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Dextran molecular size and degree of branching as a function of sucrose concentration, pH, and temperature of reaction of *Leuconostoc mesenteroides* B-512FMCM dextransucrase

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Abstract

Reactions of *Leuconostoc mesenteroides* B-512FMCM dextransucrase with increasing concentrations of sucrose, from 0.1 to 4.0 M, gave a decreasing amount of high-molecular weight dextran (HMWD) ($>10^6$ Da) with a concomitant increase in low-molecular weight dextran (LMWD) ($<10^5$ Da). At 0.1 M sucrose, pH 5.5, and 28 °C, 99.8% of the dextran had a MW $>10^6$ Da and at 4.0 M sucrose, 69.9% had a MW $<10^5$ Da and 30.1% had a MW $>10^6$ Da, giving a bimodal distribution. The degree of branching increased from 5% for 0.1 M sucrose to 16.6% for 4.0 M sucrose. The temperature had very little effect on the size of the dextran, which was $>10^6$ Da, but it had a significant effect on the degree of branching, which was 4.8% at 4 °C and increased to 14.7% at 45 °C. Both the molecular weight (MW) and the degree of branching were not significantly affected by different pH values between 4.5 and 6.0.

Keywords: Dextran; Dextransucrase; *Leuconostoc mesenteroides* B-512F; Molecular size; Percent branching

1. Introduction

Dextrans make up a class of polysaccharides that are D-glucans of various structures with contiguous α-(1→6) glycosidic linkages in the main chains and α-(1→2), α-(1→3), or α-(1→4) branch glycosidic linkages, depending on the specificity of the particular dextransucrase.[1] The enzymes responsible for the synthesis of these dextrans from sucrose are known as dextransucrases, glucansucrases, and glucosyltransferases. The enzymes are elaborated by various bacterial species from two genera, *Leuconostoc* and *Streptococcus*.

The enzymatic mechanism for synthesizing dextran has been extensively studied by Robyt and co-workers. Using a pulse of ^{14}C-sucrose and a chase with nonlabeled sucrose, Robyt and co-workers[2] found that *Leuconostoc mesenteroides* B-512F dextransucrase added D-glucopyranosyl units from sucrose to the reducing-end of a growing dextran chain. A covalent glucopyranosyl–enzyme intermediate and a covalent dextranyl–enzyme intermediate are formed during synthesis and the glucopyranosyl group is added to the reducing-end of the dextranyl group. A two-site insertion mechanism was proposed for the elongation of the dextran chain.[1,2] Using similar pulse and chase techniques, Robyt and Martin[3] showed that *Streptococcus mutans* 6715 dextransucrase (GTF-S) and mutansucrase (GTF-I) also synthesized their D-glucans by a two-site insertion mechanism with D-glucose being added to the reducing-end of the growing glucan chains. Using equilibrium dialysis experiments with a sucrose substrate analogue, 6-deoxysucrose, Su and Robyt[4] showed that dextransucrase had two sucrose binding-sites, providing

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further evidence for the two-site mechanism. Ditson and Mayer[5] confirmed the synthesis of dextran from the reducing-end by *S. sanguis* GTF-S dextransucrase. In further confirmation of the mechanism, Parnaik and co-workers[6] isolated a covalent glucosyl–enzyme intermediate for *S. sanguis* GTF-S dextransucrase and Mooser and Iwaoka[7] also isolated a glucosyl–enzyme intermediate for *S. sorbrinus* GTF-S dextransucrase.

In addition to catalyzing the synthesis of dextran from sucrose, dextransucrase also catalyzes the transfer of a D-glucopyranosyl group from sucrose to other carbohydrates that are present or are added to the enzyme digest.[8,9] The added carbohydrates have been called *acceptors* and the reaction is called an *acceptor reaction*. When the acceptor is D-glucose, maltose, or isomaltose, a series of oligosaccharides with isomaltodextrins of varying number of D-glucose units are attached by an α-(1→6) linkage to the nonreducing-end of the acceptor.[10,11] Robyt and Walseth[10] showed that the acceptors were releasing the glucopyranosyl and dextranyl covalent enzyme-intermediates by a nucleophilic displacement reaction, which terminates the polymerization of dextran. Su and Robyt[4] confirmed this mechanism by showing that the active-site of B-512FM dextransucrase had a single acceptor binding-site where the acceptors were bound and could make a nucleophilic attack on the glucopyranosyl or dextranyl group to release them from the active-site and form the acceptor products.[10]

The branching of dextran does not occur from the action of a separate dextran-branching enzyme as occurs in the biosynthesis of starch. Robyt and Taniguchi[12] showed that dextran was branched by dextransucrase itself in an acceptor reaction in which a dextran chain is the acceptor that binds in the acceptor binding-site, where its C-3–OH group makes a nucleophilic attack onto C-1 of the glucopyranosyl or dextranyl group, that are covalently linked at the active-site, releasing them from the active-site and forming an α-(1→3) branch linkage with the D-glucose or with the dextran chain.

Leuconostoc species require sucrose in the culture medium, as an inducing agent for the elaboration of glucansucrases, whereas the *Streptococcus* species do not require sucrose in the medium to produce glucansucrases.[1] Kim and Robyt obtained *L. mesenteroides* mutants from strains, B-512FM, B-742, B-1299, and B-1355 that are constitutive for the elaboration of glucansucrases.[13–17] A mutant, *L. mesenteroides* B-512FMCM, was obtained that produced 13-times more enzyme than the parent mutant strain, B-512FMC, and over a 100-fold more than the original B-512F, commercial strain.[18–20]

Alsop[21] reported that as the sucrose concentration in a dextransucrase digest was increased, from 2 to 20% (w/v), the amount of low-molecular weight dextran (LMWD) increased, and with 20% sucrose, there was 43.9% LMWD, which was defined as polysaccharides and/or oligosaccharides with molecular weights below 5000 Da. There is no report on the size distribution and the degree of branching of dextrans synthesized under different enzyme reaction conditions, such as pH, temperature, and sucrose concentrations of 0.1–4 M.

In this paper, we report the size distribution and the degree of branching of dextrans synthesized by *L. mesenteroides* B-512FMCM dextransucrase, using a relatively broad concentration of sucrose, 0.1–4.0 M, with different pH values of 4.5–6.0, and different temperatures of 4–45 °C.

2. Experimental

2.1. Preparation of dextransucrase

A constitutive mutant, *L. mesenteroides* B-512FMCM, was obtained by mutating *L. mesenteroides* B-512FMC with vacuum ultraviolet radiation.[19] Dextransucrase was prepared by culturing *L. mesenteroides* B-512FMCM in a glucose medium, as described by Kim and Robyt.[13–17] The enzyme was purified as described by Kim and Kim.[20] The purified enzyme gave a single protein band on polyacrylamide gel electrophoresis that had a specific dextransucrase activity of 250 IU/mg, both of which indicate a highly purified enzyme.

2.2. Synthesis of dextrans under different conditions

The reaction digests were prepared at various sucrose concentrations (0.1–4.0 M), pH values of 4.5–6.0, and temperatures, 4–45 °C. Sucrose was dissolved in 20 mM acetate buffer (pH 5.2); 40 U of dextransucrase was added to 50 mL of buffered sucrose incubated at the various temperatures. One unit of dextransucrase was defined as the amount of enzyme that liberated 1 μmol of D-fructose per min, with the D-fructose being measured by thin-layer chromatography (TLC), using an imaging densitometer (BioRad, Model GS 710) with D-fructose standards (50–2000 ng) or by the measurement of the incorporation of 1 μmol of D-glucose into dextran, measured by the amount of ^{14}C dextran formed from [U-^{14}C]-sucrose.[1,3,4] The dextrans in the digests were precipitated by the addition of two volumes of EtOH after all of the sucrose had been consumed.

2.3. Analysis of molecular weights (MWs)

Dextran sizes and quantities were determined by measurement of multi-angle light scattering intensities, using a DAWN GPC photometer (Wyatt Technology, Santa Barbara, CA). Dextran T10, T70, T500, and

T2000 (Pharmacia, Uppsala, Sweden) (10 mg/mL) and T150 (Dextran Products Ltd., Toronto, Canada) (10 mg/mL) were used as standards.[22]

2.4. Analysis of branching

The branching of the synthesized dextrans was analyzed by hydrolysis with endodextranase and the quantitative determination of the hydrolysis products by TLC analysis. Endodextranase hydrolyzes dextran to give D-glucose, isomaltose, isomaltotriose, and branched isomaltodextrins: a tetrasaccharide (3^3-α-D-glucopyranosyl isomaltotriose to hexasaccharides (3^3-isomaltosyl isomaltotetraose and 3^3-D-glucopyranosyl isomaltopentaose)[23] and higher branched saccharides. A ratio (R) of [% branched isomaltodextrins + % unhydrolyzed dextran] ÷ [% monosaccharides + % isomaltodextrins] = [BIM + UHD] ÷ [M + IMD] = R was obtained for each synthesized dextran from the TLC analysis of the dextranase hydrolyzed dextrans. It previously has been shown by methylation analysis and ^{13}C NMR that the dextran synthesized by *L. mesenteroides* B-512F dextransucrase, reacting with 0.1 M sucrose at pH 5.5 and 28 °C, gave a dextran with 5% α-(1→3) branch linkages.[24,25] This dextran gave a ratio (R) of 0.247. This ratio was used to obtain a *branching conversion factor* by dividing the percent branching (5%) by R (0.247) or $5\% \div 0.247 = 20.24$. This factor was then used to determine the percent of branching for the various dextrans that were synthesized under the different conditions by multiplying the ratio (R) by the branching conversion factor, 20.24, or percent branching = $R \times 20.24$.

Table 1
Molecular weight distribution of dextrans synthesized by *L. mesenteroides* B-512FMCM dextransucrase at varying sucrose concentrations, pH, and temperature

Percent dextran				
Sucrose [a] (M)	10^3–10^4	10^4–10^5	10^5–10^6	$>10^6$ MW [b]
	I	II	III	IV
0.1	0.12	0	0.8	99.8
0.3	1.27	0.73	0	98.0
1.0	5.8	18.6	17.9	57.7
1.5	8.7	30.9	3.8	56.6
2.0	61.5	0	0	38.5
3.0	68.4	0	0	31.6
4.0	69.9	0	0	30.1
pH [c]				
4.5	13.2	1.2	26.8	58.8
5.0	1.6	1.1	10.4	86.9
5.2	1.3	0	10.1	88.6
5.5	0.5	0	4.5	95.0
6.0	0.3	0	0	99.7
°C [d]				
4	0	0.4	16.4	83.2
15	4.2	0	20.6	75.2
23	6.1	0	9.1	84.8
28	2.3	7.8	15.4	74.5
37	1.3	0	33.4	65.3
45	0	0	15.9	84.1

[a] Reactions conducted at pH 5.5 and 28 °C and different sucrose concentrations.
[b] Molecular weights divided into four divisions.
[c] Reactions conducted at 1.5 M sucrose and 28 °C and different pH values.
[d] Reactions conducted at 1.5 M sucrose and pH 5.5 and different temperatures.

2.5. Dextranase hydrolysis of dextran and TLC analysis in the determination of the degree of branching

Penicillium dextranase (1.0 U; D-4668, Sigma Chemical Co., St. Louis, MO) was added to 1.0 mL, containing 10 mg of the various dextrans, pH 5.5 (20 mM citrate–phosphate buffer), at 37 °C and allowed to react for 3 h. Aliquots (1–5 μL) were added to 20 × 20 cm Whatman K5F TLC plates, which were irrigated at 22 °C, using two ascents (18 cm path length) of 4:10:3 volume proportions of nitromethane–1-propanol–water. The carbohydrates were visualized on the plate by rapidly dipping the plate into a solution containing 0.3% (w/v) *N*-(1-naphthyl)ethylenediamine and 5% (v/v) H_2SO_4 in MeOH, dried, and heated at 120 °C for 10 min.[26] The carbohydrate products from dextranase hydrolysis (D-glucose, isomaltose, isomaltotriose, branched isomaltodextrins, and unhydrolyzed dextran) were separated and quantitatively determined by TLC, using an imaging densitometer (BioRad, Model GS 710) with glucose standards (50–2000 ng).[26,27]

2.6. Analysis of dextran synthesized by a high concentration of sucrose

The precipitated dextran (6 mg), obtained from a dextransucrase reaction with 3.0 M sucrose, was dissolved in 1.2 mL of water and added to a 1.5 × 100 cm column of Bio-Gel P-150 that had an exclusion volume of 150,000 Da. The column had a flow rate of 0.06 mL/min and 1.0 mL fractions were collected and the eluted dextran was detected using the micro phenol–sulfuric acid method.[28] The eluted dextran was divided into two parts, a high-molecular weight dextran (HMWD > 10^5 Da) and a LMWD (< 10^5 Da).

The HMWD was rechromatographed on a 1.0 × 60 cm Bio-Gel A-0.5m column with a flow rate of 0.06 mL/min and 1.0 mL fractions were collected. The degree of branching was determined for both HMWD

Table 2
Dextranase/TLC determination of the degree of branching of dextrans synthesized by *L. mesenteroides* B-512FMCM dextransucrase at various concentrations of sucrose, pH, and temperatures

Sucrose (M) [b]	Monosaccharide [% M]	Isomaltodextrins [% IMD]	Branched isomaltodextrins [% BIM]	Unhydrolyzed dextran [% UHD]	Ratio (R) [BIM + UHD] [M + IMD]	Percent branching [a]
0.1	31.3	48.8	12.5	7.3	0.247	5.0
0.3	33.9	44.9	14.6	6.6	0.269	5.4
1.0	30.8	43.2	21.9	4.1	0.351	7.1
1.5	31.5	39.7	24.7	4.1	0.404	8.2
2.0	25.4	42.3	30.0	2.3	0.477	9.7
3.0	16.7	44.8	36.8	1.7	0.626	12.7
4.0	18.4	36.6	37.5	7.5	0.818	16.6
pH [c]						
4.5	35.3	40.1	18.9	5.7	0.326	6.6
5.0	29.4	44.3	20.0	6.3	0.357	7.1
5.2	28.5	43.2	21.9	6.2	0.403	8.2
5.5	27.1	44.3	21.8	6.8	0.401	8.1
6.0	34.5	41.1	17.5	6.9	0.323	6.5
°C [d]						
4	23.4	57.6	14.5	4.5	0.235	4.8
15	19.8	59.3	16.0	4.9	0.264	5.3
23	17.9	57.6	18.5	6.0	0.325	6.6
28	16.4	58.3	18.9	6.4	0.339	6.9
37	16.5	53.1	20.7	9.7	0.437	8.8
45	8.7	49.2	26.6	15.5	0.727	14.7

[a] Branching for dextran synthesized from 0.1 M sucrose was assumed to be 5% from Refs. 24 and 25. A branching conversion factor was obtained by dividing 5% by the ratio (R), 0.247, giving 20.24. The percent branching for the other dextrans was then obtained by multiplying their ratios (R) by the conversion factor, 20.24.
[b] Reactions were conducted at pH 5.5 and 28 °C.
[c] Reactions were conducted at 1.5 M sucrose and 28 °C.
[d] Reactions were conducted at 1.5 M sucrose and pH 5.5.

and LMWD by dextranase hydrolysis and TLC analysis as described above.

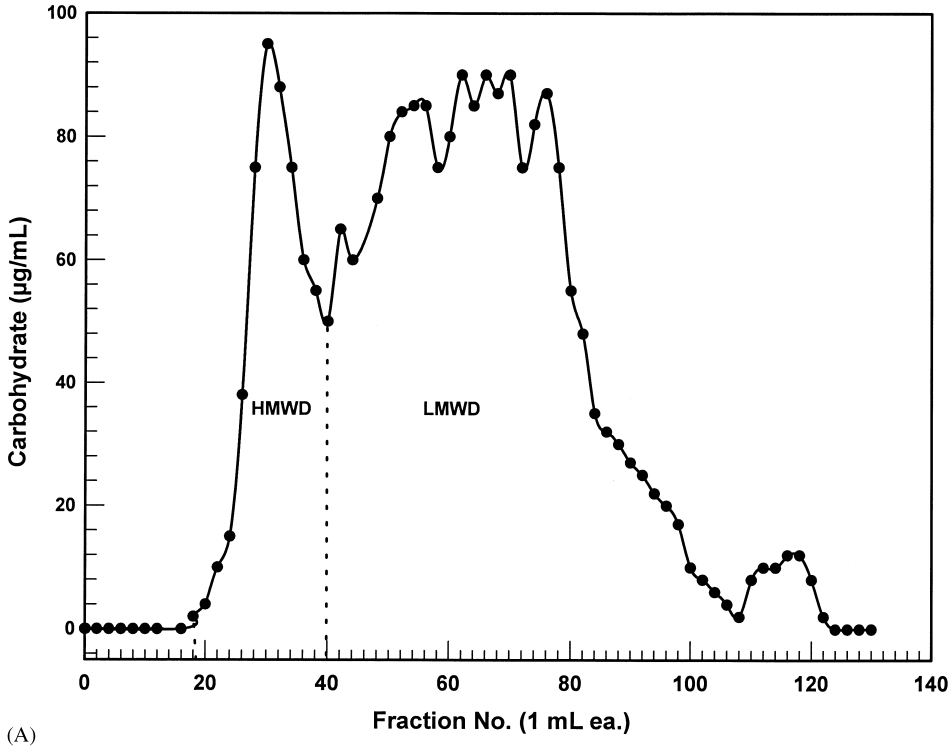
3. Results and discussion

Table 1 shows the relative percents of the different MWs of dextrans synthesized by *L. mesenteroides* B-512FMCM dextransucrase as a function of sucrose concentration, pH, and temperature. The MWs of the synthesized dextrans were determined by Wyatt Technology multi-angle light scattering intensities. At 0.1 M sucrose, 28 °C, pH 5.5, 99.8% of the dextran had a MW greater than 10^6 Da. Table 1 shows that as the concentration of sucrose was increased, the lower MW fractions were increased. At 1.0 and 1.5 M sucrose, the intermediate MWs, 10^4–10^5 and 10^5–10^6 Da increased the most, with a concomitant decrease of MWs greater than 10^6 Da. At 2.0, 3.0, and 4.0 M sucrose, the lower MWs (10^3–10^4 and 10^4–10^5 Da) increased and the intermediate MWs decreased. At these higher concentrations of sucrose, MWs was definitely bimodal, with the lowest MW dextrans (10^3–10^4 Da) predominating at the higher concentrations of sucrose and the very high MW dextrans ($> 10^6$ Da) decreasing, but remaining relatively constant between 38.5 and 30.1%.

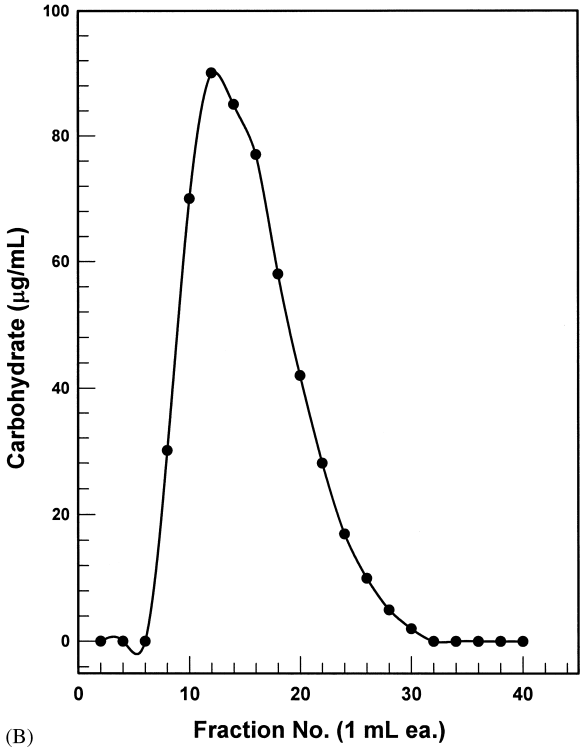
The effect of pH was studied, using 1.5 M sucrose and 28 °C. The pH had much less effect on the MWs of the synthesized dextrans (Table 1) than the sucrose concentration or the temperature. At pH 4.5, the very high MW dextran was predominate at 58.8%. There were, however, significant amounts of most of the other MW dextran fractions of 13.2–26.8%, with the exception of the 10^4–10^5 Da fraction, which was only 1.2%. As the pH was increased above 4.5, the very high MW dextran ($> 10^6$ Da) increased and all of the lower MW fractions decreased, but especially the lowest MW fractions (10^3–10^4 and 10^4–10^5 Da) was synthesized.

The effects of temperature were measured at 1.5 M sucrose and pH 5.5. At the very lowest temperatures studied (4 and 15 °C), the very high MW dextran ($> 10^6$ Da) was the major dextran synthesized (Table

1). As the temperature was increased, the very high MW dextran decreased, with modest increases in the intermediate MW dextrans being synthesized. At 37–45 °C, the predominant MWs were the very high ($>10^6$ Da) and the HMWDs (10^5–10^6 Da) were the exclusive products.



(A)



(B)

Fig. 1. (A) Gel permeation chromatography on Bio-Gel P-150 of dextran synthesized from 3.0 M sucrose, pH 5.5, and 28 °C by *L. mesenteroides* B-512FMCM dextransucrase. The dextran was divided into two fractions: HMWD and LMWD by pooling fractions 18–40 and 42–122, respectively. (B) Gel permeation chromatography on Bio-Gel A-0.5m of HMWD obtained from chromatography of Bio-Gel P-150.

From this study, the optimum conditions for the synthesis of very high MW dextran in high yields would be obtained at relatively low sucrose concentrations (0.1–0.3 M), high pH values of 5.5–6.0, and high temperature of 37–45 °C. The optimum conditions for the synthesis of LMWDs would be high sucrose concentrations of 3.0–4.0 M, low pH (4.5), and intermediate temperatures of 23–28 °C.

The effect of the concentration of sucrose on the degree of branching was studied at pH 5.5 and 28 °C (Table 2). The data clearly shows that as the concentration of sucrose was increased from 0.1 to 4.0 M, the percent of branching is significantly increased from 5.0 to 16.6%. The high sucrose concentrations gave very high degrees of branching, with 4.0 M giving one α-(1→3) branch linkage out of every six D-glucose residues. Changes in the pH had very little effect on the degree of branching, using 1.5 M sucrose and 28 °C. In going from pH 4.5 to 5.2, the degree of branching increased from 6.6 to 8.2%, and then decreased again to 6.5% at pH 6.0.

The temperature, however, had a significant effect on the degree of branching, using 1.5 M sucrose and pH 5.5. At 4 °C, the degree of branching was the lowest at 4.8%. As the temperature was increased, the degree of branching was increased to 5.3% at 15 °C, 6.9% at 28 °C, 8.8% at 37 °C, and 14.7% at 45 °C (Table 2). The effect of the increase in temperature, giving an increase in the degree of branching, might have been expected from the mechanism of branching dextran by dextransucrase. The linear dextran molecules bind at the acceptor-site and form the branch linkages by an acceptor transglycosylation reaction.[12] The binding of dextran to the acceptor-site is controlled by the diffusion of the dextran molecules, and therefore, it is temperature dependent. The higher the rate of diffusion, the higher is the degree of branching due to greater interaction between dextransucrase, sucrose, and acceptor dextran. Tanriseven and Robyt[29] reported that when the sucrose concentration was increased over 200 mM, sucrose was bound to a third, low-affinity binding-site of dextransucrase that allosterically changed the conformation of the active-site, preventing the synthesis of dextran. This could also be the cause here in both decreasing the dextran MW and increasing the degree of branching at high sucrose concentrations.

Dextran synthesized from 3.0 M sucrose at pH 5.5 and 28 °C by dextransucrase was fractionated on Bio-Gel P-150 (Fig. 1A). The dextran fractions were pooled and separated into a HMWD and LMWD as shown in Fig. 1A. The HMWD was then chromatographed on Bio-Gel A-0.5m (Fig. 1B), which showed only a single peak. The degree of branching for the two dextrans was determined by dextranase hydrolysis and TLC as described. The degree of branching for the two dextran fractions was not significantly different, with the HMWD having 10.6% branching and the LMWD having 9.8% branching, giving an average of 10.2% branching for the unfractionated dextran, about two-times the degree of branching obtained from 0.1 M sucrose at pH 5.5 and 28 °C.

From this study, the lowest degree of branching ($<4.8\%$) would be obtained by using 0.1 M sucrose, 4 °C, and pH 4.5 or 6.0 and the highest degree of branching ($\sim 16\%$) would be obtained by using a high concentration of sucrose (4.0 M), 45 °C, and pH 5.5.

Although the trends are apparent in this study for controlling both the MW and the degrees of branching of the dextran synthesized by *L. mesenteroides* B-512FMCM dextransucrase, the study could be expanded, using a more complete combinatorial study of sucrose concentration and temperature to give a pattern that could be used to select a wide range of MWs and degrees of branching. This would lead to better controling and selecting dextrans of different MWs, with different degrees of branching.

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