Powder lemon juice containing oligosaccharides obtained by dextranucrase acceptor reaction synthesis and dehydrated in sprouted bed

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Abstract Oligosaccharides can be synthesized using the sugars present in the fruit juices through the dextranucrase acceptor reaction. In the present work, the effect of reducing sugar and sucrose concentration on oligosaccharide formation in lemon juice was evaluated through response surface methodology. The oligosaccharide formation in lemon juice was favored at high concentrations of sucrose (75 g/L) and reducing sugar (75 g/L). At this synthesis conditions, an oligosaccharide concentration of 94.81 g/L was obtained with a conversion of 63.21% of the initial sugars into the target product. Oligosaccharides with degree of polymerization up to 11 were obtained. The lemon juice was dehydrated in spouted bed using maltodextrin as drying adjuvant. The powder obtained at 60°C with 20 % maltodextrin presented low moisture (2.24 %), low water activity ($A_w=0.18$) and the lowest reconstitution time (~46 s). The results showed that lemon juice is suitable for oligosaccharides enzyme synthesis and can be dehydrated in spouted bed.

Keywords Oligosaccharides · Dextranucrase · *Leuconostoc mesenteroides* · Lemon juice

Introduction

Dextranucrase is an extracellular enzyme that belongs to the glucosyltransferase group (E.C. 2.4.1.5.). This enzyme is commercially obtained from *Leuconostoc mesenteroides* B-

512 F in a medium containing sucrose as substrate. The main product of dextranucrase is dextran, a high molecular weight polysaccharide applied mainly in pharmaceutical and in food industry (Rabelo et al. 2009). When other carbohydrates, besides sucrose, are present in the reaction medium, the enzyme synthesizes prebiotic oligosaccharides (Chung and Day, 2004). This reaction is known as acceptor reaction and the main studied acceptor is maltose (Rodrigues et al., 2006, Rabelo et al. 2009). According to the acceptor reaction mechanism, the presence of other carbohydrates, besides sucrose, shifts the synthesis from the dextran formation to the oligosaccharides production (Rabelo et al. 2009, Heincke et al., 1999). Despite several strains of *Leuconostoc mesenteroides* are able to produce dextranucrase, the acceptor reaction have been extensively studied with the enzyme produced by *L. mesenteroides* B-512 F (Rodrigues et al., 2006; Rabelo et al. 2006, Heincke et al., 1999). A proper acceptor:sucrose ratio allows the minimization of dextran formation and the maximization of oligosaccharides formation. In the acceptor reaction, the glucose moieties from sucrose are deviated from the dextran formation and incorporated into the oligosaccharides, which increases their degree of polymerization. The oligosaccharides produced by dextranucrase presents α -1,6-glucosidic bonds and are considered difficult to be obtained (Rabelo et al. 2009, Rodrigues et al., 2006, Chung and Day, 2004).

The interest in oligosaccharides synthesis has increased due to their prebiotic characteristics. A prebiotic oligosaccharide is a non-digestible carbohydrate consumed by the *Lactobacillus* and *Bifidobacteria* in human colon (Madhukumar and Muralikrishna 2012). The prebiotic oligosaccharides modulates the colon bacteria growth increasing the beneficial strains counts. The increasing in *Bifidobacteria* counts lowers the intestinal pH due to the organic acid

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production. The low pH decreases the counts of harmful strains such as *Salmonella* and *Escherichia coli* decreasing the formation of toxic metabolites as ammonia, phenols and nitrosamines.

As α -amylase has no substrate specificity for α -1, 6 linkages (Böttger et al. 2013), carbohydrates containing α -1,6 linkages, such as dextran, isomaltose, panose and others, reach the intestine. The intestinal enzyme oligosaccharide α -1, 6-glucosidase (EC 3.2.1.10), also known as sucrase-isomaltase, hydrolysis of (1 $\rightarrow$ 6)- α -D-glucosidic linkages (Hauri et al., 1979; Rodríguez et al 2013). The ability of human gut bacteria to break down α -1,2 and α -1,6 glucosidic linkages in α -gluco-oligosaccharides (GOS) was investigated in vitro and in vivo (Djouzi et al., 1995). The oligomer breakdown and short chain fatty acid and gas production by the characteristic infant gut microbiota indicated the hydrolysis and fermentation of GOS. The study revealed that GOS with α -1, 2 glucosidic bonds, were more resistant than α -1,6. In conclusion, it was reported an association of beneficial bacteria and GOS consumption. Sarbini et al. (2013) reported that commercial α -gluco-oligosaccharides significantly increased the *Bifidobacterium* sp. population compared with the control (inulin) and the fermentation of α -gluco-oligosaccharides produced less gas than inulin. Isomalto-oligosaccharides (IMOs), are the most studied prebiotic oligosaccharide obtained by dextranucrase acceptor reaction. IMOs are composed of one maltose unit linked by α -1, 6 glucosidic bounds to glucose unit (Heincke et al., 1999; Rodrigues et al., 2005, Rabelo et al. 2009). The prebiotic effect of the oligosaccharides obtained by dextranucrase acceptor reaction was evaluated in vitro by (Machida Y. Fukui F and Komoto 1986) with positive effect on *bifidobacteria* growth and by (Vergara et al. 2010) with positive effect on *lactobacillus* growth. Thus, dextranucrase acceptor reaction can be applied to synthesize functional oligosaccharides because the oligomers produced are formed by α -1, 6 glucosidic bounds.

Fruit juices are considered healthy foods because they are rich in vitamins and minerals. Lemon juice is a source of vitamin C and its consumption was related to some health benefits such as anti-inflammatory and anti-allergic effect besides lowering the lipids content in the blood (González-Molina et al., 2008). Thus, the aim of the present study was to obtain a powder lemon juice containing oligosaccharides. Functional fruit juices are usually obtained by adding oligosaccharides to the juice (Koh et al., 2010). Instead of adding external oligosaccharides to the juice, the synthesis was carried out by dextranucrase acceptor reaction. As any enzyme synthesis is strongly affected by the substrate concentration, to study the effect of the sugar concentration on the oligosaccharide formation, an experimental planning was carried out. The synthesis was optimized using lemon juice as raw

material. The juice obtained at the best synthesis condition was dried in a spouted bed.

Materials and methods

Lemon juice preparation

Concentrated lemon juice (LIMONJAL®, Citro-Nutre, Rio de Janeiro-RJ-Brazil) was used as raw material. The juice was diluted with potable water at 1 volume of juice to 9 volumes of water as recommended by the manufacturer. The juice pH was determined by a potentiometer and sugar analyses (glucose, fructose and sucrose) were carried out by HPLC as described further.

Dextranucrase production

The bacteria *Leuconostoc mesenteroides* NRRL B-512 F obtained from ARS Culture Collection (NRRL Culture collection, United States Department of Agriculture, Peoria, IL, USA) was activated in an optimized synthetic medium composed of sucrose, 50 g/L (food grade); yeast extract, 20 g/L (Himedia); $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.20 g/L; $\text{MnSO}_4 \cdot 2\text{H}_2\text{O}$, 0.01 g/L; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01 g/L; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.02 g/L; NaCl, 0.01 g/L; and 20 g/L of K_2HPO_4 (anhydrous). The initial pH of the culture medium was adjusted to 6.5 with concentrated H_3PO_4 and the medium was autoclaved (121 °C/15 min). Activation was carried out in an orbital shaker (Tecnal TE 420) at 30 °C and 150 rpm (Silva et al., 2012, Rabelo et al. 2006; Rodrigues et al., 2003) for 12 hours. Three percent of this cell culture was inoculated into the fermentor to produce dextranucrase in a fed batch fermentation process.

Fermentation was carried out in a 1 L TecBio Fermentor at 30 °C with pH controlled at 6.5 (± 0.1); mechanical agitation of 150 rpm and aeration of 0.5 L/min in the same culture medium used for the strain activation, as described above. A solution containing NaOH (120 g/L) and sucrose (300 g/L) was fed into the fermentor to control de pH and sucrose level in the culture medium. After six hours, the feed flow rate was interrupted and the pH was allowed to drop to 5.2, when the process was terminated (Rodrigues et al., 2005).

The cells were harvested by centrifugation at 11,600 g for 10 minutes at 4 °C in a Sigma 6 K-15 centrifuge. The enzyme was recovered from the cell-free broth by precipitation with polyethylene glycol (PEG 1,500 50 %v/v). The partially purified enzyme was then diluted in a sodium acetate buffer (20 mM) containing 0.05 g/L CaCl_2 with pH adjusted to 5.2. The enzyme was stored frozen at -20 °C prior to use (Silva et al., 2012). The enzyme can be stored frozen for up to one year (Miller et al., 1986).

Enzyme activity was determined by quantifying the released fructose by the DNS (3,5-dinitrosalicylic acid) method

(Miller, 1959). The partially purified enzyme prepared as described above (45 μ L) was mixed with 455 μ L of a 10 % (w/v) sucrose solution in sodium acetate buffer pH 5.2 (20 mM containing 0.05 g/L of CaCl_2) and then incubated for 1 h at 30 °C. Samples of 100 L were collected every 15 min and mixed with 100 mL of the DNS reagent to stop the reaction. The enzyme activity was calculated by linear regression and expressed in IU/mL (international unit/mL). One international unit is defined as the amount of enzyme that releases 1 μ mol of fructose per minute under ideal reaction conditions (30 °C and pH 5.2) as described by (Rabelo et al. 2009). In the present study, the enzyme activity was determined prior to use to attest that the desired amount was added to the reaction synthesis.

Enzyme synthesis of prebiotic oligosaccharides in lemon juice

Syntheses were carried out at optimum enzyme synthesis conditions (30 °C and pH 5.2) in a thermostatic glass batch reactor containing 10 mL of the juice. The juice pH was adjusted to 5.2 with NaOH 1 M. Glucose, fructose and sucrose levels were changed according to a two-level central composite rotated design with three central points (Table 1). Enzyme activity was 0.5 IU/mL. When necessary, external sugars were added to the juice to reach the desired concentration. Syntheses were carried out until the total consumption of sucrose, which was calculated according to the definition of enzyme activity and confirmed by high-performance liquid chromatography (HPLC) analysis. Dextran was precipitated by adding three volumes of ethanol 96 % (v/v) (Rodrigues et al., 2005, Kooi and La Grange, 1952). The supernatant was used to quantify the carbohydrates. The precipitated dextran was diluted in distilled boiling water and assayed as total carbohydrate by phenol-sulfuric acid method (Dubois et al., 1956).

The prebiotic oligosaccharides were characterized according to its degree of polymerization by Thin Layer Chromatography (TLC) as described by Robyt (2000) for dextranase acceptor reaction products. Separation was achieved on Whatman K6 silica plates, 250- μ m thickness (Whatman, Kent, UK). Diluted samples of 5 μ L were absorbed onto the plates on a line about 1.5 cm above the lower plate edge. After drying with a hairdryer, the plate was irrigated for two ascents in a solvent mixture composed of acetonitrile:ethyl acetate:1-propanol: H_2O (85:20:50:90). To make sugars visible, the plates were sprayed to saturation with a solution containing 0.3 % (w/v) of 1-naphthylethylenediamine dihydrochloride in methanol containing 3 % (v/v) of concentrated sulfuric acid. The plates were then heated in an oven at 120 °C until the spots were visible (approximately 10 min).

The residual sugars were analyzed by high performance liquid chromatography (HPLC) in a Varian ProStar system equipped with two high-pressure pumps model ProStar 210,

refraction index detector model ProStar 355 RI and column oven (Timberline). Separation was achieved using an Aminex® (300 mm $\times$ 7.8 mm) column at 85 °C. Ultra pure water at 0.8 mL/min was used as eluent and the detector temperature was 35 °C. All samples were analyzed in triplicate. The software ProStar WS 5.5 was used to acquire and handle the data. Oligosaccharide concentration, oligosaccharide and dextran yield were calculated by mass balance through the Eqs. 1, 2, 3.

$$\text{Oligosaccharides} \left(\frac{\text{g}}{\text{L}} \right) = \text{TS}_{\text{cons}} - \text{DXT} \quad (1)$$

$$Y_{\text{OLIGO}}(\%) = \frac{\text{Oligosaccharides} \left(\frac{\text{g}}{\text{L}} \right)}{\text{TS}_{\text{consumed}} \left(\frac{\text{g}}{\text{L}} \right)} \times 100 \quad (2)$$

$$Y_{\text{DXT}}(\%) = \frac{\text{DXT} \left(\frac{\text{g}}{\text{L}} \right)}{\text{TS}_{\text{consumed}} \left(\frac{\text{g}}{\text{L}} \right)} \times 100 \quad (3)$$

Where:

TS_{cons}	total sugar consumed in the enzyme reaction (g/L)
DXT	dextran formed during the enzyme synthesis (g/L)
$Y_{\text{OLIGO}}(\%)$	oligosaccharides yield (%)
$Y_{\text{DXT}}(\%)$	dextran yield (%)

Juice dehydration in spouted bed

The prebiotic lemon juice was dehydrated in a spouted bed model LM FBD 3.0 (Labmaq do Brasil, Ribeirão Preto-SP). The drying parameters were: air flow rate of 1.70 L/m³; atomizer flow rate of 20 L/m³; atomizer pressure (80 bar; feed flow rate 4.5 mL/min); the inert particles were polystyrene spheres with 3 mm of mean diameter; inert load was 400 g and the volume of the juice dried was 200 mL. Two different drying conditions were evaluated: inlet air temperature (60 and 70°C) and three maltodextrin concentration (10, 15 and 20 %) as drying adjuvant. The powder was stored at vacuum sealed aluminized polyethylene bags. The moisture content was determined according to AOAC methodology (AOAC - ASSOCIATION OF OFFICIAL ANALYTICAL CHEMISTS 15th edition 1995). Water activity was determined using an AQUALAB® equipment model 3TE. The reconstitution time was measured according to the methodology described by

Table 1 Experimental planning and responses from the enzyme synthesis of oligosaccharides in lemon juice

Run	Suc (g/L)	RS (g/L)	Oligos (g/L)	Dextran (g/L)	Y _{OLIG} (%)	Y _{DXT} (%)	TS _{cons} (g/L)
1	25	25	15.25	1.47±0.07	30.50	2.93	16.72
2	25	75	39.88	0.15±0.02	39.88	0.15	40.03
3	75	25	58.33	0.15±0.01	58.33	0.15	58.49
4	75	75	94.81	0.10±0.02	63.21	0.06	94.91
5	14.6	50	22.75	0.11±0.02	35.22	0.17	22.86
6	85.3	50	77.75	1.52±0.00	57.46	1.12	79.27
7	50	14.6	36.17	0.06±0.05	55.99	0.10	36.24
8	50	85.3	67.01	0.19±0.01	49.53	0.14	67.20
9	50	50	44.64	0.21±0.03	44.64	0.21	44.85
10	50	50	50.60	0.14±0.01	50.60	0.14	50.73
11	50	50	50.15	0.14±0.01	50.15	0.14	50.30

Suc - sucrose concentration; RS – reducing sugar; Y_{OLIG}- oligosaccharides yield

Y_{DXT}- dextran yield; TS_{cons} – total sugar consumed

Goula and Adamopoulos (2010). The powder yield was cal-

culated based on the juice soluble solids (Brix) before drying, according to the following equation:

$$\text{Yield (\%)} = \text{weight powder} \times 100 \times \left(\frac{100}{\text{Juice weight} \times \text{soluble solids juice}} \right) \quad (4)$$

Statistics

Statistical analyses were carried out using Statistica 7.0 (Statsoft). A confidence interval of 95 % was taken into account.

Results and discussion

The lemon juice used as raw material presented 18.77 g/L of sucrose, 22.68 g/L of reducing sugar being 11.34 g/L of glucose and 11.34 g/L of fructose. Final carbohydrate concentration presented in Table 1 takes into account the sugars present in the juice. The juice pH was 2.70. As dextranase is quickly denaturized at this pH, the pH value was adjusted to 5.2 (optimum for enzyme activity) using NaOH. Table 2 depicts the effect of the independent variables (sugar concentration) on the evaluated responses.

Only the linear effects were significant on the oligosaccharide formation, oligosaccharides and dextran yield. No significant effect on consumed sugars were exerted by the initial substrate concentration. Dextran yield was affected only by sucrose linear effect. This result was expected since dextran formation is favored by high sucrose concentration. Eqs. 5 and 6 present the

fitted models for oligosaccharides concentration and yield.

$$\begin{aligned} \text{Oligosaccharides (g/L)} = & 2.42 + 4.50 \\ & \times 10^{-1} \text{Suc} - 1.16 \times 10^{-2} \text{RS} \\ & + 1.93 \times 10^{-3} \text{Suc}^2 + 2.299 \\ & \times 10^{-3} \text{RS}^2 + 4.74 \\ & \times 10^{-3} \text{Suc} \times \text{RS} \end{aligned} \quad (5)$$

$$\begin{aligned} Y_{\text{OLIG}}(\%) = & 23.32 + 7.33 \times 10^{-1} \text{Suc} - 1.67 \\ & \times 10^{-1} \text{RS} - 2.30 \times 10^{-3} \text{Suc}^2 + 2.82 \\ & \times 10^{-3} \text{RS}^2 - 1.80 \text{Suc} \times \text{RS} \end{aligned} \quad (6)$$

Where

Suc sucrose concentration (g/L)
RS reducing sugar concentration (g/L)
Y_{OLIG} oligosaccharides yield (%)

The fitted models presented in Eqs. 5 and 6 were statistically validated by ANOVA analysis and F-test at a confidence

Table 2 Estimated effects on the evaluated responses

Factor	Oligos		Dextran		Y _{OLIG}		Y _{DXT}		TScons	
	Effect	S.E.	Effect	S.E.	Effect	S.E.	Effect	S.E.	Effect	S.E.
Mean	48.45*	2.75*	0.17	0.34	48.61*	2.46*	48.45*	3.10*	0.17	0.50
Suc (L)	43.96*	3.37*	0.16	0.42	44.11*	3.02*	20.65*	3.79*	-0.38	0.61
Suc (Q)	2.41	4.01	0.64	0.49	3.05	3.59	-2.87	4.52	0.70	0.73
RS (L)	26.19*	3.37*	-0.30	0.42	25.89*	3.02*	1.28	3.79	-0.71	0.61
RS (Q)	3.73	4.01	-0.04	0.49	3.69	3.59	3.53	4.52	0.17	0.73
Suc x RS	5.92	4.77	0.63	0.59	6.56	4.27	-2.25	5.37	1.35	0.86

* Significant at 95% of confidence interval.; Y_{OLIG} – oligosaccharide yield; Y_{DXT} – dextran yield; TScons – total sugar consumed

level of 95%. The calculated F-value was 45.54 and 6.27 for Eqs. 5 and 6 respectively. As the calculated F-values were higher than the listed one ($F_{3,5}=5.05$), at the given confidence interval, the models were considered significant and were used to build the response surface graphs. The correlation coefficients (R^2) for the models were 0.98 and 0.86 for Eqs. 5 and 6 respectively. Figure 1 presents the surface graphs for oligosaccharides production in lemon juice.

According to the results presented in Fig. 1, the higher the concentration of substrates (sucrose and reducing sugar) the higher was the oligosaccharides formed during the synthesis. The higher oligosaccharide concentration was 94.81 g/L obtained using 75 g/L of sucrose and 75 g/L of reducing sugar. Similar results were obtained by Silva et al. (2012) using cashew apple juice as substrate for oligosaccharide synthesis. Figure 2 shows the oligosaccharide yield. A marked increase on oligosaccharide yield was observed at high sucrose concentrations because sucrose is the source of glucose units that

are incorporated into the oligosaccharides chain. The maximum yield (63.21 %) was obtained when 75 g/L of sucrose and 75 g/L of reducing sugars were used.

According to (Ebert and Schenk 1968), the enzyme dextranase breaks the sucrose into glucose and fructose and dextran formation occurs via the insertion mechanism, which inserts the glucose units into the dextran growing chain by a ping-pong mechanism involving two active sites. In the acceptor reaction, the acceptor molecule binds to an intermediate active site (acceptor site), and the glucose units are deviated from the dextran growing chain promoting the acceptor chain elongation (Robyt et al, 1974, Tanriseven and Robyt 1992; Rodrigues et al., 2005). Thus, the higher the amount of acceptor, more acceptor active sites are occupied and more oligosaccharides chains are initiated. As the glucose moieties incorporated into the acceptor unit comes from the sucrose break promoted by dextranase, high concentrations of acceptors and sucrose resulted in high amounts of oligosaccharides.

Fig. 1 Surface response for oligosaccharide formation in lemon juice **Fig. 2** Surface response for oligosaccharide yield in lemon juice

Fig. 3 Degree of polymerization of oligosaccharides formed in lemon juice

The oligosaccharides formed were analyzed by thin layer chromatography (TLC). Oligosaccharides with different degrees of polymerization were formed in all experimental runs with different degrees of polymerization (Fig. 3). The experimental runs 3, 4 and 6 showed the highest degree of polymerization indicating that applying high concentrations of sucrose resulted in oligosaccharides chain elongation. According to Lee et al. (2008), the higher the degree of polymerization the higher is the probability of the oligosaccharide reach the large intestine. Thus, long chain oligosaccharides are better than shorter chain ones because they are more prone to reach the large intestine, where they are metabolized by the probiotic intestinal bacteria.

The experimental run four was chosen for the drying experiments in the spouted bed due to the high concentration and yield of oligosaccharides. It was not possible to collect any powder from the drying experiments carried out with 10 % of maltodextrin. The juice powder adhered to the equipment collector and the inert particles became totally impregnated with the juice. Thus, this condition was considered inadequate

for the drying processing. This might have occurred because fruit juices are strongly susceptible to stickiness due its high content of sugars and organic acids and the addition of 10 % of maltodextrin was not sufficient to increase the glass transition temperature of the product. In products rich in sugars, the material can be turned into syrup during the drying processing leading to yield loss and adhesion (Goula and Adamopoulos, 2010). Table 3 presents the yield and powder characterization of the prebiotic lemon juice dried using 15 and 20 % of maltodextrin at the two different drying temperatures evaluated.

The lemon juice powder obtained at 60 °C with 20 % maltodextrin presented the lowest moisture and reconstitution time, probably due to the higher amount of maltodextrin. According to Goula and Adamopoulos (2010), the effect of maltodextrin on the powder reconstitution depends on the moisture and on the adherence ability of the product. Powders with low moisture present high particle superficial area in contact to the rehydration water. On the other hand, run two presented the higher yield. The water activity of the powders obtained in runs 2 and 3 was not statistically different indicating that the decrease in the temperature along with the increase on maltodextrin load did not affect the powder water activity.

Conclusions

Commercially, the oligosaccharides are obtained and added to the food matrix. However, the synthesis of these products using the food matrix as raw material is an interesting alternative since the oligosaccharides separation and purification steps is not necessary. In the present work, lemon juice showed to be a suitable raw material for oligosaccharides synthesis. The product can be preserved by dehydration in spouted bed. Dextranase acceptor reaction produces oligosaccharides with α -1,6 glucosidic bonds. These carbohydrates reach the intestine where they can be consumed by the intestinal microbiota. Thus, the lemon powder containing oligosaccharides obtained herein is a potential non-dairy prebiotic product that can be applied as ingredient in food preparation or be reconstituted and consumed as done in non-functional juice powders.

Table 3 Moisture, water activity, reconstitution time and yield of the prebiotic lemon juice powder

Run	Temperature°C/ maltodextrin	Moisture (%)	Water activity	Reconstitution (seconds)	Yield (%)
1	60 °C/15 %	3.27 ^a ± 0.11	0.25 ^a ± 0.00	47.90 ^b ± 0.00	32.03 ^b
2	70 °C/15 %	2.50 ^b ± 0.08	0.17 ^b ± 0.00	51.32 ^a ± 0.00	33.35 ^a
3	60 °C/20 %	2.24 ^c ± 0.11	0.18 ^b ± 0.00	45.49 ^c ± 0.00	27.43 ^c

Mean±standard deviation ($n=3$). Values followed by different small letters in the same column show significant difference by Tukey test ($p<0.05$)

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