

Continuous Culture System for Production of Biopolymer Levan Using *Erwinia herbicola*

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The optimal production of the fructan biopolymer levan by the bacterium *Erwinia herbicola* was investigated, including variations in nitrogen, carbon and phosphorous sources, pH, incubation time, culture methodology, and polymer processing. Levan yields up to 19% by weight were produced based on conversion of sucrose as the carbon source when grown in a continuous culture system and processed by tangential flow filtration. Product identity was confirmed with gas chromatography (GC) and ^{13}C nuclear magnetic resonance (NMR). Gel permeation chromatography (GPC) and low-angle laser light scattering (LALLS) determination of the molecular weight of the product showed a significant difference in molecular weight values dependent on the method of analysis. Analysis by GPC resulted in a molecular weight one order of magnitude lower than LALLS independent of sample, underscoring the unusual nature of this biopolymer.

Key words: levan · continuous culture · molecular weight · *Erwinia herbicola*

INTRODUCTION

Levan, a beta 2,6 fructan biopolymer with occasional beta 2,1 branching is produced by plants¹³ and microorganisms.¹ It is one of the two main forms of polyfructose which occur in nature, the other being inulin, which consists primarily of 2,1 linkages and is generally much lower in molecular weight than levan (100 residues average for inulin vs. up to 3 million residues for levan⁶).

Extracellular polysaccharides, such as dextran, xanthan, and pullulan, have a variety of uses as industrial gums while comparatively little is known about the production and properties of levan. Recently, Han and Clarke used *Bacillus polymyxa* in batch culture to produce levan and characterized the resulting polymer by infrared (IR), gas-liquid chromatography (GLC), ^{13}C nuclear magnetic resonance (NMR), and methylation/mass spectroscopy.^{7,8}

Levan is produced by several other microorganisms however, including *Bacillus subtilis*, *Streptococcus salivarius*, *Pseudomonas prunicola*, *Actinomyces* sp., and *Erwinia herbicola*.¹ In this study, *E. herbicola*, (previously *Aerobacter levanicum*⁵) was used in a continuous culture system with tangential flow purification,

and the resulting polymer characterized with GC, gel permeation chromatography (GPC), ^{13}C NMR, and low-angle laser light scattering (LALLS). In addition, it was compared with levan produced by *Bacillus polymyxa*.

MATERIALS AND METHODS

Erwinia herbicola, ATCC 15552, maintained on nutrient agar slants was used for this study. Preliminary studies were conducted with 50-mL batch cultures grown on a minimal medium⁹ and varying the carbon, nitrogen, and phosphorous sources and the pH to arrive at the optimal combination of medium components for high yield of polymer. The medium used in the continuous culture resulted from these initial batch studies and consists of the following: K_2HPO_4 , 3.0 g/L; NaCl, 2.0 g/L; urea, 0.34 g/L; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.4 g/L; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01 g/L; sucrose, 5% w/v; pH 7.2. Magnesium and Fe solutions were prepared as 100 × stocks, filter sterilized, and added aseptically after autoclaving. The sucrose solution was also prepared separately and added aseptically after autoclaving.

To inoculate cultures, a sterile inoculating loop was used to scrape growth from the nutrient agar slants on which the strain was maintained and the cells were transferred to a 250-mL DeLong flask containing 50 mL of medium. This culture was shaken at 125 rpm at 25°C for 24 h, and then a new flask was inoculated with a 2% inoculum from the growing culture and shaken again under the same conditions for 24 h. This second flask was used as the inoculum for the continuous culture.

For continuous culture, a BioFlo model C30 fermentor (New Brunswick Instrument Co., Edison, NJ) with a 1.8-L culture vessel was set with an agitation rate of 200 rpm, a temperature of 25°C, and an aeration rate of 0.5 L/min. The culture was incubated 48 h before starting the flow. Flow rates (20–40 mL/h) were controlled with a Rabbit peristaltic pump (Rainin Instrument Co., Woburn, MA) for 8 days. Cells were removed by centrifugation of the medium collected during 24–48-h periods at 23,430g for 30 min. The supernatant volume was doubled with Milli-Q water and filtered with a Millipore tangential flow filtration system, Pellicon Model

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Cassette OM-141 (Millipore, Bedford, MA). The solution was first passed through a cassette with a 30,000-MW cutoff to selectively retain the high molecular weight polymer (>30,000). The concentrated retentate was then precipitated with a 1:2 volume of acetone with stirring, and the water/acetone solution was decanted off. The precipitate was washed several times with acetone and dried over CaSO₄ in a desiccator. Microscopic examination of an aqueous solution of the resulting polymer determined it to be cell free.

Gas chromatographic analysis was performed on a Hewlett-Packard gas chromatograph, model 5880A, equipped with a Durabond DB-1 fused silica column (30 m × 0.26 mm) (J.&W. Scientific, Rancho Cordova, CA) with helium as the carrier gas and a flame ionization detector. A sample (10 mg) of the polymer was hydrolyzed by boiling in 0.1M hydrochloric acid for 2 h. The solution was neutralized [Amberlite IR 45(OH) resin, Rohm and Haas, Philadelphia, PA], lyophilized, and warmed with Tri-Sil Z (1 mL) (Pierce Chemical Co., Rockford, IL).

Gel permeation chromatography was performed on a Waters 150-C ALC/GPC gel permeation chromatograph calibrated using a series of pullulan standards ranging in molecular weight between 12,200 and 853,000 daltons (Polymer Laboratories, Church Stretton, United Kingdom). The standards were run through three Bio-Gel TSK columns (BioRad, Richmond, CA) in series: one TSK-60 and two TSK-50s. A third-order calibration curve was generated correlating molecular weight distribution with retention time on the columns. Standards and samples were solubilized at 0.1% in the carrier solvent, an aqueous solvent of 0.1M sodium acetate, acetic acid 2% (v/v), and sodium azide 0.05% (w/v). Flow rate was 1 mL/min, injection volume 200–300 μL, and run time 40 min.

¹³C NMR was performed on a Varian XL 00 Superconducting FT NMR spectrometer with a 47-kG Nb-Ti magnet. Spectra were measured for 6–10% w/v solutions in D₂O (10-mm sample tubes) at 50.3 MHz. External trimethylsilane was the reference (δ = 0) for assigning chemical shift values.

LALLS and the Zimm plot analyses were done according to the method of Rollings^{4,15} with a multiple-angle laser light scattering photometer, Dawn model F (Wyatt Technology, Santa Barbara, CA). The solvent was 2% acetic acid and 0.1M sodium acetate, pH 4.0.

RESULTS AND DISCUSSION

Initial batch studies with variable carbon, phosphorous, or nitrogen sources at pH 7.2 (Tables I–III) and batch studies at variable pH (Fig. 1) resulted in the development of a suitable levan medium for continuous culture studies. The carbon studies, as expected, showed the highest yields of polymer with fructose containing disaccharides. Because the energy from the hydrolysis of the disaccharide glycosidic bond is required for poly-

Table I. Effect of carbon source on polymer yields from *E. herbicola*.

Carbon source	Percent yield
Sucrose	7.1
Dextrose	0.3
Galactose	1.3
Fructose	0.2
Maltose	0.8
Mannose	0.8
Raffinose	4.9
Mannitol	1.4

Note: Yields are based on conversion of carbon source to polymer during 3 days incubation at 25°C with agitation at 125 rpm, pH 7.2.

Table II. Effect of phosphorous salt on polymer yields from *E. herbicola*.

Phosphorous source	Percent yield
0.2% KH ₂ PO ₄	25.7
0.3% K ₂ HPO ₄	24.0
0.4% Na ₂ HPO ₄ /NaH ₂ PO ₄	14.3
0.4% Na ₂ HPO ₄	20.3
0.4% NaH ₂ PO ₄	11.8

Note: Yields are based on conversion of sucrose to levan during 3 days incubation at 25°C with agitation at 125 rpm, pH 7.2.

Table III. Effect on nitrogen source on polymer yields from *E. herbicola*.

Nitrogen source	% Yield	Nitrogen source	% Yield
NaNO ₃	0.9	0.10% yeast extract, 0.10% peptone, 0.22% NH ₄ Cl	1.2
0.20% NaNO ₃ , 0.20% peptone	1.0	0.30% yeast extract, 0.22% NH ₄ Cl	1.2
0.10% NaNO ₃ , 0.10% peptone	0.1	0.20% yeast extract, 0.22% NH ₄ Cl	1.3
0.02% yeast extract, 0.20% peptone	0.9	0.22% NH ₄ Cl, 0.20% peptone	1.5
0.20% yeast extract, 0.20% peptone	2.1	0.22% NH ₄ Cl, 0.10% peptone	0.6
0.20% yeast extract, 0.10% peptone	3.6	0.01% (NH ₄) ₂ SO ₄ , 0.03% urea	1.8
0.10% yeast extract, 0.10% peptone	0.7	0.03% urea	3.0

Note: Yields are based on conversion of sucrose to levan during 3 days incubation at 25°C with agitation at 125 rpm, pH 7.2.

merization,¹² fructose alone yielded little polymer. Polymer yields from disaccharides not containing fructose were probably not levan, but other acetone-precipitation microbial polysaccharides, and were not analyzed further. Polymer yields from a number of different phosphate and nitrogen sources were similar. Therefore, the salts resulting in the cleanest (whitest) polymer were chosen. There was little change in polymer yield near neutrality, while at pH extremes of 5 and 8 yields dropped off to zero. A midpoint of 7.2 was chosen for the continuous culture system.

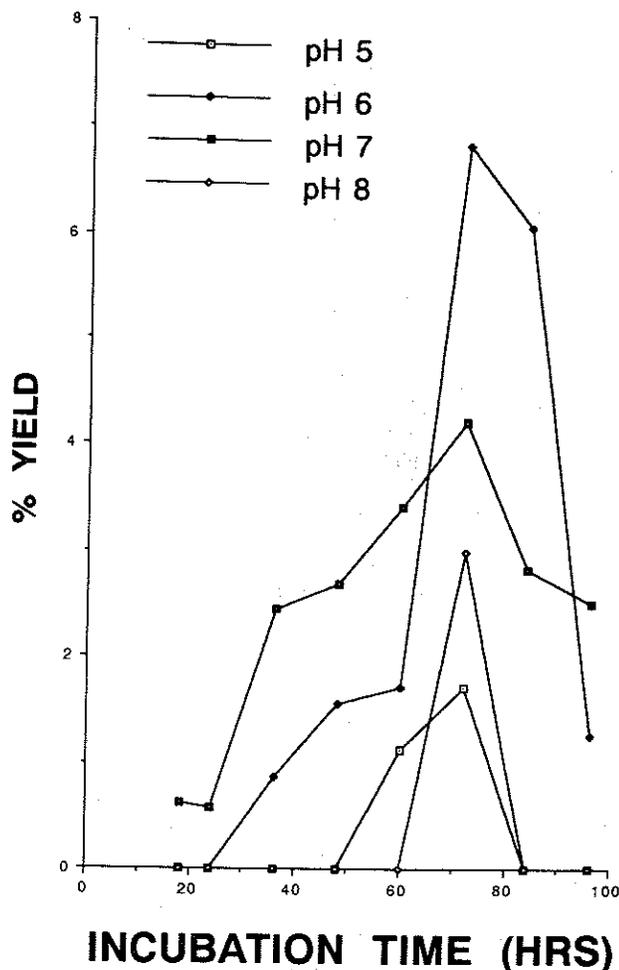


Figure 1. Effect of pH on polymer yields from *E. herbicola* over time. Yields based on conversion of sucrose to levan at 25°C with agitation at 125 rpm.

Yields of levan during continuous culture remained high throughout an 8-day incubation (above 19% based on sucrose or 38% based on fructose), and the apparent molecular weights remained fairly constant (Fig. 2). A 1:1 dilution with tangential flow filtration was used to obtain a clean, cell-free polymer. GC analysis showed four peaks, all corresponding with those obtained from silylation of equilibrated fructose, and ^{13}C NMR indicated a composition mostly of beta 2,6 linkages.

GPC analysis of the levan indicated a molecular weight of approximately 1.1×10^6 – 1.6×10^6 , although since these values are off the standard curve they are only approximate. Levan from *B. polymyxa*⁸ gave a molecular weight of 1.6×10^6 when evaluated on the same GPC system and has been reported at 2×10^6 .^{7,8} Since these measurements are made at or near the void volume of the column and no column resolving higher molecular weights is commercially available, a second method to determine molecular weight was used. Both levans were analyzed for molecular weight by LALLS, which involves no arbitrary assumptions about molecular shape, distribution, or homogeneity, an important consideration in determining the molecular weight of

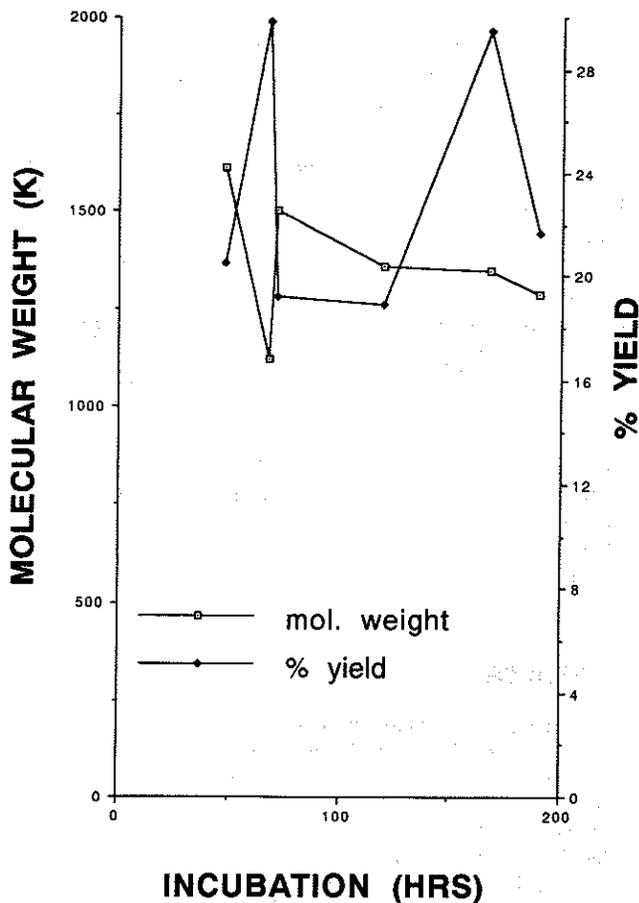


Figure 2. Production of levan from *E. herbicola* under continuous culture conditions. Molecular weights were determined by GPC and yields are based on conversion of sucrose to levan.

polysaccharides.^{4,15} This procedure, which is probably more accurate, gave a molecular weight for the *Erwinia* levan of 2.97×10^7 with a radius mean squared of 43.8 nm and a second virial coefficient of 0. For the *B. polymyxa* levan, the LALLS analysis showed a molecular weight of 2.07×10^7 with a radius mean squared of 40.3 nm and a second virial coefficient of 0.

The discrepancy between the GPC and LALLS in the above numbers points to the importance of determining molecular weight by several methods when working with polysaccharides. It is clear from the analysis that the levan from the two different organisms are quite similar while the molecular weights determined by light scattering bring the molecular weights determined by GPC into question. The molecular weight separation limit of the TSK-60 column used is the highest commercially available, limiting the use of aqueous GPC for molecular weight determination of this polymer. A survey of the literature shows molecular weights of levan varied with the method of analysis. A crude method, methanol fractionation, gives molecular weights for bacterial levan under 200,000 daltons.¹¹ GPC analysis gives values ranging from 1×10^6 to 20×10^6 daltons for levan from *S. salivarius*¹⁰ and *E.*

herbicola.³ Viscosity, light scattering, and sedimentation methods give values for *S. salivarius* levan from 18×10^6 to 100×10^6 daltons.^{2,14,16}

Bacillus polymyxa yields were reported at 50% based on available fructose,⁶ which translates to 25% based on available sucrose. The *E. herbicola* yields in this study in batch culture varied with media, with a high of 27.1% based on sucrose. However, the high yield of the continuous culture system (18.9–29.9%) coupled with the simplicity of tangential flow purification suggest that the *Erwinia* system described in this study may be useful for large-scale production of this beta fructan.

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