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Production of insoluble dextran using cell-bound dextransucrase of *Leuconostoc mesenteroides* NRRL B-523

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Abstract

Water-insoluble, cell-free dextran biosynthesis from *Leuconostoc mesenteroides* NRRL B-523 has been examined. Cell-bound dextransucrase is used to produce cell-free dextran in a sucrose-rich acetate buffer medium. A comparison between the soluble and insoluble dextrans is made for various sucrose concentrations, and 15% sucrose gave the highest amount of cell-free dextran for a given time. *L. mesenteroides* B-523 produces more insoluble dextran than soluble dextran. The near cell-free synthesis was validated in a batch reactor, by monitoring the cell growth which is a small (10^6–10^7 CFU/mL) and constant value throughout the synthesis.

Keywords: *Leuconostoc mesenteroides*; Dextransucrase; Insoluble dextran; Cell-free synthesis

1. Introduction

Dextran has been widely used as a blood volume expander, in the pharmaceutical industry,[1] in the food industry and as chromatographic media[2] due to its non-ionic character and good stability under normal operating conditions. The production of dextran by bacteria differs from the formation of most other exopolysaccharides in that the process is essentially extracellular and does not require the monosaccharide to be activated.[3] But, there is a stringent substrate requirement satisfied by sucrose and a small number of related oligosaccharides. Many lactic acid bacterial strains possess the ability to produce extracellular dextran when grown in the presence of sucrose. The dextran produced by a given species or strain of bacterium may be either a water-soluble or a water-insoluble capsular dextran.

The term dextran collectively describes a large class of bacterial extracellular hydrocolloid homopolysaccharides consisting of α-D-glucopyranosyl units polymerized predominantly in a α-(1→6) linkage.[4] The degree of branching (non-α-1→6 bonds) and molecular weight affect the water solubility and other rheological properties of the dextran.[5] Dextran is synthesized by the enzyme, dextransucrase (sucrose: 1,6-α-D-glucan 6-α-glucosyltransferase, EC 2.4.1.5), from sucrose. Although many different species of the genera *Leuconostoc*, *Lactobacillus* and *Streptococcus* are known to synthesize an extracellular dextransucrase under appropriate growth conditions, the dextran product (and hence the enzyme) is strain specific. For example, when grown on sucrose, *Leuconostoc mesenteroides* releases the enzyme dextransucrase, which polymerizes the remaining sucrose to dextran with molecular weights of several million:

$$n\underset{\text{sucrose}}{C_{12}H_{22}O_{10}} \xrightarrow{\text{dextransucrase}} \underset{\text{dextran}}{(C_6H_{12}O_6)_n} + n\underset{\text{fructose}}{C_6H_{12}O_6}$$

There have been numerous studies on the production of dextransucrase and water-soluble dextran from *L. mesenteroides* NRRL B-512F.[6,7] Landon et al.[8] proposed a method of producing cell-free soluble dextran by concentrating and purifying dextransucrase produced by the strain NRRL B-512F. However, there are very few reports dealing with insoluble dextran production by *L. mesenteroides* NRRL B-523. Recent studies have shown that not only water-soluble dextrans, but also water-insoluble dextrans are of high industrial value.

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Unlike water-soluble dextrans, water-insoluble dextrans are more effective in controlling the viscosity or stability of fluids. They can also effectively control the permeability of porous media.[9] We have focused on the strain NRRL B-523, because it produces a considerable amounts of insoluble dextran, and the growth kinetics has been well studied.[10] Dextran synthesis from the strain NRRL B-523 was first reported by Jeanes.[11] The kinetic behavior of soluble and insoluble forms of dextran from *L. mesenteroides* NRRL B-1299 has been investigated with sucrose as substrate.[12] A kinetic model for the production of cells and polysaccharides has also been developed for the strain NRRL B-523,[13] but the production of insoluble dextran under cell-free conditions has not been attempted. This strain showed promising results in bacterial profile modification for enhanced oil recovery.[14]

Accordingly, our study focused on synthesizing insoluble dextran in a cell-free system that would exhibit physiochemical properties compatible with these various roles. In this work we report on studies that examined concentrations of dextransucrase and both soluble and insoluble dextrans of *L. mesenteroides* NRRL B-523 in batch reactors. Due to the difficulties in separating the cell-bound dextransucrase, a small amount of the cell pellet, containing cells and the cell-bound enzymes, was used to produce the cell-free insoluble dextran. It has been verified that there is no further cell growth in the dextran synthesis medium resulting in a negligible number of cells with a large amount of dextran, which is defined as a near-cell-free condition. Therefore, the method used in this study can provide a possible alternative to cell-free exopolymer production using cell-bound enzymes.

2. Experimental

Bacteria.—*L. mesenteroides* NRRL-B523 (ATCC 14935) was obtained from the American Type Culture Collection (Rockville, MD). It is a facultative anaerobe that grows under mesophilic conditions and was placed in the freezer for long-term storage. Every 30 days, an aliquot was transferred from the freezer, streaked onto slants prepared using Difco Lactobacillus MRS broth, grown for 24 h, and stored at 4 °C. The slants were discarded after 30 days of 4 °C storage.

Growth conditions.—*L. mesenteroides* inoculum was prepared by transferring the culture from slants into a medium containing 9 g/L glucose, 9 g/L fructose, and 11.11 g/L yeast extract (Sigma, St. Louis, MO) solution diluted in a phosphate-buffered saline solution. The mineral salt solution contained NaCl (0.07 M), NH_4Cl (0.6 M), CH_3COONa (0.06 M), ascorbic acid (0.5 g/dm^3), and trace minerals (Fisher Scientific, Pittsburgh, PA).[15] The combination of glucose and fructose can maintain cell growth without inducing the production of exopolymers. After 22 h of growth at 25 ± 1 °C, 10 mL of inoculum was aseptically transferred to a 500-mL batch reactor containing 200 mL of the growth medium. The growth medium is the same phosphate-buffered saline solution with 2% sucrose as the carbon source instead of glucose and fructose. The initial cell concentration was $8.1 \times 10^7 \pm 0.1 \times 10^7$ cells/mL. All batch reactions were conducted at 25 ± 1 °C and 150 rpm in a rotary shaker.

Analytical measurements.—Bacterial growth was followed by a UV–Vis spectrophotometer (660 nm).[16] When the OD reached 1.0, the inoculum was transferred to the culture medium containing sucrose and other nutrients. An inoculum of 5% was used for all the experiments, because it gave high OD values compared to 10% inoculum transfer. Experiments were carried out in triplicate.

Enzyme production.—Suspended dextransucrase was obtained from the growth medium by centrifuging the sample at 3000*g* for 20 min after 22 h of inoculation. The cell-free supernatant that contained the suspended dextransucrase was stored at 4 °C for enzyme assay.

Soluble enzyme assay.—Dextransucrase activity of cell-free culture supernatant was determined by measuring the initial rate of fructose production using the dinitrosalicylic acid method (DNS method).[17] Reactions were carried out 30 °C in 20 mM acetate buffer (pH 5.4) containing 100 g/L. Exactly 100 μL of an enzyme solution was added to 500 μL of a solution of sucrose (400 g/L), 200 μL of sodium acetate buffer (pH 5.4) and 200 μL of $CaCl_2$ (0.5 g/L). The reaction mixture without dextransucrase was incubated for 5 min in a thermostated reactor at 30 °C. The reaction was started by the addition of dextransucrase. At time intervals of 5 min, 100-μL samples were removed from the reaction mixture and added to 100 μL of reagent (DNS). The reaction was immediately terminated because of the high pH of the reagent.

The tubes of samples and the tubes of standards were placed in a water bath for approximately 5 min so that the colorimetric reaction could take place. After 15 min of incubation on ice, samples were mixed with 1.5 mL of water, and absorbance was measured at 540 nm. One unit of dextransucrase activity (DSU) is defined as the amount of enzyme that catalyzes the formation of 1 μmol of fructose per minute at 30 °C in 20 mM sodium acetate buffer (pH 5.4) with 100 g/L sucrose and 0.05 g/L $CaCl_2$. It can be inferred that 1 DSU = 1/21 U according to the international definition of enzyme activity. The enzyme activity was calculated using the standard curve prepared with fructose. Experiments were carried out in triplicate.

Soluble dextran recovery.—The cell-free supernatant was shaken with 2 vols of 70% EtOH to precipitate soluble dextran. The precipitate was collected by cen-

trifugation at 3500*g* for 30 min, followed by filtration on a sterile 0.45-μm Millipore filter. The precipitate (soluble dextran) was saved for concentration measurement.

Insoluble cell-free dextran synthesis.—The insoluble biomass pellet obtained from centrifuging the growth medium after 24 h was washed with deionized water and used for the production of insoluble cell-free exopolymer. A small amount of the pellet (approximately 2 mL) was transferred into a 500-mL batch reactor containing 200 mL of sodium acetate buffer (pH 5.4) containing 130 μM $CaCl_2$ and 15% sucrose as the initiator and substrate for dextransucrase, respectively. After 22 h, the entire medium was centrifuged at 3500*g* for 20 min to collect the cell-free insoluble dextran as a pellet at the bottom. The pellet (insoluble dextran) was saved for concentration measurement.

Dextran assay.—The exopolymer (a glucose polymer chain) concentration was measured by degrading this glucose polymer into glucose monomers and assaying the glucose concentration with a phenol–H_2SO_4 assay.[18]

Prior to the phenol–H_2SO_4 assay, separation and hydrolysis of the soluble and insoluble exopolymers were carried out according to the procedures described by Jeanes et al.;[19] aliquots of sample (5 mL) were taken from the batch reactors and centrifuged (6000*g*) for 20 min to separate insoluble and soluble parts. After 20 min centrifugation, an insoluble dextran pellet had collected at the bottom. The supernatant (soluble exopolymers and metabolic byproducts) was saved for the soluble exopolymer assay. The pellet was rinsed with deionized water and centrifuged again.

Precipitated soluble and insoluble exopolymers were treated in the following manner.[20] Hydrochloric acid (2 M) was added to the precipitates to adjust the pH to 1 ± 2, and the solution was heated for 3 h in a boiling water bath with caps tightly closed. After boiling, the pH of the sample was adjusted to 6.5 with 10% KOH. This solution was then assayed for glucose using the phenol–H_2SO_4 assay by UV spectroscopy at 490 nm. The amount of dextran, measured as glucose, was calculated from the standard curve prepared with glucose.

3. Results and discussion

Fermentation parameters affecting dextran production.—When *L. mesenteroides B-523* was grown in batch culture with sucrose as an energy source under controlled conditions of temperature (25 °C) and agitation (150 rpm), the dextransucrase activity (5%) in the phosphate buffer medium parallels bacterial growth (Fig. 1). As shown in Fig. 1, the maximum cell concentration ($OD_{660\ nm}$ 1.75) and the enzyme activity (120 DSU/mL) were obtained at the late exponential growth phase and beginning of the stationary phase (24 h). The dextransucrase activity of the culture decreased during the period following the stationary phase, possibly due to the absence of alkaline earth metal ions (particularly Ca^{2+}). Tsuchiya et al.[21] found that dextransucrase production is optimal at pH values between 7–8, but the enzyme denatured irreversibly when maintained at that pH range for an extended period of time. The activity of dextransucrase was preserved at 4 °C for 24 h, when the pH of the medium was kept between 5.0 and 5. This was confirmed by pH of the basal medium after 20 h, which decreased to 4.9 during the culture growth due to release of various acids.

The culture was grown for 20 h until the early stationary phase and then transferred to the batch reactor containing 2% sucrose medium. Fig. 1 shows OD curves for 5 and 10% inoculum. An inoculum of 5% gave the maximum OD value (1.75) at 22 h, compared to 10% inoculum (1.63) at 28 h. The high value of enzyme activity (120 DSU/mL) indicates the possibility of a large amount of dextran production. However, precipitation (66%) of the culture supernatant by ethanol produced small amounts of soluble dextran.

Cell-free synthesis.—In order to produce cell-free dextran, the medium must be free of any bacterial cells or it must contain as small a number of cells as possible (10^6–10^7 CFU/mL). Sodium acetate buffer (20 mM, pH 5.4) was chosen as the medium for cell-free insoluble dextran synthesis. Calcium chloride ($CaCl_2$, 130 μM) was added to the buffer to activate the cell-bound enzyme. A small amount of cell pellet (~ 2 mL) obtained from the growth medium was transferred to the acetate buffer because the bound enzyme could not be separated from the bacterial cells. As shown in Table 1,

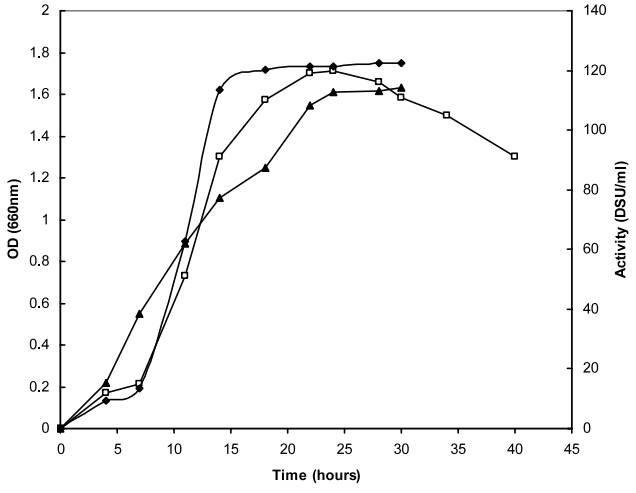

Fig. 1. Dextransucrase production by cultures of *L. mesenteroides* NRRL B-523. Culture conditions: batch fermentation using 20 g/L sucrose as carbon source at 25 °C. □, enzyme activity (5%); ◆, 5% inoculum; ▲, 10% inoculum.

Table 1
Cell growth in different acetate buffer mediums containing 2, 5, 10 and 15% sucrose

Sucrose (%)	CFU/mL $\times 10^{-7}$					
	Time (h)					
	0	8	12	18	24	36
2	2.50	2.60	2.61	2.50	2.52	2.40
5	1.40	1.41	1.45	1.38	1.40	1.30
10	1.10	1.24	1.20	1.18	1.20	1.04
15	2.30	2.30	2.18	2.14	2.20	2.30

there is no cell growth in the synthesis medium for all sucrose (2, 5, 10 and 15%) concentrations tested. This confirms that bound enzyme polymerizes the substrate sucrose to dextran without the need for additional cell growth.

The cell growth is prevented by the absence of growth factors such as yeast extract, phosphates and trace minerals. Fig. 2 shows a comparison of cell growth in the two media, phosphate buffer and sodium acetate buffer. As clearly shown, the cell growth in phosphate buffer follows the lag, exponential, stationary and decay phases. While in acetate buffer medium, there is no growth of the added cells.

The insoluble dextran synthesis was performed for various sucrose concentrations viz. 2, 5, 10 and 15% (Fig. 3). All concentrations showed an increase in exopolymer concentration, but 15% sucrose medium gave the highest amount of dextran for a given time. A small amount of soluble dextran (~ 0.6 mg/mL) was produced by the suspended enzymes in the supernatant.

It can be concluded that dextransucrase B-523 produces more insoluble dextran than soluble dextran and most of the dextransucrase are cell-bound. Therefore, a method to produce insoluble dextran using *L. mesenteroides* B-523 requires the use of cell-bound dextransucrase rather than the suspended enzymes. We have examined a method for *L. mesenteroides* B-523 insoluble dextran production, using cell-bound enzymes. The conditions for the near cell-free synthesis have been validated by the low and constant cell count. Under these conditions, dextransucrase showed a high degree of enzyme activity that can be utilized for continuous insoluble dextran synthesis.

4. Conclusions

While attempts were made to obtain the dextran production entirely cell-free, the resulting dextran included a few cells because of the difficulty in removing the bound enzymes from the cells. We believe that the current results provide a framework for further studies on the rheological properties of cell-free insoluble dextran, which are critical for bacterial profile modification, biofilm immobilization in a biofiltration column and food process applications. Although, the basic rheological properties of pure insoluble dextran should be measured without the interference of cells, it is difficult to obtain a cell-free insoluble dextran because of enzyme-cell binding. The method introduced in this paper provides an alternative way to produce cell-free insoluble dextran using cell-bound enzyme.

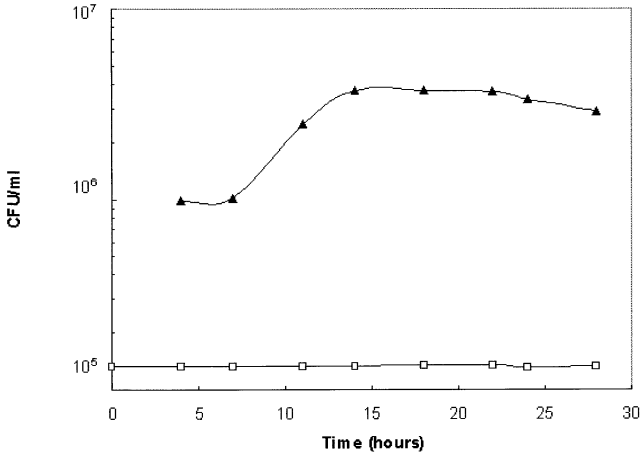

Fig. 2. Comparison of cell number of *L. mesenteroides*. CFU/mL in the phosphate buffer medium after inoculation. CFU/mL of *L. mesenteroides* during insoluble dextran synthesis in acetate buffer medium. ▲, phosphate buffer; □, acetate buffer.

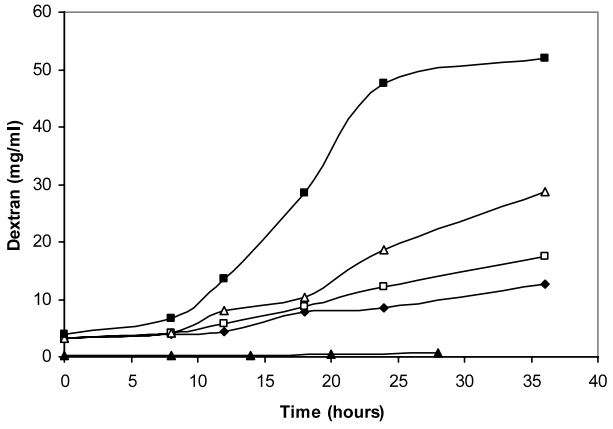

Fig. 3. Comparison of insoluble dextran synthesis using cell-bound dextransucrase with soluble dextran of *L. mesenteroides* NRRL B-523 for various sucrose concentrations. ▲, soluble dextran; ◆, 2% sucrose; □, 5% sucrose; △, 10% sucrose; ■, 15% sucrose.

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