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Enzymatic Synthesis of Prebiotic Oligosaccharides

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Abstract

Prebiotic oligosaccharides are nondigestible carbohydrates that can be obtained by enzymatic synthesis. Glucosyltransferases can be used to produce these carbohydrates through an acceptor reaction synthesis. When maltose is the acceptor a trisaccharide composed of one maltose unit and one glucose unit linked by an α-1,6-glycosidic bond (panose) is obtained as the primer product of the dextransucrase acceptor reaction. In this work, panose enzymatic synthesis was evaluated by a central composite experimental design in which maltose and sucrose concentration were varied in a wide range of maltose/sucrose ratios in a batch reactor system. A partially purified enzyme was used in order to reduce the process costs, because enzyme purification is one of the most expensive steps in enzymatic synthesis. Even using high maltose/sucrose ratios, dextran and higher-oligosaccharide formation were not avoided. The results showed that intermediate concentrations of sucrose and high maltose concentration resulted in high panose productivity with low dextran and higher-oligosaccharide productivity.

Index Entries: Enzymatic synthesis; optimization; factorial design; surface response analysis; prebiotic oligosaccharides.

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Introduction

A prebiotic is a nondigestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, improving the host's health *(1)*. Isomaltooligosaccharides with a degree of polymerization in the range of 2–8 have shown significant increasing growth activity of bifidobacteria in human intestines. These bacteria are known as useful microorganisms having an important role in maintaining human health: preventing the increase of microorganisms that produce harmful substances, such as amines and ammonia, in the intestines.

Prebiotic carbohydrates have the advantage of being selectively utilized by bifidobacteria and not by noxious microorganisms such as *Salmonella* and *Escherichia coli (2–7)*.

Panose is a trisaccharide composed of one maltose unit and one glucose unit linked by an α-1,6-glycosidic bond. This carbohydrate, considered to be prebiotic, can be obtained from sucrose and maltose by dextransucrase acceptor reaction. Panose can be used as an antifading agent in food pigments and as a food antioxidant, as well as in orally used products, including food and drinks. Panose presents anticariogenic properties, because it is not fermentable by oral microorganisms *(8,9)*.

Dextransucrase (EC 2.4.1.5) is a bacterial extracellular glucosyltransferase, which promotes the synthesis of dextran. Fructose is a natural side product released when the enzyme polymerizes glucose from sucrose into dextran. The high enzyme stability, under optimum synthesis conditions, allows its use in industrial scale *(10)*.

When an acceptor is also fed into a reactor, some of the glucose moieties are deviated from dextran formation to the production of acceptor products *(11–15)*. Panose is the prime acceptor product formed when maltose is used as acceptor. Figure 1 presents the dextransucrase acceptor reaction with maltose for panose production *(11)*.

When a sufficient amount of panose exists and sucrose is still available, panose acts as an acceptor, forming isomaltosyl-1,6-α-*O*-D-maltose, which also acts as an acceptor, forming a homologous series (higher oligosaccharides). The yield and the degree of polymerization of these oligosaccharides depend on the availability of the acceptors and sucrose in the reactor *(15,16)*.

Despite all potential applications of panose, a process for its large-scale production is still not available. Fermentation processes can be applied to produce prebiotic carbohydrates *(5,6)*, but enzymatic synthesis presents higher productivities with easier process control.

The main goal of the present work was to study the effect of maltose and sucrose concentration on panose productivity using maltose as acceptor and dextransucrase from *Leuconostoc mesenteroides* B512F. A partially purified enzyme was used in order to reduce the costs of enzyme purification. Synthesis was optimized with the aim of high panose productivity and low dextran and higher-oligosaccharide formation.

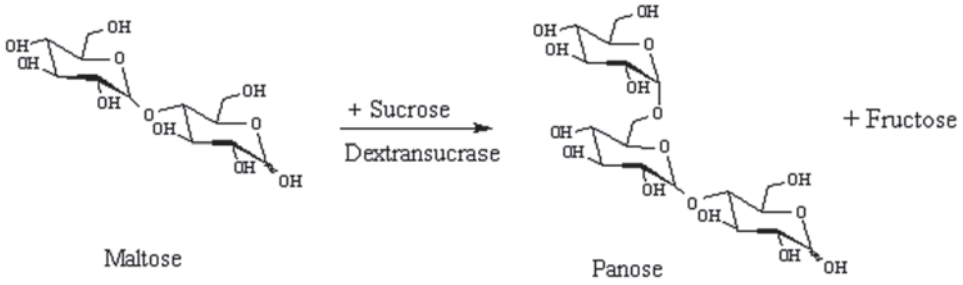

Fig. 1. Dextransucrase acceptor reaction for panose production.

Materials and Methods

Preparation of Enzyme

A strain of *L. mesenteroides* B512F obtained from ARS Culture Bacterial Collection (NRRL Culture Collection, United States Department of Agriculture, Peoria, IL) was employed to produce dextransucrase in a fed-batch fermentation process. The culture medium was composed of 50 g/L of sucrose (food grade), 20 g/L of yeast extract (Merck), 0.20 g/L of $MgSO_4 \cdot 7H_2O$, 0.01 g/L of $MnSO_4 \cdot 2H_2O$, 0.01 g/L of $FeSO_4 \cdot 7H_2O$, 0.02 g/L of $CaCl_2 \cdot 2H_2O$, 0.01 g/L of NaCl, and 20 g/L of K_2HPO_4 (anhydrous) (*14,17*).

A mixed solution composed of NaOH (120 g/L) and sucrose (300 g/L) was fed into the fermentor to control the pH and sucrose level in the culture medium. Fermentation was performed in a fermentator BioFlo 3000 (New Brunswick Scientific) at 30°C and the pH was controlled at 6.7 (±0.1) for 6 h with mechanical agitation of 150 rpm and aeration of 0.5 L/min. After this period, the feed flow rate was interrupted and the pH was allowed to drop to 5.2, when the process was considered finished.

The cells were harvested by centrifugation (11,806*g* for 10 min). The enzyme was recovered from the culture broth by precipitation with polyethylene glycol (PEG 1500). The partially purified enzyme was diluted in a pH 5.2 sodium acetate buffer (20 m*M*) containing 0.05 g/L of $CaCl_2$. The enzyme was recovered immediately after cell harvesting, because the enzyme in crude fermented broth is not stable unless it is frozen at –80°C *(17)*.

Enzyme Activity Assay

Enzyme activity was determined by quantification of the fructose released during the reaction by the dinitrosalicylic acid method *(18)*. The enzymatic preparation (55 µL) was mixed with 455 µL of a 10% (w/v) sucrose solution in an acetate buffer, pH 5.2 (20 m*M* containing 0.05 g/L of $CaCl_2$), and then incubated for 1 h at 30°C *(17)*. The enzyme activity was expressed in dextransucrase units (DSU) per milliliter. One dextransucrase unit is the amount of enzyme that converts 1 mg of sucrose into dextran in 1 h under ideal reaction conditions (30°C and pH 5.2) *(11)*.

Table 1
Experimental Planning Showing Responses
(Panose, Higher–Oligosaccharide, and Dextran Productivities)
and Maltose/Sucrose Ratio

Run	Ratio (M/S)[a]	Sucrose (m*M*)	Maltose (m*M*)	*P*DXT (g/[L·h])	*P*Pan (g/[L·h])	*P*HO (g/[L·h])
1	1.00	25	25	5.00	4.53	8.76
2	8.00	25	200	6.13	8.20	4.54
3	0.25	100	25	2.95	1.43	0.69
4	2.00	100	200	2.25	6.62	1.84
5	4.50	25	112.5	7.47	6.78	6.62
6	1.13	100	112.5	2.67	6.06	1.21
7	0.40	62.5	25.0	3.75	1.25	2.25
8	3.20	62.5	200	2.63	8.40	2.15
9	1.80	62.5	112.5	3.99	6.56	1.72
10	1.80	62.5	112.5	4.00	6.55	1.80

[a]M/S, maltose/sucrose.

Enzymatic Synthesis of Panose

Syntheses were carried out at optimum synthesis conditions (30°C and pH 5.2) in a thermostated glass batch reactor (10 mL). Maltose and sucrose levels were changed according to a two-level central composite design with two central points (Table 1). The carbohydrate levels in the experimental planning were chosen in order to maintain a low viscous reaction medium. Enzyme activity was 22 DSU/mL.

Syntheses were performed until 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 3 vol of 96% ethanol. The supernatant was used to quantify the carbohydrates. The precipitated dextran was diluted in distilled boiling water and assayed as total carbohydrate *(19)*. Syntheses were done in replicate and all analyses were done in duplicate.

Product Analysis

The supernatant containing panose, higher oligosaccharides, fructose, and unreacted maltose was analyzed by HPLC. A Varian Pro Star system equipped with two high-pressure pumps (model 210), refraction index detector (model 350), and an Eldex CH model 150 column oven was used to carry out the analysis. Separation was achieved in an Aminex®HPX-87 C (300 × 7.8 mm) column at 85°C. Ultrapure water at 0.3 mL/min was used as eluent and the detector temperature was 45°C. All samples were analyzed in duplicate. The software ProStar WS 5.5 was used to acquire and handle the data. Oligosaccharide concentration was calculated from peak areas using glucose as the standard *(5)*.

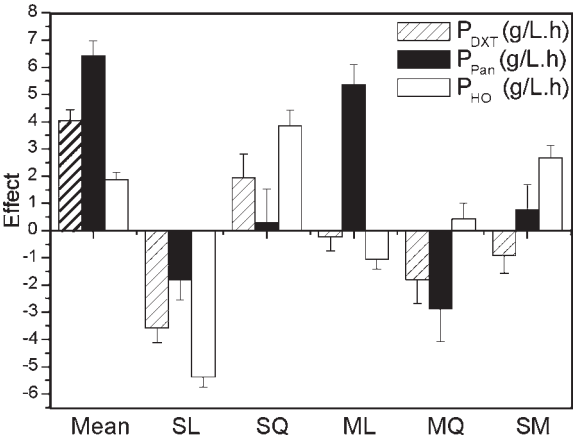

Fig. 2. Calculated effects for each model (*see* Eqs. 1–3). SL, sucrose linear effect; SQ, sucrose quadratic effect; ML, maltose linear effect; MQ, maltose quadratic effect; SM, sucrose and maltose interaction effect.

Results and Discussion

HPLC analysis showed that maltose was not entirely consumed in any of the assays. The viscosity of the medium at the end of the synthesis was low, because low amounts of dextran were obtained when the acceptor was employed as the second substrate. Dextran production and higher oligosaccharides were not avoided in any of the assays.

Table 1 provides the experimental planning (sucrose and maltose concentrations) and the responses: dextran, panose, and higher-oligosaccharide productivities. Maltose/sucrose molar ratios are also presented.

All main effects, linear and quadratic, and interactions were calculated for each model. Figure 2 presents a comparative graph of these effects. The most relevant variable concerning dextran productivity is sucrose, and maltose concentration is significant for panose productivity. Sucrose and maltose concentrations, as well as their interaction, are significant for higher-oligosaccharide productivity. The statistical models for *P*DXT, PPan, and *P*HO are expressed by Eqs. 1–3, respectively, within 95% of confidence:

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$$P_{DXT} = 7.38 - 1.18 \times 10^{-1}S + 6.89 \times 10^{-4}S^2 - 3.41 \times 10^{-2}M - 1.19 \times 10^{-4}M^2 - 1.39 \times 10^{-4}SM \quad (1)$$

$$P_{Pan} = 3.34 - 5.06 \times 10^{-2}S + 1.08 \times 10^{-4}S^2 + 6.55 \times 10^{-2}M - 1.88 \times 10^{-4}M^2 + 1.16 \times 10^{-4}SM \quad (2)$$

$$P_{HO} = 15.62 - 2 + 89 \times 10^{-1}S + 1.37 \times 10^{-3}S^2 - 3.79 \times 10^{-2}M + 3.01 \times 10^{-5}M^2 - 4.1 \times 10^{-4}SM \quad (3)$$

Table 2
ANOVA of Dextran Productivity (*see* Eq. 1)

Source of variation	Sum of squares	Degrees of freedom	Mean of squares	*F* value
Regression	23.62	5	4.72	10.94
Residual	1.72	4	0.43	
Total	25.34	9		
Correlation coefficient	0.932			
F listed value (95%)				*F*5,4 = 6.26

Table 3
ANOVA of Panose Productivity (*see* Eq. 2)

Source of variation	Sum of squares	Degrees of freedom	Mean of squares	*F* value
Regression	53.05	5	10.61	12.23
Residual	3.47	4	0.87	
Total	56.52	9		
Correlation coefficient	0.939			
F listed value (95%)				*F*5,4 = 6.26

Table 4
ANOVA of Higher-Oligosaccharide Productivity (*see* Eq. 3)

Source of variation	Sum of squares	Degrees of freedom	Mean of squares	*F* value
Regression	61.8	5	1.35	66.75
Residual	0.78	4	0.20	
Total	52.56	9		
Correlation coefficient	0.987			
F listed value (95%)				*F*5,4 = 6.26

Tables 2–4 depict the analysis of variance (ANOVA) for the studied responses. According to these tables, the models can be considered statistically significant according to the *F*-test within 95% of confidence, because they are higher than the listed *F* value.

Figures 3–5 present the surface responses obtained with the presented regression equations. According to the surface response obtained for dextran productivity (Fig. 3), sucrose strongly affected this response at low sucrose concentrations ($S < 50$ m*M*), whereas at high sucrose concentrations ($S > 70$ m*M*), this variable did not significantly affect dextran productivity. At moderate sucrose concentration ($50 < S < 70$ m*M*), dextran productivity was slightly affected. High maltose concentrations decreased dextran productivity. However, the effect of maltose was lower than the

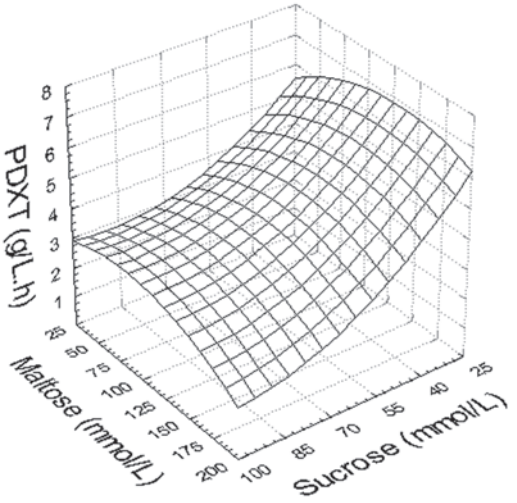

Fig. 3. Fitted surface response for dextran productivity (*see* Eq. 1).

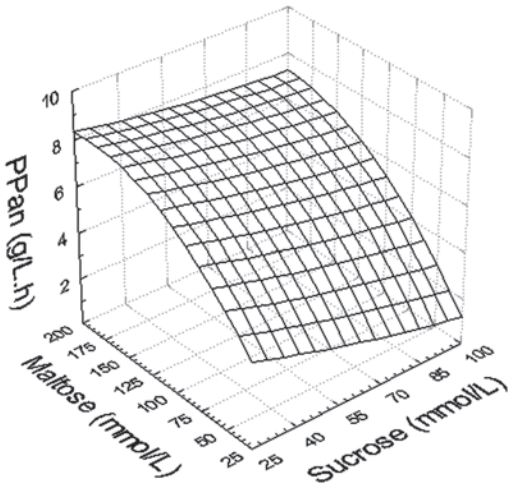

Fig. 4. Fitted surface response for panose productivity (*see* Eq. 2).

effect of sucrose on dextran productivity. According to Fig. 3, using sucrose concentrations higher than 50 m*M* and high maltose concentrations can minimize dextran productivity.

Figure 4 presents the surface response obtained for panose productivity (the desired product). According to Fig. 4, sucrose affected panose productivity at low sucrose concentrations ($S < 50$ m*M*) very slightly. On the other hand, maltose concentration strongly affected panose productivity, because when high maltose concentrations were loaded into the reactor high panose productivities were obtained. Although panose productivity could be maximized by employing high maltose concentrations, the effect

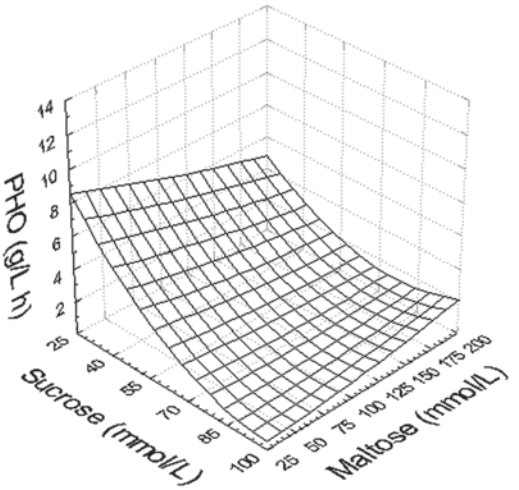

Fig. 5. Fitted surface response for higher-oligosaccharide productivity (*see* Eq. 3).

of maltose was limited, and at maltose/sucrose ratios of 6 and above (S = 25 mM), panose productivity tended toward a constant value even when high maltose concentrations were used (Fig. 4). Figure 5 presents the surface response obtained for higher-oligosaccharide productivity. According to Fig. 5, maltose almost did not affect higher-oligosaccharide productivity, and only a slight increase on this response was observed when high maltose concentrations were employed. However, sucrose strongly affected high oligosaccharide productivity, especially when low concentrations were loaded into the reactor (S < 50 mM). Figure 5 demonstrates that employing high sucrose concentrations minimized higher-oligosaccharide productivity.

The results presented in Figs. 3–5 combined demonstrate that high maltose concentrations minimized dextran and maximized panose productivity (Figs. 3 and 4). Even though this operating condition could slightly increase higher-oligosaccharide productivity (Fig. 5), the strong effect of maltose on panose productivity makes high maltose concentration a good operating condition toward high panose productivity. Low sucrose concentrations maximized higher-oligosaccharide productivity (Fig. 5) and increased panose productivity (Fig. 4). This operating condition also strongly improves dextran productivity. This effect is attributed to the maltose-accelerating effect on the overall reaction rate *(15)*. Therefore, moderate sucrose concentration seems to be the best condition for high panose productivity with low dextran and higher low-oligosaccharide productivities.

According to the results presented in Table 1, runs 2 and 8 are the conditions that result in higher panose productivities (8.20 and 8.40 g/[L·h] respectively). Comparison of the results obtained in run 8 with those obtained in the other runs (Table 1) demonstrates that run 4 presented

lower dextran and lower higher-oligosaccharide productivities than the ones obtained in run 8. However, dextran and higher-oligosaccharide productivities obtained in run 4 are only 16% lower than those obtained in run 8, and panose productivity is 28% lower in run 4. Other results also showed lower oligosaccharide productivities when compared with the one obtained in run 8, but at the expense of higher dextran productivities and lower panose production. Therefore, the best operating condition that simultaneously minimizes dextran and higher-oligosaccharide productivities is the one used in run 8 (high maltose concentration and moderate sucrose concentration).

A previous study regarding the acceptor reaction of dextransucrase concluded that by increasing the maltose/sucrose ratio, dextran synthesis could be suppressed (*20*). In that study, only panose was obtained when using a maltose concentration 20 times higher than sucrose. However, to obtain high panose amounts at this operating condition, a large amount of nonreacted maltose (low substrate conversion) remains in the reactor. High viscous reaction medium, owing to high carbohydrate concentration, would derive from this condition. Because of this limitation such a process is not interesting from an industrial point of view. In addition, in this former work, the strain used to obtain the enzyme was a mutant of the wild strain used in the present work and a purified enzyme (high-cost enzyme) was used.

Conclusion

The effect of sucrose and maltose concentration on panose productivity by acceptor reaction synthesis with dextransucrase from *L. mesenteroides* B512F was investigated. A wide range of maltose/sucrose ratios was evaluated, and dextran and higher-oligosaccharide formation was always detected even when high maltose/sucrose ratios were employed. Several investigators have studied acceptor reaction with dextransucrases *(5,6,11–16,20–24)* and observed that high acceptor/sucrose ratios led to higher acceptor products and lower dextran formation. However, few works concerning process optimization and the application for large-scale production have been reported.

In the present work, a partially purified enzyme was used in order to reduce the process costs, because enzyme purification is one the most expensive steps in enzymatic synthesis. The results presented clearly show that neither dextran nor higher oligosaccharides can be avoided as byproducts even when employing very high maltose/sucrose ratios (run 2). However, it is possible to optimize the synthesis, and the surface response analysis of the results indicates that high panose productivities can be obtained with low dextran and higher-oligosaccharide formation by employing high maltose concentrations and moderate sucrose concentrations, which correspond to a maltose/sucrose molar ratio of 3.2, for the range of experimental conditions studied.

Nomenclature

PDXT = dextran productivity (g/[L·h])
PHO = productivity of higher oligosaccharides (g/[L·h])
PPan = productivity of panose (g/[L·h])
S = initial concentration of sucrose (mmol/L)
M = initial concentration of maltose (mmol/L)

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