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Beet sugar syrup and molasses as low-cost feedstock for the enzymatic production of fructo-oligosaccharides

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Running title: Fructo-oligosaccharides synthesis from syrup and molasses

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ABSTRACT

Sugar syrup and molasses from beet processing containing 620 and 570 mg/ml sucrose, respectively, were assayed as low-cost and available substrates for the enzymatic synthesis of fructo-oligosaccharides (FOS). A commercial pectinase (Pectinex Ultra SP-L, from *Aspergillus aculeatus)* characterised by the presence of a transfructosylating activity, was used as biocatalyst. The FOS production increased when lowering the initial pH value of syrup (7.5) and molasses (8.9) to 5.5. Sugar syrup and molasses were diluted in order to reduce substrate viscosity; interestingly, the percentage of FOS with regards to total sugars remained almost constant, which indicated a high transferase to hydrolase ratio for this enzyme. Kinetics of FOS production was analysed. Using approx. 10 *U* transfructosylating activity per g sucrose, the FOS concentration reached a maximum of 388 mg/ml after 30 h using syrup and 235 mg/ml in 65 h with molasses. These values corresponded to approx. 56% and 49% (w/w), respectively, of the total amount of carbohydrates in the mixture. The enzyme was also covalently immobilised on an epoxy-activated polymethacrylate-based polymer (Sepabeads® EC-EP5). We found that immobilised Pectinex Ultra SP-L can be efficiently applied to the synthesis of FOS using syrup and molasses as substrates.

Keywords: β-Fructofuranosidase, Fructosyltransferase, Transglycosidase, Sucrose, Prebiotics, Polymethacrylate, Pectinex Ultra SP-L, Animal feed.

1. INTRODUCTION

Fructo-oligosaccharides (FOS) of the inulin-type constitute one of the most established groups of prebiotics in the world (*1*). Addition of such molecules to food products may help to prevent illness, control calcium balance and contribute to the reduction of antibiotic consumption (*2,3*).

FOS of the inulin-type are fructose oligomers with a terminal glucose group, in which 2-4 fructosyl moieties are linked via β(1→2)-glycosidic bonds (*4*). Commercial FOS are mainly composed of 1-kestose (GF_2), nystose (GF_3) and 1^F-fructosylnystose (GF_4). The properties of other types of FOS, such as those of the levan-type (*5*) and the neo-FOS (*6*), are very promising, but they are not commercially available yet.

FOS are industrially produced through fructosyl transfer from pure sucrose using a fungal enzyme (*7*). FOS-synthesising enzymes are present in many higher plants (asparagus, chicory, onion, Jerusalem artichoke, etc.) and microorganisms, especially fungi (*Aureobasidium pullulans, Aspergillus niger, Aspergillus oryzae*, etc.) (*4;8-10*). FOS-producing enzymes are assigned as hydrolases (β-fructofuranosidases, EC 3.2.1.26) or fructosyltransferases (transfructosidases, EC 2.4.1.9). The maximal FOS production for a particular enzyme depends on the relative rate of transfructosylation and hydrolysis (*11*).

In addition to their use in human food products, the administration of FOS to the diets of some animals results in improvement in feed efficiency and a reduction of diarrhoea and smell in feces (*12*). In order to extend the use of FOS as animal feed additives, it is necessary to minimise production costs. Beet sugar syrup and molasses are cheap and available sources of sucrose, and are adequate feedstock for FOS production of animal-feed grade.

Pectinex Ultra SP-L is a commercial enzyme preparation from *Aspergillus aculeatus* used in the food industry for fruit juice processing to reduce viscosity. It contains different pectinolytic and cellulolytic enzymes (e.g. endo-poly-galacturonase, endo-pectinylase and

pectin esterase) (*13*). Interestingly, Pectinex Ultra SP-L also contains a transfructosylating activity (*14,15*).

In this work, we have assayed Pectinex Ultra SP-L, soluble or immobilised on a epoxy-activated polymethacrylate (Sepabeads EC-EP), for transformation of beet syrup and molasses into FOS.

2. EXPERIMENTAL PROCEDURES

Materials

The beet sugar syrup and molasses were kindly donated by Azucarera Ebro (Valladolid, Spain). Pectinex Ultra SP-L (batch No. KRN05401) was kindly donated by Novozymes A/S. Sepabeads EC-EP5 (batch No. E407P094) was kindly provided by Resindion S.R.L. (Mitsubishi Chemical Corporation, Milan, Italy). Glucose and dinitrosalicylic acid were from Sigma. Sucrose and fructose were purchased from Merck. 1-Kestose and nystose were from TCI Europe (Zwijndrecht, Belgium). 1^F-Fructosylnystose was from Megazyme (County Wicklow, Ireland). All other reagents and solvents were of the highest available purity and used as purchased.

Standard activity microassay

The enzymatic activity towards sucrose was measured following the initial rate of reducing sugars production by the dinitrosalicylic acid (DNS) method. The spectrophotometric assay was adapted to a 96-well microplate scale as described (*16*). One unit *(U)* of activity was defined as that catalysing the formation of 1 μmol reducing sugar per minute under the above conditions.

Immobilisation of fructosyltransferase

Pectinex Ultra SP-L (20 ml) and Sepabeads EC-EP5 (8 g) were mixed and incubated for 24 h at room temperature with roller shaking. The ratio protein/support was approx. 45 mg protein per gram of carrier. The biocatalyst was then filtered, washed (3 x 30 ml) with 50 mM sodium acetate buffer (pH 5.6), dried under vacuum and stored at 4ºC.

Batch production of fructo-oligosaccharides

Soluble or immobilised Pectinex Ultra SP-L was added to diluted beet sugar syrup and molasses, previously filtered through a glass microfibre filter. Total reaction volume was 5 ml.

The biocatalyst was added to a final activity in the mixture of 1 *U*/ml or 5 *U*/ml (determined by the standard DNS microassay). The mixtures were incubated at 60 °C in an orbital shaker (Stuart Scientific) at 200 rpm. At different times, 40 µl aliquots were extracted from the reaction mixture, diluted with 160 µl water and incubated 10 min at 90 °C to inactivate the enzyme. Samples were centrifuged 5 min at 6000 rpm using an eppendorf with a 0.45 µm Durapore® membrane (Millipore), and analysed by HPLC.

HPLC analysis

The concentration of the different products was analysed by HPLC with a quaternary pump (Delta 600, Waters) coupled to a Lichrosorb-NH2 column (250 x 4.6 mm) (Merck). The mobile phase was acetonitrile:water (75:25 v/v), conditioned with helium, and used at a flow rate of 0.7 ml/min. The column temperature was kept constant at 25 ºC. A differential refractometric detector (model 9040, Varian) was used and set to a constant temperature of 30 ºC. The data obtained were analysed using the Millennium Software.

3. RESULTS AND DISCUSSION

Composition of sugar syrup and molasses

Sugar syrup is the sugar juice obtained after beet juice evaporation, from which sucrose is crystallised. Molasses is the main by-product of the sugar industry. Both sources are notably less expensive than pure sucrose for production of FOS. The use of molasses for FOS synthesis has only been explored by Shin *et al.* in cultures of *Aureobasidium pullulans* cells (*9*) whereas, to our knowledge, the sugar syrup has not been assayed as an alternative substrate in this process.

Sugar syrup and molasses are mixtures of sucrose and other carbohydrates. Molasses has also more unidentified components and some particulate materials (esp. carbonates). Table 1 shows the specifications of beet syrup and molasses that have been used in this study. Sugar syrup and molasses were viscous solutions containing 620 and 570 mg/ml sucrose and 0.6% and 5.6% (w/w) of betaine (trimethyl glycine), respectively. The weight distribution of carbohydrates in syrup was 74.7% sucrose, 13.6% 1-kestose, 7.5% glucose, 3.5% nystose and 0.7% fructose; molasses contained 95.7% sucrose, 2.9% glucose and 1.4% of other carbohydrates. It is noteworthy the presence of a considerable amount of FOS (17.1%) in the syrup. The 1-kestose content depends on the origin of the raw material and the type of technology adopted in beet processing (*17,18*), and may cause shape and kinetic modifications of the final sucrose crystals. On the other hand, kestoses are becoming increasingly important due to their properties as prebiotic compounds. Other trisaccharides such as raffinose and neokestose were also present, especially in sugar syrup, but at a lower concentration (<0.2% w/w) compared with 1-kestose.

Effect of pH and substrate concentration on FOS production

The presence of a fructosyl-transfer activity in commercial Pectinex Ultra SP-L from Novozymes A/S (used in juice clarification) was first reported by Hang and Woodams (*14*). Using Pectinex Ultra SP-L, we found that fructose was formed in a very small scale

compared with that of glucose, which indicated that this activity was better defined by a transfructosidase than a β-fructofuranosidase (*16*). Optimum pH and temperature for production of fructooligosaccharides from sucrose using Pectinex Ultra SP-L were reported 5.5 and 60 °C, respectively (*19*). Recently we have purified the enzyme responsible for this transfructosidase activity (data not shown).

The initial pH of the sugar syrup and molasses was 7.5 and 8.9, respectively. We observed that FOS production from syrup increased from 30.6 to 36.4% when moving from the starting pH (7.5) to pH 5.5 (by the addition of glacial acetic acid), whereas for molasses an increment from 3.5 to 14.8% was observed when lowering pH from 8.9 to 5.5.

In order to reduce the viscosity of the feedstock, which may cause technological difficulties, several dilutions from syrup and molasses were prepared by adding distilled water to the substrate and adjusting pH to 5.5. This allowed us to investigate the effect of substrate concentration on FOS production.

The synthesis of fructooligosaccharides from sucrose is a kinetically controlled reaction that involves a fructosyl-enzyme intermediate. The nucleophiles H_2O and sucrose compete for the fructosyl-enzyme intermediate. When H_2O is the nucleophile, the enzyme acts as a hydrolase (releasing glucose and fructose). When sucrose is the nucleophile, the enzyme acts as a transfructosidase. The first condensation product (1-kestose) can also be hydrolysed by the enzyme. The reaction time must be carefully controlled to stop the process when the maximum yield of condensation products is achieved. The maximum yield of FOS depends on two parameters: the concentration of sucrose and the intrinsic transferase/hydrolase ratio of the enzyme. In order to improve the yield of transfructosylating products, a high sucrose concentration must be used to increase the ratio $k_3 \cdot [\text{Sucrose}]/k_2 \cdot [H_2O]$ (*20*).

Different diluted solutions of syrup and molasses containing Pectinex Ultra SP-L (5 *U*/ml) were incubated at 60 °C for 24 h, and FOS content analysed. Table 2 summarises the initial sucrose concentration, as well as the sucrose conversion and FOS production in 24 h.

It is noteworthy that the FOS percentage (referred to the total amount of carbohydrates) maintained a value close to 53-57% (w/w) for syrup and 41-46% (w/w) for molasses, which confirmed that the transferase/hydrolase ratio for this enzyme must be considerably high. As expected, the maximum FOS production (460 mg/ml for syrup and 245 mg/ml for molasses) was obtained without dilution. In contrast, 1^F-fructosylnystose (GF4) production increased with substrate dilutions (up to 12-fold with syrup and 42-fold with molasses, Table 2). In fact, at lower sucrose concentrations the formation of high polymerisation degree products is favoured, as there exists more competition between sucrose and reaction products to accept a fructosyl unit.

Batch production of fructooligosaccharides

We analysed the kinetics of FOS batch production of fructooligosaccharides (FOS) from sugar syrup and molasses. To have an efficient mixing, dilutions (1:1.2) from syrup and molasses were utilised. Fig. 1 shows the progress of the process and Fig. 2 represents the percentage and concentration (mg/ml) of total FOS. In the case of syrup, the FOS concentration reached a maximum value of 388 mg/ml after 30 h (228 mg/ml 1-kestose, 149 mg/ml nystose and 9 mg/ml 1^F-fructofuranosylnystose). At this reaction time, the percentage of FOS was 56.0%, referred to total carbohydrates in the mixture. For molasses, the maximum concentration of fructooligosaccharides was about 235 mg/ml (49.2 % FOS) after 65 h (62 mg/ml 1-kestose, 143 mg/ml nystose and 30 mg/ml 1^F-fructofuranosyl-nystose). After 140 h, reactions were close to equilibrium, with a FOS percentage of 49% and 42% for syrup and molasses, respectively (Fig. 2). Similar results using pure sucrose as substrate were also obtained in our laboratory with soluble and immobilised Pectinex Ultra SP-L (*16*).

Immobilisation of fructosyltransferase on Sepabeads EC-EP5

Sepabeads EC are polymethacrylate-based carriers for enzyme immobilisation. The series Sepabeads EC-EP is epoxy-activated, with a high reactive group density. The porosity of these materials is very suitable to obtain biocatalysts with a high volumetric activity. The

chemistry for attachment enzyme/support is straightforward. Compared with other epoxy acrylic polymers, Sepabeads EC-EP carriers possess high mechano-osmotic stability, low compressibility and high resistance to microbial attack. Furthermore, the raw materials applied for the production of these supports are included in the EU list of resins allowed for the processing of foodstuffs (*21*).

Fructosyltransferase enzyme in Pectinex Ultra SP-L was immobilised on Sepabeads EC-EP5. Immobilisation was carried out by mixing the Pectinex and the polymer, without any buffer or pH adjustment (*16*). The protein concentration in commercial preparation of the enzyme was 17.8 mg/ml. The activity towards sucrose, using the DNS assay, was 321 *U*/ml. Protein content of the immobilised biocatalyst was 36 mg/g of support and its activity 21.0 *U*/g biocatalyst.

Fructosyltransferase immobilised on Sepabeads FP-EC5 was utilised for FOS production. Pure sucrose (630 mg/ml), sugar syrup and molasses were comparatively used as substrates. Fig. 3 shows the FOS production and FOS percentage –referred to total carbohydrates in the mixture–, with the different starting materials. It is interesting to note that these values correlated well with those obtained with the soluble enzyme. The highest percentage of FOS in the final mixture (61%) was found with pure sucrose. This value is very close to that we obtained with the related support Sepabeads EC-EP3 (*16*), and comparable to the reported with other immobilised transfructosidases (*22*). The FOS percentage determined with sugar syrup and molasses (53% and 37.5%) can be considered very satisfactory. It is interesting to point out that, in terms of FOS production, the highest value was found with sugar syrup (440 mg/ml) compared with 385 mg/ml and 215 mg/ml obtained with pure sucrose and molasses, respectively. This is a simple consequence of the presence of FOS in the starting syrup.

As syrup and molasses are coloured materials and the FOS specifications for human nutrition are very restrictive in this subject, the FOS obtained by the methods described in this work could be easily employed for animal feed. In the recent past years, some relevant changes have been introduced in the nutrition of farmed animals, in order to cover the needs

of essential nutrients and/or to optimise feed utilisation. Examples include the administration of immuno-stimulants or natural substances having an anti-bacterial effect. Prebiotics are being already employed to control pathogenic bacteria, reduce faecal odour, and enhance growth performance. Research to date indicates positive effects of prebiotics on health status and performance of companion animals, livestock, and poultry (*23-25*). The process for FOS synthesis described here is inexpensive, simple, efficient and, employing the immobilised biocatalyst, easy to scale-up to different reactor configurations.

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Table 1. Some specifications of the beet sugar syrup and molasses used in this work.

	Sugar syrup	**Molasses**
Brix units[a]	69.3	75.1
pH	7.5	8.9
Density (g/ml)	1.30	1.40
Sucrose (%) [b]	47.5	40.7
[Sucrose] (mg/ml) [c]	620	570
Betaine (%) [d]	0.6	5.6

[a] Grams of dry matter per 100 g of sample, provided by the manufacturer.

[b] Grams of sucrose per 100 g of sample, determined by HPLC.

[c] Determined by HPLC.

[d] Grams of betaine per 100 g of sample, provided by the manufacturer.

Table 2. Effect of dilution of sugar syrup and molasses on production of FOS [a].

Substrate	Dilution (ml H_2O added per ml substrate)	Sucrose consumed (%)[b]	1-Kestose (%)[c]	Nystose (%)[c]	1^F-fructosylnystose (%)[c]	Total FOS (%)[c]	FOS production (mg/ml)
Syrup	0.0	73	37.7	16.5	1.5	55.7	460
Syrup	0.2	79	21.1	26.9	6.9	55.0	380
Syrup	0.5	83	12.7	26.9	14.3	53.8	295
Syrup	1.0	86	16.0	22.0	18.7	56.7	235
Molasses	0.0	45	33.5	7.8	0.3	41.6	245
Molasses	0.2	57	20.6	20.7	2.5	43.8	215
Molasses	0.5	55	15.2	22.5	7.8	45.6	180
Molasses	1.0	61	9.0	19.7	12.6	41.3	125

[a] Experimental conditions: 5 *U*/ml fructosyltransferase, 60 °C, 220 rpm, pH 5.5. Values refer to 24 h reaction.

[b] Percentage of the initial sucrose that is transformed into carbohydrates in 24 h.

[c] The values refer to percentage of FOS with respect to the total amount of carbohydrates in the mixture, after 24 h reaction. The initial percentage of 1-kestose and nystose in sugar syrup were 13.6% and 3.5%, respectively.

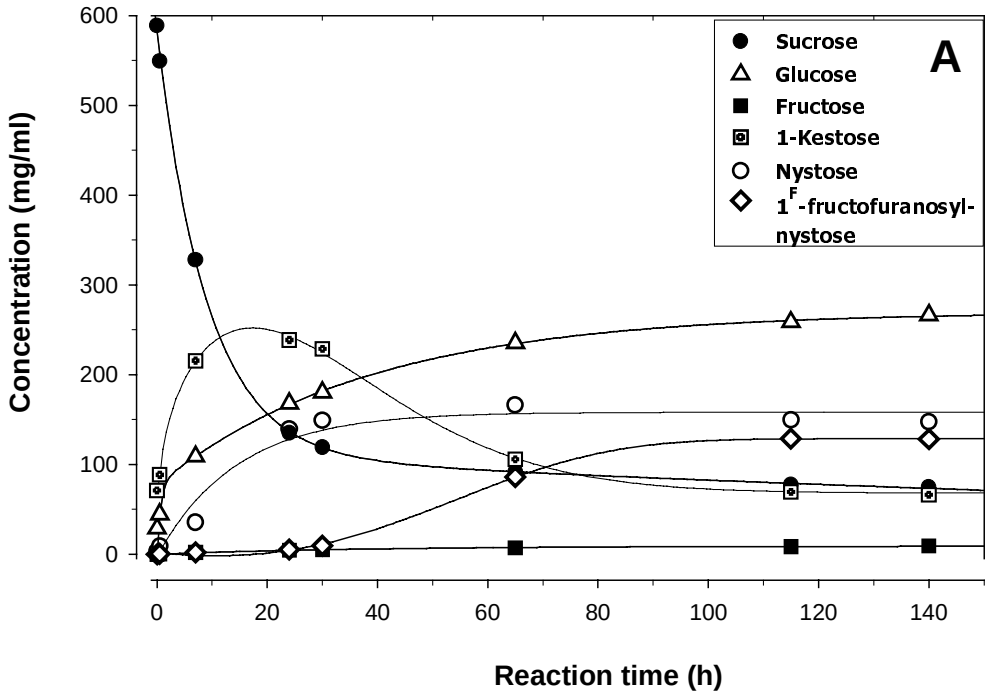
Figure Legends

Fig. 1. Time dependency of FOS batch production from (A) beet sugar syrup and (B) molasses, catalysed by soluble Pectinex Ultra SP-L. Experimental conditions: pH 5.5, 5.0 *U*/ml (standard DNS assay), 60 °C. The substrate was diluted by adding 0.2 ml H_2O per ml.

Fig. 2. (A) Kinetics of FOS production and (B) percentage of FOS referred to the total amount of carbohydrates using sugar syrup (○) and molasses (●) Experimental conditions: pH 5.5, 5.0 *U*/ml (standard DNS assay), 60 °C. The substrate was diluted by adding 0.2 ml H_2O per ml.

Fig. 3. (A) FOS production and (B) percentage of FOS referred to the total amount of carbohydrates using sucrose (630 mg/ml), beet sugar syrup and molasses, catalysed by immobilised Pectinex Ultra SP-L in Sepabeads EC-EP5. Experimental conditions: 1.0 *U*/ml (standard DNS assay), pH 5.5, 60 °C. The initial percentage of FOS in sugar syrup was 17.1% (142 mg/ml) -indicated by a black bar-.

Fig. 1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Glucose
Fructose
1-Kestose
Nystose
1F-fructofuranosyl-nystose
Concentration (mg/ml)
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400
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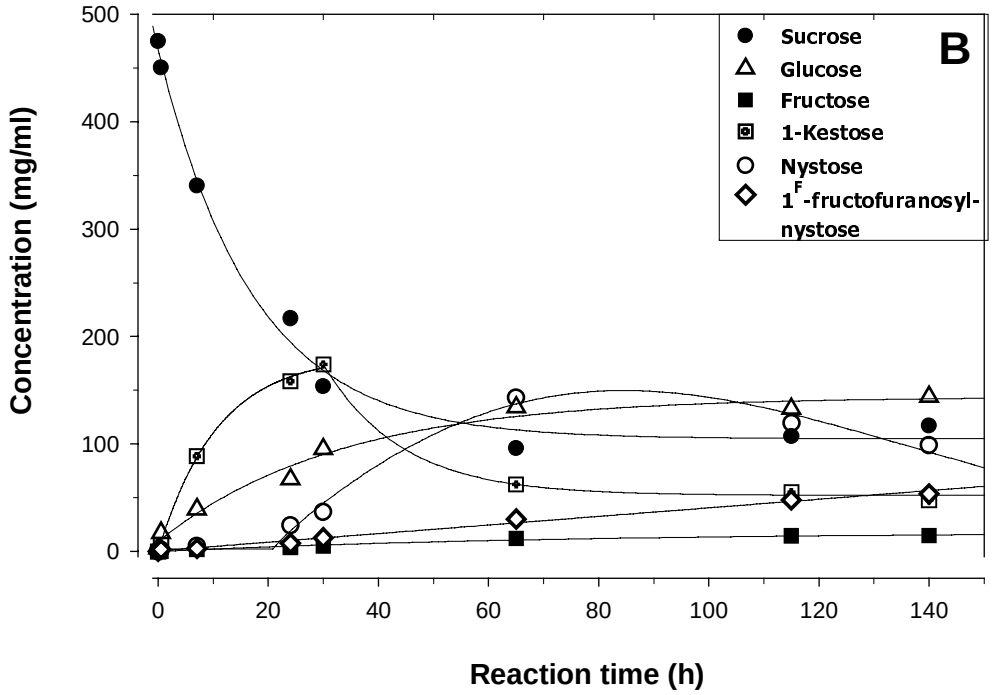
40
60
80
100
120
140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Sucrose
Glucose
Fructose
1-Kestose
Nystose
1F-fructofuranosyl-nystose
Concentration (mg/ml)
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0
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60
80
100
120
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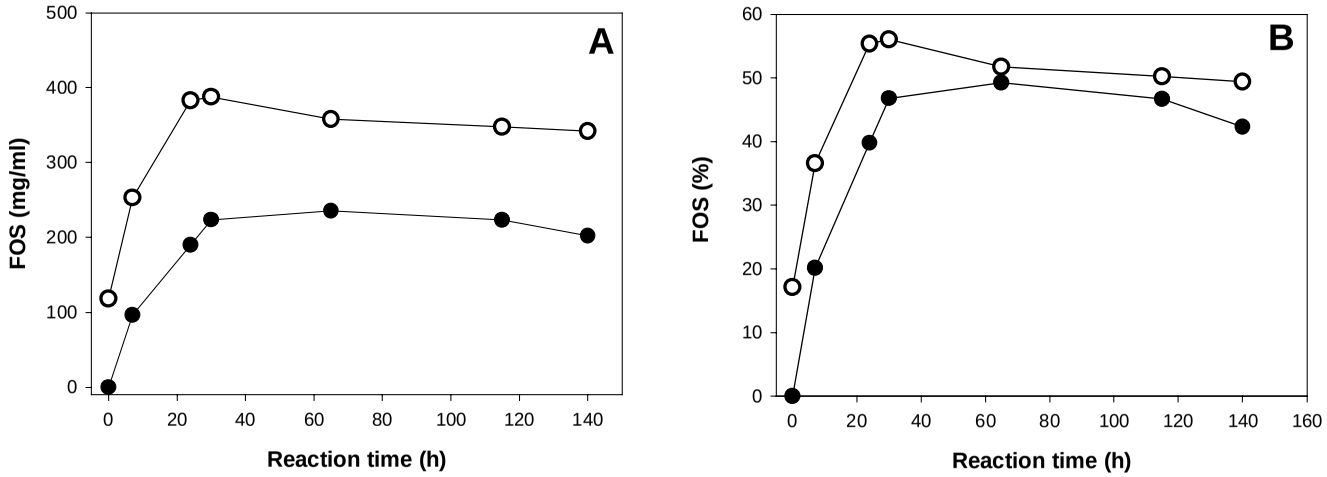
Fig. 2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400
300
200
100
0
0
20
40
60
80
100
120
140
Reaction time (h)
B
FOS (%)
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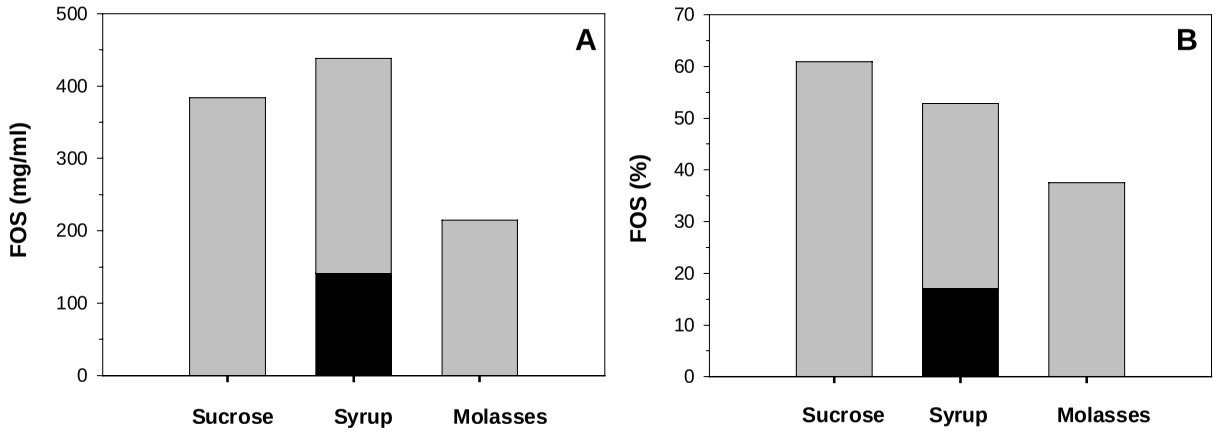
50
40
30
20
10
0
0
20
40
60
80
100
120
140
160
Reaction time (h)

Fig. 3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FOS (mg/ml)
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400
300
200
100
0
Sucrose
Syrup
Molasses
B
FOS (%)
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60
50
40
30
20
10
0
Sucrose
Syrup
Molasses