Production and Characterization of Microbial Levan

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A levan-producing bacterium was isolated from soil. Cultural and physiological characteristics of the isolate identified the organism as a strain of *Bacillus polymyxa*. The organism's characteristics for polysaccharide synthesis were studied. The bacterium produced polysaccharide in high yield when grown on sucrose solution. Hydrolysis and subsequent analysis showed the product to consist entirely of D-fructose. ^{13}C NMR and methylation analyses indicated the product to be a $\beta(2\rightarrow6)$-linked polymer of fructose, with 12% branching. The polysaccharide has a molecular weight of about 2 million and is readily soluble in water.

Fructans are polymers of fructose that occur in nature in two general forms, distinguished by the type of linkage between the fructose molecules (Figure 1). Inulin, the form found in many plants, is formed by $\beta(2\rightarrow1)$-linked fructose molecules. Levan, found primarily in microbial products, although recently also found in various grasses and woody-stemmed plants (Nilsson, 1988), is a $\beta(2\rightarrow6)$-linked fructose polymers, with some branching through the O1 site. While inulins often have about 100 residues, microbial levans are much larger, containing as many as 3 million residues (French, 1989).

Microbial levans are produced from sucrose-based substrates by a variety of microorganisms: *Bacillus subtilis* (Dedonder, 1966; Tanaka et al., 1979), *Bacillus polymyxa* (Hestrin et al., 1943), *Aerobacter levanicum* (Evans and Hibbert, 1946), *Streptococcus sp.* (Corrigan and Robyt, 1979; Shimamura et al., 1987), *Pseudomonas sp.* (Fuchs, 1956), and *Corynebacterium laevaniformans* (Dias and Bhat, 1962).

Early reports on levans were obscured by incomplete description of impure products, and yields were too low for industrial application (Pontis and Del Campillo, 1985). Levans and dextrans are known in the sugar industry as microbial products that cause sucrose loss and filtration problems. Although extensive studies have been made on dextrans, relatively little is known about levans with regard to their production, properties, and industrial applications. This paper will present the characteristics of levan production by a strain of *B. polymyxa* and describe the composition and properties of the levan produced by the organism.

MATERIALS AND METHODS

Production of Levan. A soil isolate, identified as strain *B. polymyxa* (NRRL B-18475), was used for production of levan. The organism was grown on a defined medium, which consisted of sucrose (80 g), peptone (2 g), yeast extract (2 g), K_2HPO_4 (2 g), $(NH_4)_2SO_4$ (2 g), and $MgSO_4$ (0.3 g) in 1 L of water. Sugarcane juice with no added nutrients was also used as a growth medium. After several days of growth on a rotary shaker at 30 °C, the culture medium was centrifuged to remove the bacterial cells and the levan was precipitated with 1.5 volumes of ethanol or isopropanol. The precipitate was collected on freeze-dried or vacuum-dried. The amount of levan was determined by HPLC (Waters Associates, Milford, MA) with a refractive index detector and Aminex HPX-87C column (Bio-Rad Corp., Richmond, CA) at 85 °C with deionized water as a mobile phase. Qualitative determination of levan was also made by weighing the air-dried precipitate. Optical rotation was determined before and after hydrolysis with 0.5% oxalic acid.

Characterization of Levan. ^{13}C NMR spectroscopy was performed at 53.0 MHz with a Varian VXR-200 spectrometer. The decoupled spectra were obtained with a 45° pulse width and 1-s recycle time. Samples of levan and inulin were dissolved in D_2O solution, heated for 15 min at 60 °C, and poured into 5-mm tubes. After the spectra were run, about 100 transients were acquired in the presence of a sealed external capillary of neat tetramethylsilane to allow referencing of the chemical shifts. Methylation analysis was run by the method of Hakomori (1964) followed by hydrolysis with trifluoroacetic acid, sodium borohydride reduction, and acetylation, in which partially methylated monomers were converted to alditol acetate. Gas–liquid chromatography was performed on a Hewlett-Packard 5970, used as an inlet for the mass spectrometer. Molecular weight was determined on a Sephacryl S-500 column (2.6 × 70 cm), with deionized water as solvent, upward flow of 2.75 mL/min, and detection by a refractive index monitor, Model R-401 (Waters Associates). Optical rotation was measured on a polarimeter (Type AA-10, Optical Activity Ltd., Cambridgeshire, England) with a sodium lamp and a 100-mm sample tube. The sample of inulin used for NMR and infrared analyses was provided by A. French, Southern Regional Research Center, USDA, New Orleans.

RESULTS AND DISCUSSION

Isolation and Identification of Levan-Producing Organisms. A levan-producing bacterium was isolated from soil and identified as a strain of *B. polymyxa*. The organism has been registered at the Northern Regional Research Center, USDA, Peoria, IL, and identified as NRRL B-18475. The isolated bacterial cells were rods with 2–10-μm length and 0.5–1.0-μm width; they occurred singularly are were motile with peritrichous flagella. Cells stained Gram-variable. Colonies became gummy and adhered to the agar surface, especially on sucrose media. Sporulation was rare, but some subterminal spores were observed after 30 days of cultivation on nutrient agar. Cells had swollen sporangia. Cells grew at temperatures ranging from 25 to 37 °C, and gas and acid were formed on glucose, a characteristic of *B. polymyxa*. Other physiological and nutritional characteristics of the isolate were similar to that of *B. polymyxa*, described in *Bergey's Manual of Systematic Bacteriology* (1986). Details of isolation and characterization of the organism was reported elsewhere (Han, 1989).

Production of Levan. The *B. polymyxa* (NRRL B-18475) produced a large quantity of extracellular polysaccharides when grown on 4–16% sucrose solution. Fig-

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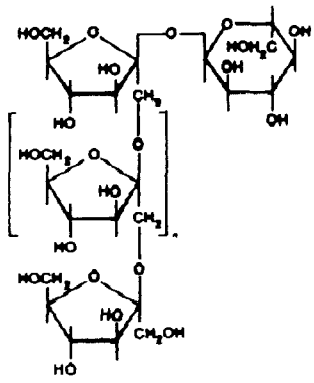
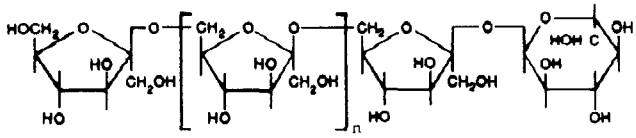


a. Inulin (F2-1F2-1F2-1F2-1G)



b. Levan (F2-6F2-6F2-6F2-1G)

Figure 1. Chemical structure of inulin (a) and levan (b).

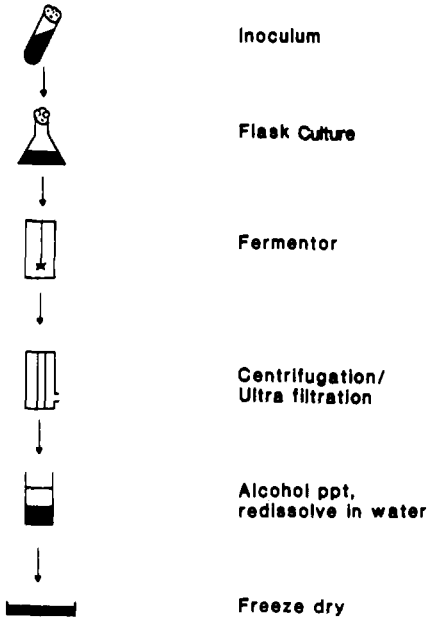


Figure 2. Scheme for production of levan by *B. polymyxa*.

ure 2 presents a levan production scheme. The levan production was noted after a few days of cell growth, and the level reached a maximum after cell growth reached the stationary phase (Figure 3). Usually a least a 10-day cultivation time was needed for a maximum yield. The pH of the growth medium fell from 7.0 to 4.7 due to acid production. Optimum temperature for growth and levan production was around 30 °C. In a typical fermentation, the isolate produced about 3.6 g of levan in 100 mL of 15% sucrose medium in 10 days (about 50% yield on available fructose). The polysaccharide production was especially pronounced when the culture was gently shaken during the cultivation period. Vigorous agitation and aeration inhibited levan production. A small amount of microbial polysaccharide (alcohol precipitate) was also produced when the organism was grown on lactose, maltose, and raffinose, but not on glucose or fructose. The organism produced polysaccharide from sugarcane juice, but the yield was much less than that obtained from the basal medium containing the same concentration of sucrose.

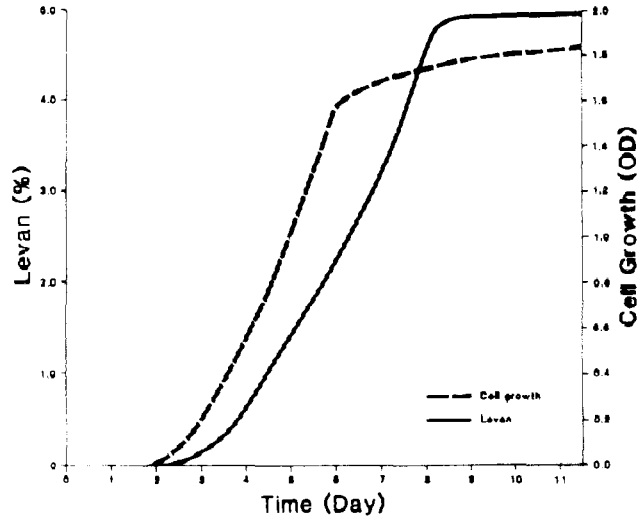


Figure 3. Time course of cell growth and levan production by *B. polymyxa*.

Levan was harvested by precipitation from the culture broth by addition of ethanol or 2-propanol. The yield and consistency of the product varied depending on the amount of alcohol added. The levan started to precipitate at the medium to alcohol ratio of 1:1.2, and the yield peaked at the ratio of about 1:1.5. Further increase in the ratio resulted in hardening of the levan, making the product less fluid. Slightly less 2-propanol was needed than ethanol to precipitate levan. Although most of the bacterial cells, unfermented sugars, and other solubles remained in the aqueous alcohol phase, preremoval of microbial cells by centrifugation was desired to obtain a pure form of levan. The final product was a brownish white, gummy material that could be freeze-dried or vacuum-dried. This was further purified by dialysis or ultrafiltration of a second precipitation with 75% ethanol.

Composition and Properties. The levan produced by the isolate *B. polymyxa* consisted of about 98% fructose as revealed by HPLC of the acid hydrolysate. The product was readily soluble in water and insoluble in 75% alcohol at room temperature. In contrast to low solubility of inulin ($\beta(2\rightarrow1)$ linkage), the high solubility of the product may be a characteristic of $\beta(2\rightarrow6)$-linked levans. The product was very susceptible to hydrolysis in boiling 0.5% oxalic acid. Since the initial molecule in levan formation is sucrose, terminal glucose groups are expected to be present in levan chains. However, because of the small portion of terminal groups in their high molecular weight levan, essentially no glucose was observed on hydrolysis or on methylation analysis.

A 5% aqueous solution of crude levan, after dialysis through a membrane with 12 000-Da cutoff, gave a single, sharp clean peak just below 2×10^6 Da on Sephacryl S-500. It should be noted that this peak is sharper (narrower molecular weight range) than those of the commercially available dextrans used as GPC standards. The uniformity of the product is perhaps due to the result of a long fermentation period (up to 15 days). The compound is stable in aqueous solution at pH 4.5 for up to 36 h when monitored by HPLC analysis. The levan has an optical rotation $[\alpha]^{24}_0$ of −42.0. It is nonhygroscopic, which is unusual in view of its high solubility. Lyo-

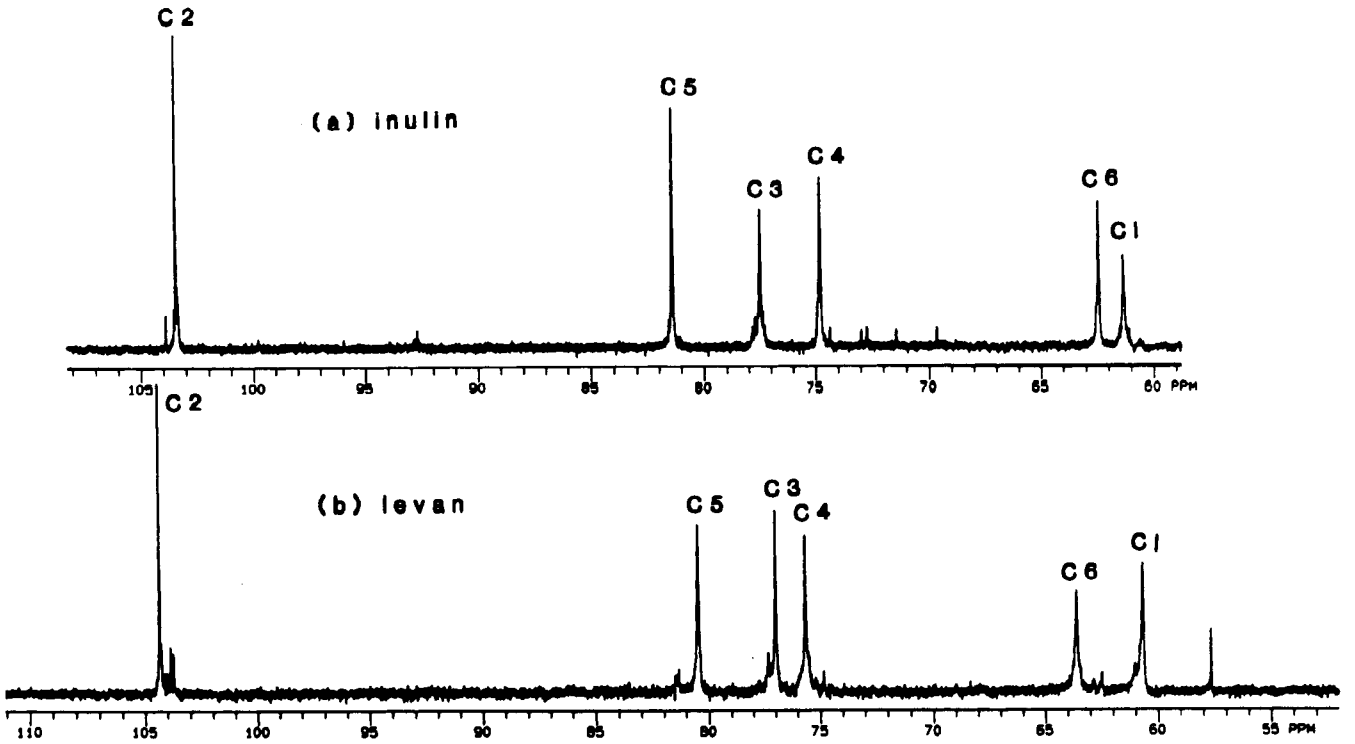


Figure 4. ^{13}C NMR spectra of inulin (a) and levan (b).

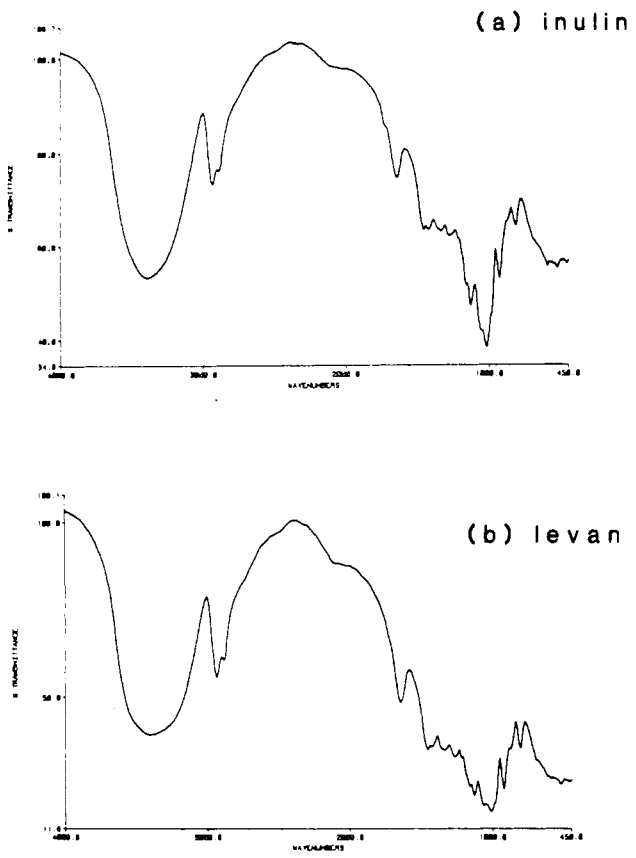


Figure 5. Infrared spectra of inulin (a) and levan (b).

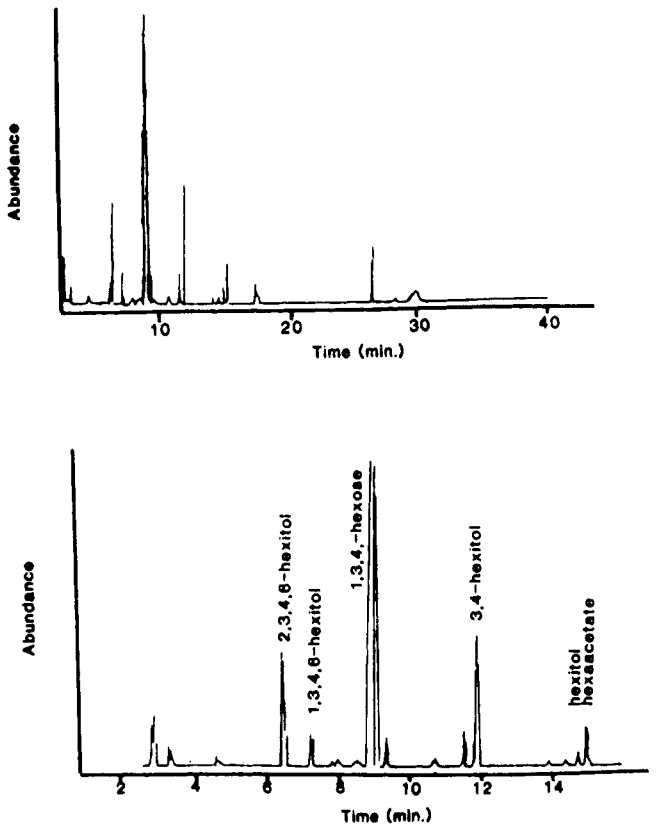


Figure 6. GLC of methylated bacterial levan (a, top) and identification of alditol acetate peaks (b, bottom).

philized sheets of levan have been maintained under atmospheric condition for up to 6 months.

In Figure 4, NMR peaks from levan are compared to peaks from inulin. The ^{13}C NMR spectra showed six main resonances at 104.2, 80.5, 77.0, 75.7, 63.6, and 60.7 ppm, which is almost identical with the peak positions for levan previously identified by Shimamura et al. (1987). The peak positions vary slightly from literature values, but the relative spacings are similar between the values obtained from experimental levan and that from literature. The T^4 anomeric peak (C2) is at about 104 ppm for both. The primary carbons (C1 and C6) are more closely grouped in inulin, and the ring carbons (C3, C4, and C5) are more closely grouped in levan, characteristic of the published differences between inulin and levan (French, 1989). Data clearly show the polysaccharide pro-

Table I. Linkage Types As Indicated by Methylation Analysis

linkage type	%
$\beta(2\rightarrow6)$-linked fructose	71
branch points (at 1, 2, and 6)	12
terminal groups (1- or 2-posn)	13
free hexose	4

duced by the isolate to be levan type with the linkage of $\beta(2\rightarrow6)$fructofuranoside. The infrared spectra of inulin and bacterial levan showed similar characteristics between them (Figure 5).

After methylation, analysis on GLC provided the chromatogram shown in Figure 6a with the area of methylated monomer peaks expanded and shown in Figure 6b. Branch points are indicated by the presence of 3,4-dimethyl-substituted fructose, and the degree of branching of 12% is supported by the observation of 13% terminal groups, indicated by monomethylated fructose residues, substituted at the 1- or 2-positions (Table I). The branches are formed by $\beta(1\rightarrow2)$ linkage with side chains of $\beta(2\rightarrow6)$-linked residues. The degree of branching in levans has been shown to range from 5 to 20% (Lindberg et al., 1973). The free hexose probably resulted from hydrolysis during methylation, or from incomplete methylation, or from contamination by another polysaccharide.

CONCLUSION

A soil isolate, identified as a strain of *B. polymyxa*, produced a large quantity of extracellular polysaccharide in a sucrose medium. The polysaccharide was identified as a levan, which consisted entirely of fructose, with residues linked through $\beta(2\rightarrow6)$-fructofuranoside linkages. Once the properties are fully elucidated, this levan may be useful in food and industrial applications.

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Registry No. Levan, 9013-95-0.

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