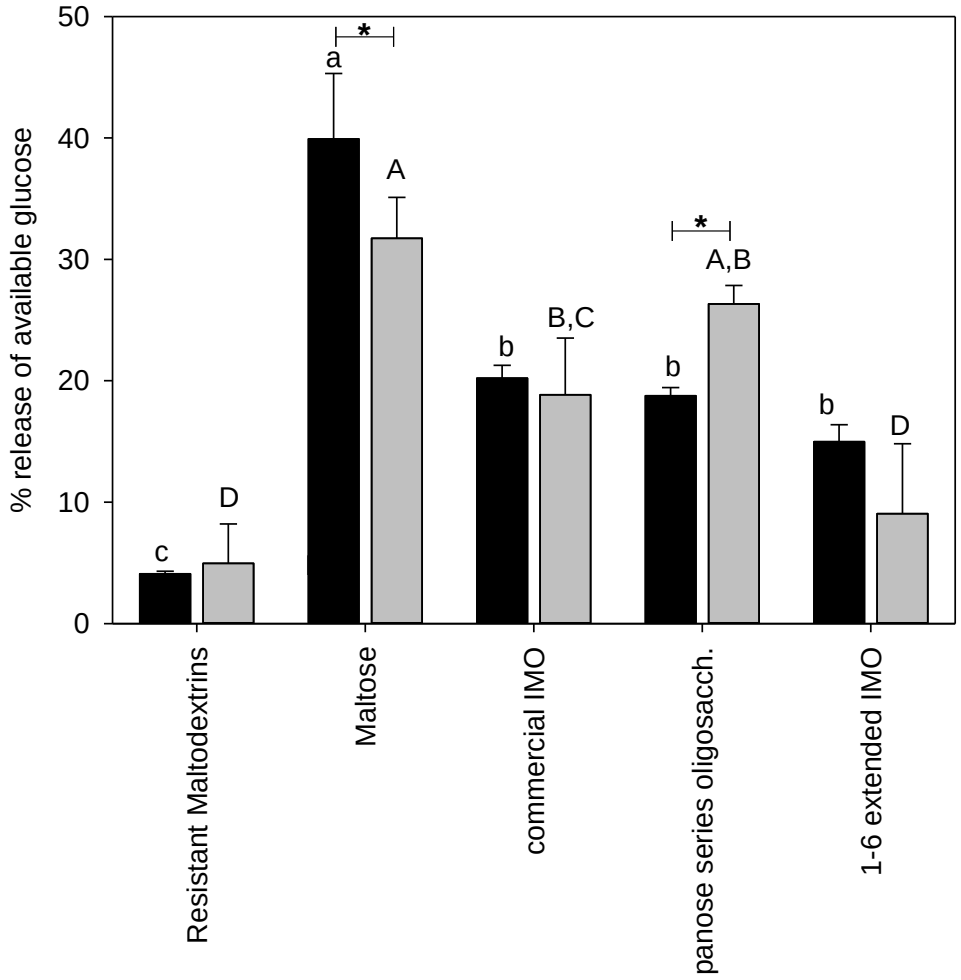

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