Effect of temperature on production of oligosaccharides and exopolysaccharides by *Weissella cibaria* 10M

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Abstract

The formation of HoPS and oligosaccharides in sourdough fermentation improves bread quality but is dependent on the expression of glycansucrases by lactic acid bacteria. Data on the expression of dextransucrases by *Weissella* spp., however, are limited. This study therefore aimed to assess dextansucrase expression in *W. cibaria* 10M, focusing on the effect of temperature. The effect of temperature on growth, oligosaccharide and dextran synthesis by *W. cibaria* 10M was determined and the expression and activity of cell-associated dextransucrase from *W. cibaria* 10M were investigated. The oligosaccharides profiles were measured by thin layer chromatography and high performance anion exchange chromatography coupled to pulsed amperometric detection. Dextran formation was quantified by size exclusion chromatography. *W. cibaria* grew fastest at 30 °C but oligosaccharide formation was highest at 20 °C or less. Dextransucrase expression as measured by reverse transcription quantitative PCR, SDS-PAGE, and activity of cell-associated dextransucrase were maximal at 15 °C. Cold shift incubation, characterized by incubation at 30 °C to obtain biomass, followed by shift to 6 °C to induce dextransucrase expression, supported high dextransucrase activity in laboratory media. Cold shift fermentation of wheat and sorghum sourdoughs supplemented with 15 or 30% sucrose increased the yields of oligosaccharides, and resulted in formation of 16 and 12 g / kg dextran in wheat and sorghum sourdoughs, respectively. Dextran formation was decreased in favour of oligosaccharide formation when doughs were supplemented with maltose. In conclusion, cold shift fermentation of sourdough with *W. cibaria* supports high dextran yields or formation of oligosaccharides without excess acidification.

Keywords: Isomalto-oligosaccharides; exopolysaccharides; dextran, dextransucrase, *Weissella cibaria*; sourdough, sorghum.

1. Introduction

The formation of extracellular homopolysaccharides (HoPS) by lactic acid bacteria (LAB) during sourdough fermentation improves texture and storage life of bread (Galle and Arendt, 2014; Tieking and Gänzle, 2005). HoPS formation is accompanied by formation of oligosaccharides, which add nutritional functionality to baked goods through their prebiotic activity (Yan et al., 2018; Schwab et al., 2008). HoPS and oligosaccharide formation by lactic acid bacteria is dependent on the availability of sucrose, and is mediated by extracellular fructansucrases or glucansucrases (Tieking et al., 2003; van Hijum et al., 2006). These enzymes frequently occur in the species *Streptococcus mutans*, in species of the *L. reuteri* group, and in the genera *Leuconostoc* and *Weissella* (Bounaix et al., 2010; Galle et al., 2010; Tieking et al., 2003; van Hijum et al., 2006). Strains of the vertebrate host-adapted *L. reuteri* group are commonly isolated from type II sourdoughs; *Leuconostoc* spp. and *Weissella* spp. occasionally occur in spontaneous sourdoughs or in type I sourdoughs (Gänzle and Ripari, 2016; Gobbetti et al., 2016; Latttanzi et al., 2013). Among HoPS producing lactic acid bacteria, the use of *Weissella* spp. is considered advantageous because *Weissella* ssp. produce high molecular weight dextrans (Chen et al., 2016; Katina et al., 2009). Moreover, *Weissella cibaria* and *W. confusa* generally do not express mannitol dehydrogenase activity and thus accumulate less acetic acid when compared to *Leuconostoc* spp. or lactobacilli (Galle, et al 2010).

Glucansucrases are GH70 enzymes and are categorized based on the linkage types of the glucans formed (van Hijum et al., 2006). Dextran, a α-(1→6) linked glucan with α-(1→2,6) or (1→3,6) branching points, is produced by dextransucrases of *Leuconostoc* spp., *Weissella* spp. and lactobacilli (Bounaix et al., 2010; Naessens et al., 2005; van Hijum et al., 2006). Dextran is one of the first biopolymers produced on an industrial scale and is applied in medicine, separation

technology, and as food hydrocolloid (Naessens et al., 2005). Dextransucrases transfer the glycosyl moiety from sucrose to acceptor sugars or to a growing glucan chain to produce α-(1→6) linked glucan-oligosaccharides or dextran, respectively (van Hijum et al., 2006). The ratio of oligosaccharides to polysaccharides depends on the concentration of suitable acceptor carbohydrates (Hu et al., 2017; Robyt and Eklund, 1983). Maltose, an abundant sugar in wheat sourdough but not in sorghum sourdoughs, is a preferred glucosyl acceptor for dextransucrases (Galle et al., 2010; Hu et al., 2017). Oligosaccharide formation in sourdough does not impact bread texture, however, α-(1→6) linked gluco-oligosaccharides with a DP higher than 3 increase the fibre content of bread (Yan et al., 2018; Hu et al., 2017; Schwab et al., 2008).

The formation of HoPS and oligosaccharides in sourdough fermentation is also dependent on the expression of glucansucrases by lactic acid bacteria. Reuteransucrases in *L. reuteri* are expressed constitutively (Schwab et al., 2006) while dextransucrase expression in *Leuconostoc mesenteroides* is induced by sucrose (Quirasco et al., 1999). Data on the expression of dextransucrases by *Weissella* spp., however, are limited (Bounaix et al., 2010). This study therefore aimed to assess dextansucrase expression in *W. cibaria* 10M, focusing on the effect of temperature, a major parameter for fermentation control in industrial and artisanal fermentations (Gänzle et al., 1998). Oligosaccharide formation was quantified by high performance anion exchange chromatography with pulse amperometric detection (HPAEC-PAD) and thin layer chromatography (TLC) was used as a fast analysis method; dextran was quantified by size exclusion chromatography and its size was assessed by asymmetric flow field flow fractionation coupled to multi-angle laser scattering. Dextran- and oligosaccharide formation during growth in laboratory media was compared to product formation in wheat and sorghum sourdoughs as substrates differing in the content of maltose as glucosyl acceptor.

2. Materials and Methods

2.1 Strains and growth conditions

Weissella cibaria 10M expressing the dextransucrase DsrM (Chen et al., 2016) was routinely cultivated on modified de Man Rogosa Sharpe (mMRS) agar (Schwab et al. 2008) from glycerol stock stored at -80 °C. For preparation of working cultures, colonies were picked from the agar plate, cultured at 30 °C in mMRS broth containing 24 mM maltose, 22 mM glucose and 22 mM fructose for 16 h, and subcultured in MRS containing 85 mM sucrose (sucMRS) as sole carbon source for 16 h. Media were prepared by adding filter sterilized sugar solutions to autoclaved basal media.

2.2 Growth curve of W. cibaria *10M*

W. cibaria 10M was subcultured with a 1% inoculum in mMRS broth and incubated at 6, 20, and 30°C. Samples were taken periodically for determination of the pH and the optical density (OD). The optical density was determined with 200 µL samples at 600 nm in a micotitreplate reader.

2.3 Effect of temperature on dextran synthesis by W. cibaria *10M in the presence of 0.5 M sucrose*

W. cibaria 10M was subcultured at 1% inoculum in MRS broth with 0.5 M sucrose, incubated at 30°C to the exponential phase of growth, corresponding to an optical density (OD) at 600nm of 0.2-0.4, and further incubated at 6, 15, 20, 25, or 30 °C for 24 h. Cells were removed by centrifugation for 20 min at 4 °C and polysaccharides in the supernatant were precipitated by addition of two volumes of cool ethanol, followed by incubation at 4 °C overnight. Polysaccharides were re-dissolved in water, insoluble material was removed by centrifugation, and polysaccharides in the supernatant were precipitated again with ethanol, collected by

centrifugation, and freeze dried. The size distribution of polysaccharides was analyzed by size exclusion chromatography using a Superdex 200 column (GE Healthcare Life Sciences, Mississauga, ON, Canada) eluted with water at a flow rate of 0.3 mL / min. Polysaccharides were quantified with a refractive index (RI) detector using dextran preparations (Sigma-Aldrich, Oakville, ON, Canada) with a molecular weight ranging from 5×10^6 to 4×10^7 as standard. Reactions were carried out in duplicate or triplicate.

2.4 Effect of temperature on oligosaccharide synthesis by W. cibaria *10M in the presence of 0.5 M sucrose and 0.25 M maltose*

W. cibaria 10M was subcultured at 1% inoculum in MRS broth with 0.5 M sucrose and 0.25 M maltose and incubated at 20, 25, 30 °C for 24 h. Cells were removed by centrifugation and the supernatant was heated to 90 °C for 10 min to inactivate extracellular dextransucrase. Samples were diluted 100-fold with water, filtered by 0.22 µm filter, and oligosaccharides were analyzed by a by high performance anion exchange chromatography coupled to pulsed amperometric detection (HPAEC-PAD) on a Carbopac PA20 column (Dionex, Oakville, Canada) as described (Schwab et al., 2008). Other microbial metabolites were quantified with an RI detector after separation on an Aminex 87H column as described (Galle et al., 2010).

2.5 Expression and activity of cell-associated dextransucrase from W. cibaria *10M*

W. cibaria was subcultured at 1 % inoculum in MRS broth with 0.5 M sucrose and incubated at 30 °C to the exponential phase of growth, corresponding to an OD_{600nm} of ~0.4. Cultures were then transferred to 6, 15, 20, 25, or 30 °C and further incubated for 24 h. Cells were harvested by centrifugation, resuspended in 1 mL phosphate buffered saline (pH 7.4) and the OD_{600nm} was adjusted 10**.** Cells were lysed by a bead beater. Expression of cell-associated DsrM was assessed by SDS-polyacrylamide gel electrophoresis using a 4-16 % Mini-PROTEAN® TGX™ Precast

Gel (BIO-RAD, Mississauga, Canada) and Coomassie staining. DrsM activity was determined with 50 µL of cell lysate added to 0.45 mL 20 mM Na Acetate (pH 5.2) containing 100 mM sucrose. Reactions were incubated at 4, 6, 15, 20, 25 and 30 °C for 1 - 6 h, stopped by heating to 95 °C for 10 mins, and DsrM activity was determined by enzymatic quantification of fructose (Fructose Assay Kit, Sigma-Aldrich). DsrM activity was normalized by using the protein concentration of the cell lysate as determined by the absorption at 280 nm. The specific hydrolysis and total activity of DsrM (1 U) was defined as release of 1 µmol / min of free fructose or glucose, respectively, released per 1 mg cellular protein.

2.6 Quantification of dsrM *mRNA in cultures of* W. cibaria *10M*

The expression of *dsrM* from *W. cibaria* 10M was quantified by reverse-transcriptase-quantitative PCR (RT-qPCR). Bacteria were grown in the MRS broth containing sucrose at different temperature (6, 15, 20, 25, 30 ^{o}C) to an OD_{600nm} of 0.4. RNA was extracted using RNAprotect Bacteria Reagent and a RNeasy Minikit (Qiagen, USA) and was subjected to DNAase treatment with RQ1 RNase-Free DNase Kit (Promega,Madison, USA) to eliminate genomic DNA. The treated RNA was reverse transcribed to cDNA using QuantiTect Reverse Transcription Kit (Qiagen, USA). qPCR was performed on 7500 Fast Real-Time PCR System (Applied Biosystems, Life Technologies, Burlington ON) with QuantiFast SYBR Green (Qiagen). DNase-treated RNA samples served as negative controls. Relative gene expression was calculated according to Pfaffl (2001) as follows:

$$relative\ gene\ expression = \frac{2^{\Delta Cp_{target}(control-sample)}}{2^{\Delta Cp_{reference}(control-sample)}};$$

where ΔCP is the difference in CP values for control and sample conditions. The gene coding for 16S rRNA was used as reference gene; gene expression was normalized to expression at 30 °C.

The primers used for the quantification of gene expression of *dsrM* are: DSR-F': AGACTGGTGAACGCTTGTATC and DSR-R': CTGCCTGAACTTGTGGATTTG. Results are shown as means ± standard error of the mean of 2 biological replicates each analysed in duplicate or triplicate. Statistical analysis was performed by using one way Anova with Holm-Sidak post hoc analysis (SigmaPlot, version 12.5).

2.7 Production of exopolysaccharides and oligosaccharides by W. cibaria *10M after temperature-shift incubation.*

W. cibaria 10M was subcultured at 1% inoculum in MRS broth with 0.5 M sucrose and 0.25 M maltose and incubated at 30°C until OD_{600nm} of 0.2-0.4. One aliquot of the culture incubated at 30 °C for 48 h, other aliquots were removed, cooled to 6 ºC prior to incubation for 48 h. All reactions were carried out in duplicate or triplicate. Samples were diluted 100-fold with water, filtered by 0.22 um filter, then analyzed by a Carbopac PA20 column coupled to an ED40 chemical detector (Dionex, Oakville, Canada). The carbohydrates in the reaction mixtures for different incubation time with *W. cibaria* 10M in MRS contained 0.25 M sucrose and 0.125 M maltose were also identified by TLC using silica gel plates (Sigma). Glucose, fructose, sucrose, maltose, panose, isomaltotriose (Sigma) and commercial IMO preparation (BioNeutra North America, Edmonton, Canada), served as standards. TLC plates were developed with a solvent system of n-propanol: methanol: ammonia: water =5: 4 : 2: 1 (v/v). Carbohydrates were visualized by spraying 0.5 % diphenylamine and 0.5 % aniline in 95 % ethanol solution and by heating them in an oven at 80 ◦C for 30 min.

2.8 Sourdough fermentation

Working cultures of *W. cibaria* 10M were washed twice with sterile tap water, and re-suspended in 10 mL sterile tap water (10^7 CFU/mL). Dough was prepared with 10 g of commercial whole

wheat flour or sorghum flour, 10 g of culture in tapwater, and different level of sucrose (15 or 30 % relative to the amount of flour used), and with addition of maltose or glucose (equimolar addition relative to the amount of sucrose added). Control doughs were chemically acidified with 4 parts lactic acid (80 %) and one part glacial acetic acids (100 %) and adjust to pH 4. Sourdoughs were fermented at room temperature for 1-2 h (Galle et al., 2012) and then shifted to 6 ^{o}C for 1 d. Cell counts and pH were determined at 0 h and 24 h of fermentation. After 24 h of fermentation, oligosaccharides were extracted with water at 80 °C for 2 h and then analyzed by HPAEC-PAD. Plating of sourdoughs on suc-MRS was employed to confirm the identity of fermentation microbiota with the inoculum.

2.9 Determination of the size distribution of exopolysaccharides produced during sourdough fermentation

To determine the molecular mass of polysaccharides, EPS was extracted from dough as previously described (Tieking et al., 2003). Briefly, EPS was precipitated form aqueous dough extracts with ethanol, dialyzed against distilled water, and lyophilized. The freeze-dried samples were dissolved in distilled water to a concentration of 10 g / L. To investigate the effect of different concentration of maltose or glucose on the molecular weight of the products, the molar mass determination of dextran was performed with AF4 coupled to MALLS and RI detectors (Postnova, Salt Lake City, UT, USA) (Hu et al., 2017; Rühmkorf et al., 2012). The regenerated cellulose membrane of the accumulation wall had a molecular weight cut off of 10 kDa. Poly-styrolsulphonate standard and BSA were used for calibration of detectors. Samples were diluted 10-fold with 10 mM NaCl and injected at a flow rate of 0.2 mL min^{-1} and a cross flow of 1 mL / min for 6 min. After injection, the cross flow rate remained consistent 2 min, linearly decreased to 0.1 mL / min over 10 min, and was then maintained at 0.1 mL / min for 10 min. The molar

mass was determined from the 7 laser scattering signals and RI signal by AF 2000 software (Postnova). To determine the concentration of EPS, the RI detector was used as concentration dependent detector with an increment (dn/dc) value of 0.146 mL / g (Vilaplana and Gilbert, 2010). Aqueous extracts from sourdough were analysed by AF4-RI and the yield of high molecular weight EPS was calculated from two biological repeats.

3. Results

3.1 Growth of W. cibaria *10M at different temperature*

W. cibaria was previously identified as dextran producing organisms but produced only low levels of dextran or gluco-oligosaccharides during growth in wheat sourdough at reference conditions, 30 °C (data not shown). To determine whether this relates to impaired growth at 30 °C, growth and acidification of *W. cibaria* 10M was monitored at 6 °C, 20 °C and 30 °C (Figure 1). Growth and acidification were fastest at 30 °C; after 6 and 12 h of incubation at 30 and 20 °C, respectively. The pH value decreased to below 4.8, thus decreasing dextransucrase activity, which is optimal at pH 5.4 (Kang et al., 2009). Consistent with prior observations (Galle et al., 2010), *W. cibaria* produced only small amounts of acetate and no mannitol during growth (data not shown). Growth at 6°C was observed only after the incubation time was extended to 7 d (data not shown).

3.2 Effect of temperature on oligosaccharide synthesis by W. cibaria *10M*

To determine the effect of temperature on dextransucrase-mediated oligosaccharide production, *W. cibaria* was grown at 20, 25, and 30 °C and oligosaccharide production in Suc-Mal-MRS was monitored by HPAEC-PAD (Figure 2). With sucrose as glycosyl donor and maltose as the glycosyl acceptor medium, panose-series oligosaccharides were observed (Figure 2; Hu et al.,

2017; Robyt and Eklund. 1983). After growth at the lower incubation temperature of 20 °C, less sucrose remained in the growth medium after 24 h and more panose-series oligosaccharides were produced (Figure 2), indicating higher oligosaccharide production at incubation temperatures that are below the growth optimum of *W. cibaria*.

3.3 Oligosaccharides produced by W. cibaria *10M after cold shift incubation.*

Because growth and biomass production of *W. cibaria* was fastest at 30 °C but dextransucrase-mediated oligosaccharide production increased at lower temperature, we determined whether "cold shift" incubation could maximize dextransucrase activity (Figure 3A). *W. cibaria* 10M was incubated at 30 °C for differing intervals of time and then shifted to 6 °C, followed by 48 h of further incubation. The cold shift protocol decreased the temperature to the target temperature in about 1 h (data not shown). Oligosaccharide production was initially assessed by thin layer chromatography (Figure 3). Panose and α-(1→6) extended panose-series oligosaccharides with a DP of 4, 5, 6, 7, 8, 10, 11, and 12 had a Rf of ~ 0.49, 0.40, 0.30, 0.22, 0.15, 0.1, 0.07, and 0.05, respectively (Figure 3). During growth at 30 °C, *W. cibaria* produced panose-series oligosaccharides with a DP of up to 6 (Figure 3B, left panel). Cold shift of early exponentially phase cells to 6 °C after 2 h of incubation at 30 °C resulted in virtually complete utilization of sucrose and maltose and formation of panose-series oligosaccharides with a high degree of polymerization, indicating high dextransucrase activity after cold shift (Figure 3B, right panel). Cold shift after 6 and 24h of growth at 30°C, corresponding to mid-exponential and stationary phase cells, reduced the molecular weight of oligosaccharides produced by *W. cibaria* when compared to cold shift after 2h of incubation (Figure 3B). Results obtained by thin layer chromatography were confirmed by analysis with HPAEC-PAD (Figure S1). Cold shift after 2 h to 6 h of incubation at 30 °C thus benefits transglucosylation by dextransucrase. For subsequent

experiments, cultures of *W. cibaria* were incubated at 30 °C to an OD_{600nm} of 0.4 and then shifted to 6 °C.

Quantitative analysis of panose and panose-series oligosaccharides with a DP of 4 – 8 after growth of *W. cibaria* 10M in mMRS was achieved by HPAEC-PAD. Because standards for higher panose-series oligosaccharides are not available, the method provides only a relative quantification of oligosaccharides with a DP of 4 or higher. Based on the initial experiments analysed by TLC (Figure 3), cultures were shifted from 30°C to 6°C after 2 – 10 h of incubation (Figure 4). The concentration of higher oligosaccharides was calculated by using the panose standard curve. An incubation time of 2 to 4 h prior to cold shift to 6 °C generated the highest concentration of oligosaccharides (Figure 4B). The concentration of products decreased in the order DP4 > DP5 > panose > DP6, DP7, and DP8 (Figure 4B).

3.3 Effect of temperature on DsrM expression by W. cibaria *10M*

To determine whether the increased oligosaccharide synthesis by *W. cibaria* at cold temperature relates to dextransucrase expression, DsrM expression was quantified at the level of mRNA abundance, protein abundance, and dextransucrase activity (Figure 5). RT-qPCR demonstrated that *dsrM* expression increases with decreasing incubation temperature; the abundance of the *dsrM* transcript was significantly increased at 15°C when compared to higher incubation temperatures (Figure 5 A). The cell-associated DsrM expression was also visualized by SDS-PAGE. *W. cibaria* was grown to the exponential phase of growth at 30 °C and further incubated for 24 h at the temperature specified. Whole cellular proteins were isolated from cultures that were standardized to an optical density of 10 (Figure 5B); DsrM was visible as a band at the predicted molecular weight of around 162 kDa. The band intensity corresponding to DsrM was highest after incubation at 6 and 15 °C (Figure 5B). The cell-associated dextransucrase activity

was also quantified with cells of *W. cibaria* that were grown to the exponential phase of growth at 30 °C and further incubated for 24 h at the temperature specified. The cell-associated dextransucrase activity was estimated by the release of fructose; the activity increased after decreasing the incubation temperature to 15 °C or less. Remarkably, reducing the incubation temperature to 6 °C increased the hydrolase activity as measured by the release of glucose without further increasing total activity. It is noteworthy that crude cellular extract of *W. cibaria* also contains other enzymes that are active on sucrose. In particular sucrose phosphorylase cleaves sucrose to fructose and glucose-1-phosphate and thus contributes to the apparent transferase activity but not the apparent hydrolase activity. Taken together, the three different approaches for assessment of the effect of temperature on DsrM activity consistently indicated an increased expression and activity at low temperature.

3.4 Production of exopolysaccharides by W. cibaria *10M after cold shift.*

The influence of cold shift to temperatures ranging from 6 to 25 °C on dextran production was determined in mMRS containing 0.5 M sucrose (Figure 6). The highest volumetric yield of dextran was observed after cold shift to 25 °C. This result conforms to the effect of temperature on oligosaccharide production (Figure 2) and is explained by the opposite effects of temperature on growth and biomass production and dextransucrase expression. Growth and biomass production is highest at 30 °C (Figure 1) while dextransucrase expressions is highest at 15°C or lower (Figure 5). Moreover, temperature and pH effects on dextransucrase activity need to be considered. Dextransucrase activity is highest at 30 °C, however, this temperature also favours rapid acidification, inhibiting DsrM activity.

3.6 Oligosaccharide and exopolysaccharide formation during sourdough fermentation

To determine whether cold shift fermentation optimizes dextran formation in sourdough, *W. cibaria* 10M was employed as starter cultures for wheat and sorghum sourdoughs supplemented with sucrose, sucrose and glucose, or sucrose and maltose. During growth at 30 °C, the pH decreased from 5.4 to 3.8 after 24 h of incubation and only low levels of oligosaccharides were formed (data not shown). Cold shift to 6 °C after 2 h of incubation at 20 °C limited the pH decrease to values ranging from 4.6 to 4.9; i.e. the sourdough pH remained high enough to sustain DsrM activity. Oligosaccharide production during growth in sourdough was analysed by HPAEC-PAD (Figure 7). Low oligosaccharide levels were detected in chemically acidified dough (Fig. 7); the formation of oligosaccharides increased after addition of sucrose or sucrose and glucose. Glucose addition increased formation of isomaltotriose (IM3) and panose was additionally detected. Panose co-elutes with isomaltohexose, however, maltose was available as glucosyl-acceptor but IM5 was not, suggesting that the peak represents panose (Fig. 7). Addition of 15% maltose further increased the oligosaccharide levels and resulted particularly in formation of panose and panose-series of oligosaccharides with DP 3 - 8 (Fig. 7). These results conform to prior data that maltose is a better acceptor carbohydrate than glucose for oligosaccharide production by *W. cibaria* 10M (Hu et al., 2017).

AF4 was employed to separate water soluble polysaccharides in sourdough (Figure 8). The MALS signal and the retention time demonstrated that the chemically acidified control dough contained no water soluble polysaccharides (Fig. 8). Wheat sourdoughs fermented with *W. cibaria* 10M in presence of sucrose contained high molecular weight polysaccharides, demonstrating that water soluble polysaccharides in sourdough are attributable to EPS formation by *W. cibaria* 10M. The addition of maltose or glucose did not decrease the molecular weight of the EPS but decreased the EPS concentration (Fig. 8). The yield of EPS with different

concentration of sugars in wheat and sorghum sourdoughs was evaluated with the RI as concentration dependent detector (Figure 9). The EPS yield was generally higher in wheat sourdoughs when compared to sorghum sourdoughs; increasing the sucrose concentration from 15 to 30 % increased the EPS yield more than 2 fold (Fig. 9). The highest yield of EPS, about 16 g / kg, was observed in wheat sourdough with 30% sucrose. Addition of acceptor carbohydrates generally decreased the EPS yield; however, the yield of EPS in sorghum sourdoughs containing 30% sucrose was unchanged by addition of 15% glucose.

4. Discussion

Weissella species grow optimally at temperatures higher than 30°C, however, optimum dextran synthesis was observed at lower temperatures. This study employed a cold shift method to optimize production of dextran and oligosaccharides by *W. cibaria* 10M. Dextran and oligosaccharides are alternative products of dextransucrase activity which improve textural and nutritional properties of bread, respectively (Galle and Arendt, 2014; Yan et al., 2018). Initial incubation at ambient temperature allows growth of *W. cibaria* to produce sufficient biomass; the subsequent temperature shift optimized dextransucrase expression and deferred acidification while supporting dextransucrase activity during prolonged incubation.

Temperature is a key parameter for control of the activity and composition of sourdough microbiota (Gänzle and Ripari, 2016) and fermentation at temperatures ranging from 12 – 16°C is used in artisanal baking to defer growth and acid production by sourdough lactic acid bacteria (unpublished observations of the authors). Moreover, fermentation or storage of sourdough at 4°C is used in production of Panettone (Venturi et al., 2012), a sweet baked product where dextran formation is considered to be essential for the quality and storage life (Lacaze et al.,

2007). Cold shift fermentation, however, has not been deliberately employed to optimize dextran or oligosaccharide formation by lactic acid bacteria in sourdough.

W. cibaria grows optimally at 36°C and pH 6.6; the minimum pH and temperature of growth were determined as 3.8 and less than 10°C, respectively (Riccardi et al., 2009). Other *Weissella* species grow at refrigeration temperature and contribute to meat spoilage (Silva et al., 2017). *W. cibaria* does not grow at 4°C (Björkroth et al., 2002); our data demonstrate that refrigeration temperatures of 4 – 6 °C represent the growth / no growth interface for this species. *W. cibaria* thus has the exceptional ability to grow at less than 10°C as well as more than 45 °C (Riccardi et al., 2009; this study). This broad temperature of growth matches the relevance of *Weissella* spp. as part of the commensal microbiota of cold water fish and warm blooded animals (Fusco et al., 2015).

The temperature and pH optimum of dextransucrases from *W. cibaria* and *W. confusa,* 30 – 40°C and pH 5.4, match the optimum growth conditions (Amari et al., 2013; Baruah et al., 2017; Kajala et al., 2015; Kang et al., 2009). Dextransucrases of *Weissella* spp. are unstable, however, when the temperature exceeds 25 °C. For example, the half-live of *W. confusa* dextransucrase decreased from 33 h to 7.4 h when the temperature was increased from 23 to 42 °C (Kajala et al., 2015). The effect of cold shift on dextran synthesis is thus not explained by the temperature optima of the producing strain *W. cibaria* 10M or the dextransucrase DsrM; we therefore explored whether temperature dependent gene expression may contribute to the increased dextran yield after cold shift.

In lactic acid bacteria, sucrose conversion by glucansucrases contributes to carbohydrate metabolism, generates soluble and insoluble carbohydrates as part of a biofilm matrix, and contributes to stress resistance. The host adapted organisms *L. reuteri* and *S. mutans* form

biofilms in the upper intestinal tract of animals and the oral cavity of humans, respectively (Klein et al., 2015; Walter et al., 2008). Glucansucrase expression in *L. reuteri* and *S. mutans* is constitutive and not dependent on sucrose availability in the medium (Arsköld et al., 2007; Schwab et al., 2006 and 2007). In contrast to *L. reuteri* and *S. mutans*, dextransucrase expression in *L. mesenteroides* is induced by sucrose (Bounaix et al., 2010; Neubauer et al., 2003; Quirasco et al., 1999). Stress induced expression of glycansucrases was observed in *L. reuteri* and *L. mesenteroides*. In *L. reuteri*, fructansucrase expression is induced by membrane perturbations increasing the membrane lateral pressure (Schwab et al., 2006). In *L. mesenteroides*, the glucansucrase Gsy, which forms a water insoluble glucan, is induced by oxidative stress in addition to sucrose (Yan et al., 2016). Only few reports provide data on the regulation of dextransucrase in *Weissella* spp.. Different from *L. mesenteroides*, dextransucrase expression by *Weissella* spp. was constitutive and not induced by sucrose (Bounaix et al., 2010). This study demonstrates that dextransucrase expression is induced by cold temperatures; highest levels of DsrM expression and activity were observed in the temperature range of 6 – 15 °C. Dextran production by *Leuconostoc gelidum* during growth at refrigeration temperature contributes to spoilage of ready-to-eat meat and fish products (Lyhs et al., 2004) but has not been described for *Weissella* spp.. *W. cibaria* occurs in a wide range of habitats and has been isolated from the feces and milk of warm blooded animals and from the feces of salmon and trout (Fusco et al., 2015). Induction of dextran synthesis in *W. cibaria* may support biofilm formation in cold (water) habitats.

The overexpression of DsrM by *W. cibaria* was employed in this study to increase dextran and oligosaccharide levels in wheat sourdoughs by cold shift fermentation. Initial studies on HoPS formation in sourdough reported glucan levels of 2 – 5 g / kg flour after fermentation of wheat

sourdough with addition of 10% sucrose and glucan-forming lactobacilli (Tieking et al., 2003). Fermentation of wheat and sorghum sourdoughs with addition of 20% sucrose and *W. cibaria* MG1 as starter culture accumulated 5 and 8 g dextran / kg flour, respectively (Galle et al., 2012a and 2012b). However, *W. cibaria* 10M accumulated only 0.8 g / kg dextran during growth in sorghum sourdoughs at 30 °C (Schwab et al., 2008). Higher dextran yields in sorghum sourdoughs were attributed to the higher maltose levels in wheat sourdoughs (Galle et al., 2010). Maltose is an excellent glycosyl-acceptor for dextransucrase, supporting formation of panose-series oligosaccharides, and high maltose levels thus divert dextransucrase activity towards oligosaccharide formation (Gänzle, 2014; Hu et al., 2017). Accordingly, heat treatment of wheat flour to inactivate maltogenic amylases increased the dextran yield in *W. confusa* sourdoughs to 16 g / kg (Katina et al., 2009). Up to 150 g / kg flour dextran were generated when incubating heat treated wheat bran with dextransucrase and 30% sucrose (Kajala et al., 2015). Enzyme inactivation of wheat flour or wheat bran, however, also inactivates pentosanases and proteases that improve texture and flavor of bread by solubilizing arabinoxylans, and by accumulating amino acids as flavor precursors (Gänzle, 2014). The present study achieved dextran levels of up to 16 g / kg flour with untreated, enzyme active wheat flour by addition of 30% sucrose and cold-shift fermentation. Dextran produced by DsrM significantly improved bread texture when added at a level of 1 to 2 g / kg (Chen et al., 2016). *W. cibaria* 10M sourdoughs produced with addition of sucrose and cold shift fermentation thus provide sufficient dextran for improved bread quality without adding excess acidity. Sourdoughs produced with addition of sucrose and maltose may accumulate a sufficient quantity of panose-series oligosaccharides to improve the nutritional functionality of bread (Hu et al., 2017; Schwab et al., 2008; Yan et al., 2018).

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Figure legends.

Figure 1. Growth (closed symbols) and acidification (open symbols) of *W. cibaria* 10M during incubation at 30 °C (Δ), 20 °C (□), and 6 °C (○).

Figure 2. Production of oligosaccharides after growth of *W. cibaria* at different temperatures (20 °C, 25 °C, or 30 °C) in mMRS containing 0.5 M sucrose and 0.25 M maltose. Oligosaccharides were analysed by HPAEC-PAD and chromatograms were offset by 20 µC.

Figure 3. Panel A. Schematic overview on the cold shift method for oligosaccharide production by *W. cibaria* 10M. **Panel B.** TLC separation of oligosaccharides produced by *W. cibaria* 10M in mMRS 0.5 M sucrose and 0.25 M maltose after different incubation times at 30 °C (left panel), or after incubation at 30 °C for the time indicated, followed by 2 d of incubation at 6 °C (right panel).

Figure 4. Time course of oligosaccharide production by *W. cibaria* 10M in mMRS with 0.5 M sucrose and 0.25 M maltose. **Panel A.** *W. cibaria* was grown at 30 °C for the time indicated on the x-axis. **Panel B.** *W. cibaria* was grown at 30 °C for the time indicated on the x-axis, followed by incubation at 6 °C for 2 d. The following oligosaccharides were quantified by HPAEC-PAD: ●, panose; ○, G'-panose; ▼, G'G'-panose; Δ, G'G'G'-panose; ■, G'G'G'G'-panose; □, G'G'G'G'G'-panose. All oligosaccharides were integrated using the panose standard curve. Data are shown as means ± standard deviation of two biological replicates

Figure 5. Panel A: Expression of *dsrM* of *W. cibaria* 10M incubated at different temperature to the exponential phase of growth. Expression was calculated relative to the expression of 16S rRNA using cells grown at 30 °C as reference conditions. Panel B: Visualization of DsrM expression by *W. cibaria* by SDS-PAGE of whole cell lysates. Cells were grown to OD_{600nm} 0.4

at 30 °C and incubated in mMRS-sucrose for 24 h at the temperature specified. Cells were standardized to OD_{600nm}=10 and then lysed. Panel C: Dextransucrase activity of whole cell lysate of *W. cibaria*. Cells were grown to OD_{600nm} 0.4 at 30 °C and incubated in mMRS-sucrose for 24 h at the temperature specified. Cells were standardized to OD_{600nm}=10, lysed, and the hydrolase and transferase activity per mg cellular protein were determined.

Figure 6. Production of dextran by *W. cibaria* grown at different temperatures in mMRS containing 500 mM sucrose. Cells were grown to OD_{600nm} 0.4 at 30°C and incubated at different temperatures in mMRS-sucrose for 24 h. The formation of dextran with high molecular weight was measured by HPLC-RI. Dextran was quantified with dextran with a M_W of 5×10^6 - 4×10^7 as external standard.

Figure 7. Separation of oligosaccharides produced by *W. cibaria* during growth in wheat sourdoughs**.** Isomalto-oligosaccharides (IM3, isomaltotriose) and panose-series oligosaccharides were separated by HPAEC-PAD. Sourdoughs were fermented with addition of 15 % sucrose, 15 % sucrose and 7.5 % glucose, or 15 % sucrose and 15 % maltose. Sourdoughs were incubated for 2 h at 20 °C prior to incubation for 48 h at 6 °C. Chemically acidified dough were prepared with addition of 15 % sucrose.

Figure 8. Separation of water soluble polysaccharides extracted from in wheat sourdoughs fermented with *W. cibaria*. Polysaccharides were separated by asymmetric flow-field flow fractionation (AF4) coupled to multi-angle laser scattering detector; shown is the signal at 35°. Sourdoughs were fermented with addition of 15 % sucrose, 15 % sucrose and 7.5 % glucose, or 15 % sucrose and 15 % maltose. Sourdoughs were incubated for 2 h at 20 °C prior to incubation for 48 h at 6 °C.

Figure 9. Formation of water soluble polysaccharides in wheat (**Panel A**) and sorghum (**Panel B**) sourdoughs. The concentrations of water soluble polysaccharides were measured by AF4 with RI as detector. Sourdoughs were fermented with addition of 15 % or 30 % sucrose without addition of acceptor carbohydrates (white bars), with addition of equimolar amounts of maltose (gray bars), or with addition of equimolar amounts of glucose (black bars). Sourdoughs were incubated for 2 h at 20 °C prior to incubation for 48 h at 6 °C. Data represent means ± standard deviation of two biological repeats.

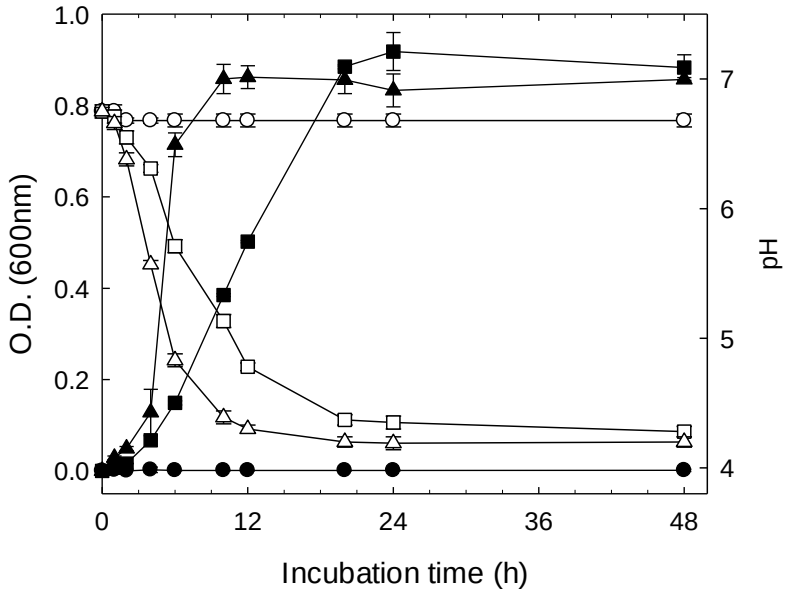


Figure 1.

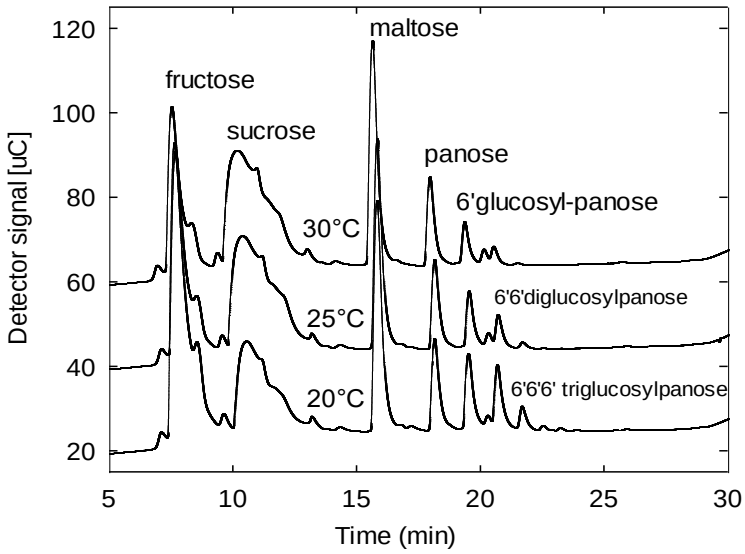

maltose
fructose
sucrose
panose
6'glucosyl-panose
30°C
6'6'diglucosylpanose
25°C
6'6'6' triglucosylpanose
20°C
Detector signal [uC]
Time (min)
120
100
80
60
40
20
5
10
15
20
25
30

Figure 2.

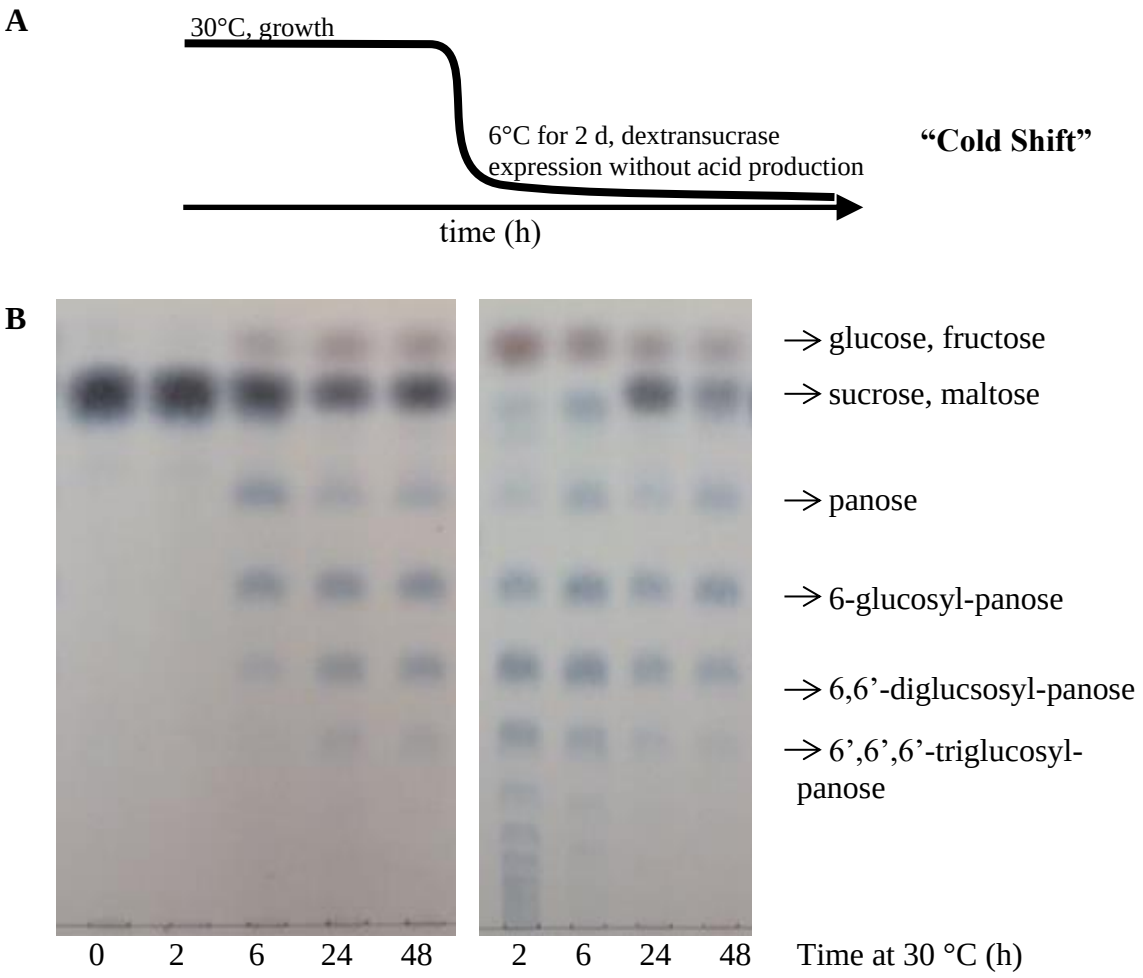

A
30°C, growth
6°C for 2 d, dextransucrase expression without acid production
"Cold Shift"
time (h)
B
→ glucose, fructose
→ sucrose, maltose
→ panose
→ 6-glucosyl-panose
→ 6,6'-diglucsosyl-panose
→ 6',6',6'-triglucosyl-panose
0 2 6 24 48 2 6 24 48
Time at 30 °C (h)

Figure 3.

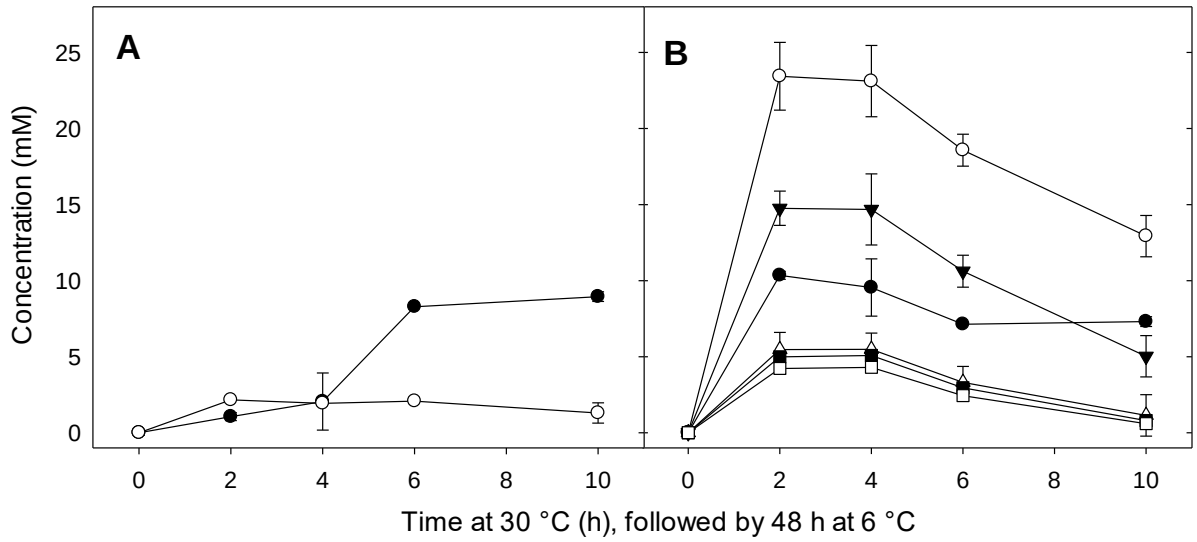
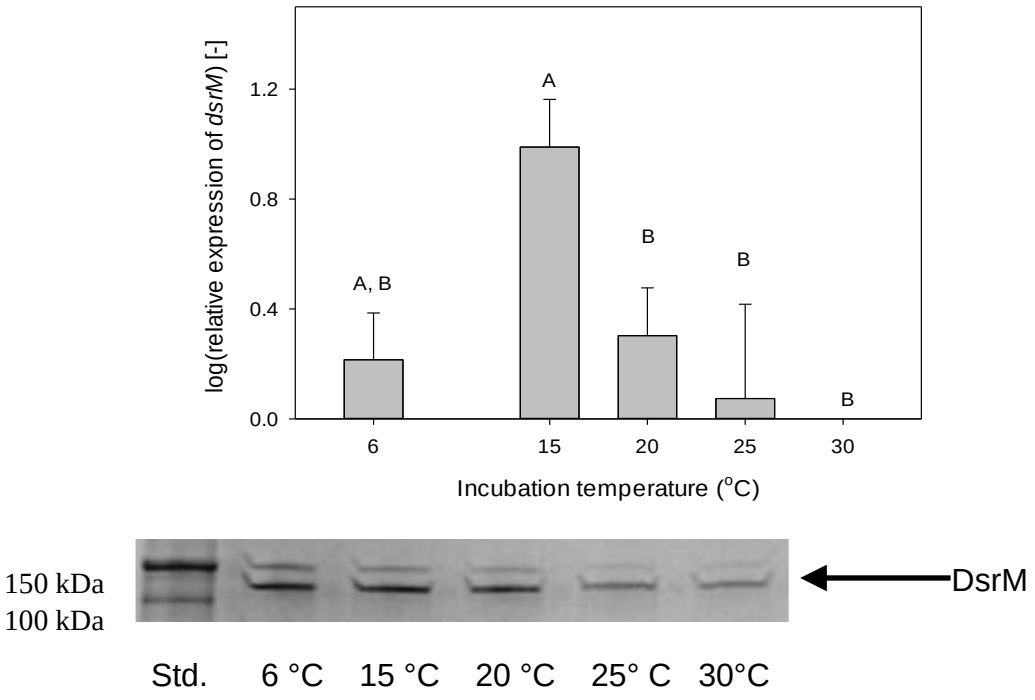

A
B
Concentration (mM)
0
5
10
15
20
25
0
2
4
6
8
10
Time at 30 °C (h), followed by 48 h at 6 °C

Figure 4.


log(relative expression of dsrM) [-]
1.2
0.8
0.4
0.0
A
A, B
B
B
B
6
15
20
25
30
Incubation temperature (°C)
150 kDa
100 kDa
DsrM
Std.
6 °C
15 °C
20 °C
25° C
30°C

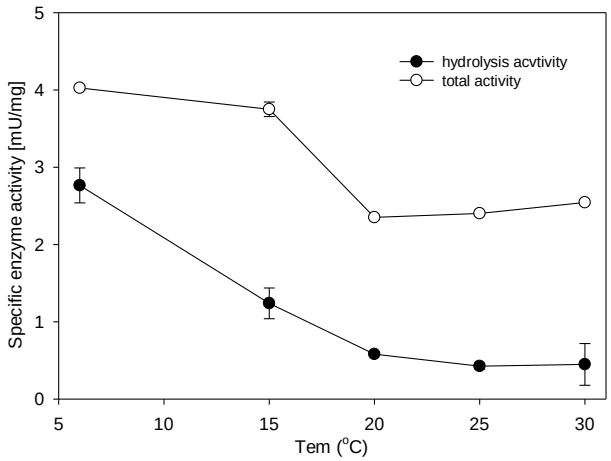

hydrolysis acvtivity
total activity
Specific enzyme activity [mU/mg]
5
4
3
2
1
0
5
10
15
20
25
30
Tem (°C)

Figure 5.

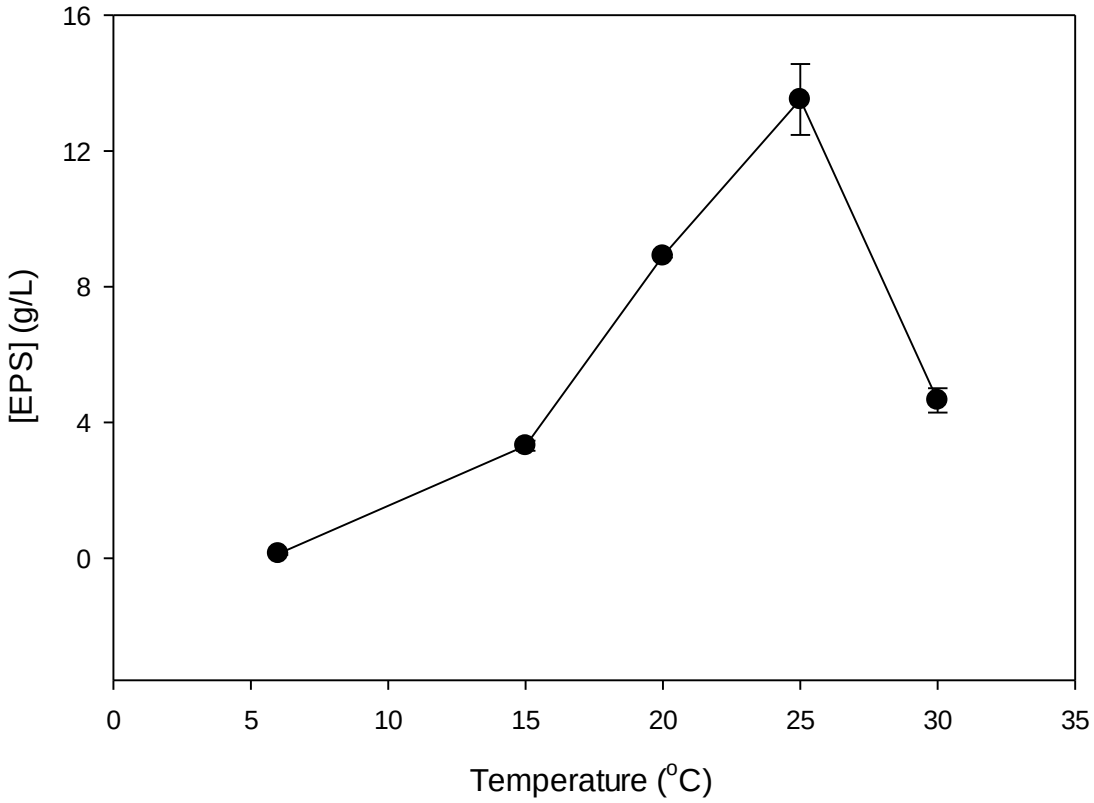


Figure 6.

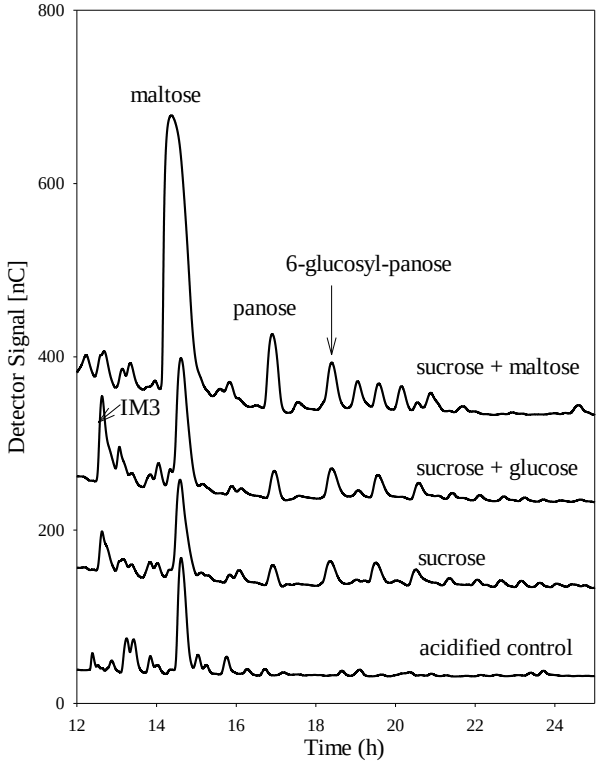

800
600
400
200
0
Detector Signal [nC]
maltose
6-glucosyl-panose
panose
IM3
sucrose + maltose
sucrose + glucose
sucrose
acidified control
12
14
16
18
20
22
24
Time (h)

Figure 7.

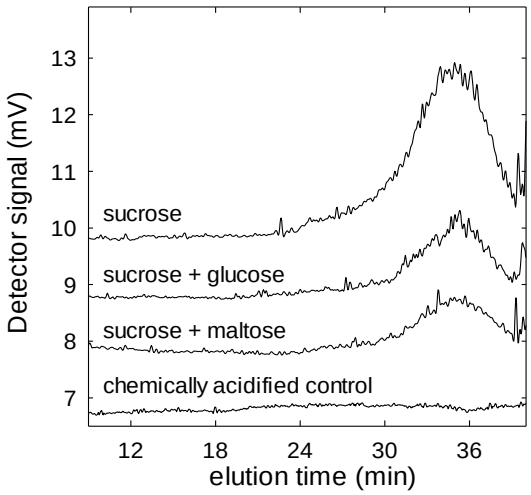

Detector signal (mV)
13
12
11
10
9
8
7
sucrose
sucrose + glucose
sucrose + maltose
chemically acidified control
12
18
24
30
36
elution time (min)

Figure 8.

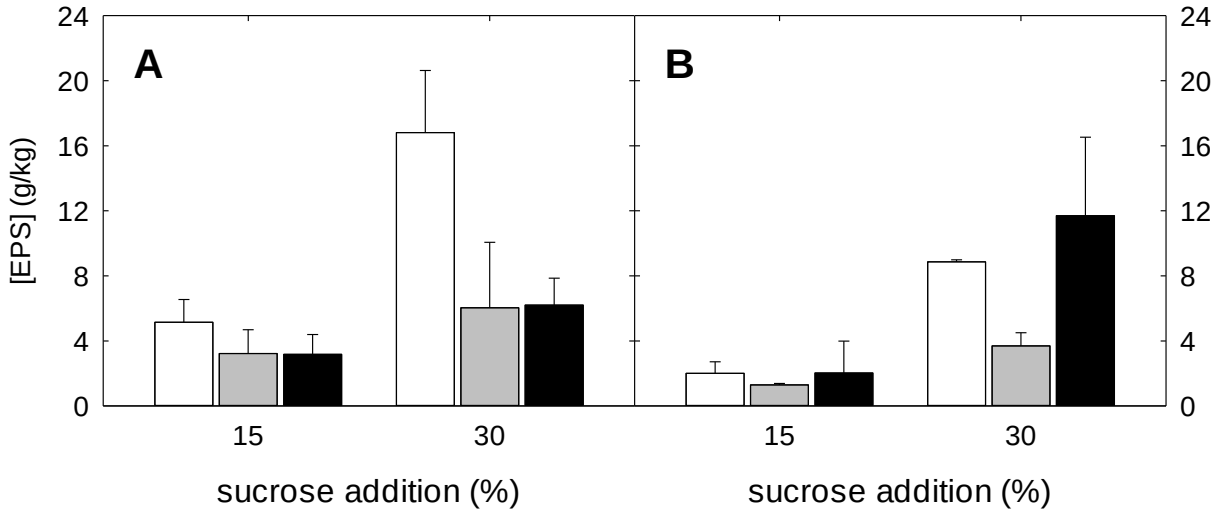


Figure 9.