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# Levan production by *Bacillus polymyxa*

Youn W. Han

*USDA, Southern Regional Research Center, New Orleans, LA, U.S.A.*

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## SUMMARY

A levan-producing bacterium was isolated from soils and its characteristics for polysaccharide synthesis were studied. A series of enrichment and plating techniques enabled the isolation of a levan-producing bacterium from closely related contaminants. Cultural and physiological characteristics of the isolate identified the organism as a strain of *Bacillus polymyxa*. The organism produced about 40 g extracellular polysaccharide per liter of sucrose medium, which was about three times more yield than levan obtained from known levan producers. The highest amount of polysaccharide was on a 8% sucrose medium. Hydrolysis of the product showed that the polysaccharide consisted entirely of D-fructose, and $^{13}$C.n.m.r. spectra confirmed that the product was levan, a fructose polymer linked by B-(2→6) fructofuranosyl linkage.

## INTRODUCTION

Levans are polyfructans linked by B-(2→6) linkages, and found in many plants and microbial products. Despite their widespread occurence, they are little known and poorly understood. Plant levans (phleins) have shorter residues (about 100 residues) than microbial levans that contain up to 3 million residues [8]. Microbial levans are produced from sucrose-based substrates by a variety of microorganisms: *Bacillus subtiles* [3,11], *Bacillus polymyxa* [7], *Aerobacter levanicum* [7], *Streptococcus* sp. [2], *Pseudomonas* sp. [6], *Corynebacterium laevaniformans* [4]. Early reports on levan were obscured by incomplete description of impure products, and yields were too low to consider for industrial applications. Hydrolysis of levan yielded D-fructose, and analysis of levan agreed with the empirical formula $(C_6H_{10}O_5)_n$. Levan is closely related to inulin, plant polyfructans linked by B-(2→1) linkage.

Extracellular polysaccharides produced by microorganisms offer a variety of useful and potentially low-cost industrial gums. A host of gums with widely different properties are available for food, cosmetic, and industrial applications. It is expected

Correspondence: Y.W. Han, USDA, Southern Regional Research Center, New Orleans, LA 70179, U.S.A.

that fermentation gums eventually will be developed to fulfill many industrial needs at low cost as microbial and fermentation technology advances. Dextrans and levans are produced from sucrose substrates by bacteria by a similar mechanism but, unlike dextran, relatively little is known concerning the production, properties, and industrial applications of levan. In this study, an organism capable of producing levan was isolated and its characteristics for extracellular polysaccharide synthesis were studied.

## MATERIALS AND METHODS

### *Media*

Mineral salt solution containing sucrose 150 g, peptone 2 g, yeast extract 2 g, $K_2HPO_4$ 2 g, $(NH_4)_2SO_4$ 2 g, $MgSO_4$ 0.3 g in 1 liter of water was used as a basal medium for production of levan. Trypticase soy agar, Czapek solution agar, malt extract, and nutrient broth, obtained from Difco Laboratories, Detroit, MI., were used for growth and maintenance of the culture.

### *Organisms*

To compare the levan producing ability the following bacteria were obtained from USDA, Northern Regional Research Center, Peoria, Illinois and American Type Culture Collection, Rockville, MD. *Bacillus polymyxa* (NRRL-B68, B130, B510, B4317), *Bacillus subtilis* (NRRL-B477, B577, B644, B675, B744a, B2612), *Aerobacter levanicum* (NRRL-B1678), *Acetobacter pasteurianus* (ATCC 11142), *Microbacterium laevaniformans* (ATCC 15953).

### *Isolation of the organism*

About 1 g of rotting sugar cane stalks and the adhering soil particles were added to 100 ml of basal medium and incubated at 30°C with constant shaking. Subsequently, one ml of growth culture was transferred to fresh media every 7–10 days. After several successive transfers, the culture was plated on the same medium solidified with agar. Those organisms which utilize sucrose as a sole source of carbon and thrive in high osmotic pressure (15% sucrose solution) would be enriched by the consecutive transfers into new media. Bacterial colonies were separated from fungal and yeast colonies and transferred into a fresh medium and incubated until full growth. A portion of the growth medium was withdrawn and centrifuged to remove the cells and other insolubles. One and half volume of ethanol or isopropanol was added to the supernatant and the resulting precipitate collected by gently syphoning off the supernatant. The precipitates were then hydrolyzed in boiling oxalic acid (0.5%) for 30 min and the polarization (optical rotation) of the solution determined. The samples showing negative polarization were tentatively selected as positive for levan production. Repeated enrichment, plating and product identification were continued until a pure culture of a levan producer was obtained.

### *Production of levan*

The levan producing organisms were cultivated on a defined media as described above. The inoculum was successively transfered to larger volumes and the final culture was in 1500 ml media in fernbach flasks. The cultures were incubated at 30°C on a rotary shaker (170 rpm) for 10 days. After the growth, the culture was centrifuged to remove bacterial cells, dialyzed to remove unfermented sugars and fermentation products with small molecular weight, and finally the levan was precipitated with 1.5 volume of ethanol or isopropanol. The precipitate was collected and freeze dried or vacuum dried.

### *Determination of levan*

One ml of culture broth was passed through a column (5.0 ml) of cellulose gel filtration media (Amicon Matrex Cellufine, GH-25 medium, Amicon Corp. Davers, MA) and the first 2 ml of filtrate was collected and hydrolyzed by boiling in 0.5% oxalic acid for 30 min. The degree of optical rotation was determined by a polarimeter (type AA-10, Optoelectronic Design Engineers, Ltd., U.K.) with a sodium lamp and a 100 mm sample tube. The amount of fructose in the levan hydrolysate was determined by comparing the degree of optical rota-

tion produced by the sample and the standard fructose solution. The specific rotation of levan $[\alpha]^{24}$ was $-42.0$. All the measurements were run in triplicate. Alternatively, levan was also determined by the weight of the precipitate formed by addition of 1.5 vol of ethanol or isopropanol to the culture filtrate. The precipitate was collected by syphoning off the supernatant; excess alcohol was removed by blotting and the air-dried sample was weighed. Quantitative determinations of levan were conducted by HPLC (Waters sugar analyzer, Waters Asso. Inc., Milford, MA) with a refractive index detector and Aminex HPX-87C column (Bio-Rad Corp, Richmond, CA). Deionized water was used as a mobile phase. The levan produced by the isolated culture was used as a standard, after it was purified by centrifugation, dialysis and lyophilization. The amount of levan measured by precipitation by alcohol and that by HPLC did not coincide, because of other coprecipitants in the former.

*$^{13}C$-n.m.r. spectroscopy:*

$^{13}$C-n.m.r. spectra were run 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 $D_20$ 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 TMS to allow referencing of the chemical shifts. The sample of inulin was provided by A. French of USDA, Southern Regional Research Center, New Orleans.

## RESULTS AND DISCUSSION

*Identification of isolate*

The levan-producing bacterial isolate was grown aerobically at 30°C on tryptic soy broth and maintained on the same medium solidified with agar. The bacterial cells were straight rods with 2–10 $\mu$m length and 0.5–1.0 $\mu$m width; they occurred singularly and were motile with peritrichous flagella. Broth-grown cells stained Gram-negative and agar-grown cells were Gram-negative/variable. Colonies became gummy and adhered to the agar surfaces. Sporulation was extremely rare but some subterminal spores were observed on nutrient agar and on 0.25% soil extract agar after 30 days. Cells grew at temperatures ranging from 25 to 37°C. Cells had swollen sporangia and gas and acid were formed on glucose; the traits are characteristics of *Bacillus polymyxa* in contrast to *Bacillus subtilus* [10]. Other physiological and nutritional characteristics of the isolate were similar to that of *B. polymyxa* described in Bergey's Manual [1].

*Production of levan*

The organism grew well on tryptic soy broth, Czapek solution, malt extract, and a mineral solution. However, the organism grew poorly on nutrient agar. When the organism was grown on 4–16% sucrose, a large quantity of extracellular polysaccharides was produced and the medium became viscous. The pH of the growth medium fell from 7.0 to 5.5 due to acid production during a 10-day fermentation period. Optimum temperature for growth and levan production was 30–35°C. The isolate produced a varying amount of levan under various conditions. In a typical fermentation, the isolate produced about 3.6 g of levan in 100 ml of 15% sucrose medium in 10 days. During the same period, other bacteria known to produce levan yielded lesser amounts or did not produce at all (Table 1). The polysaccharide production was especially pronounced when the culture was gently shaken during the cultivation period. About three times more levan was produced on shake culture than still culture (Table 2). However, vigorous cultural agitation and aeration in a fermentor produced little or no levan. Illumination of light had little effect on levan production.

Levan production by the organism was dependent on the kind and concentration of sugar. The highest amount of levan (57.3 g/l) was produced on the medium containing about 8% sucrose, while the yield decreased at higher or lower sucrose concentrations (Table 3). A small amount of microbial polysaccharide (alcohol precipitate) was also produced when the organism was grown on lactose, maltose, and raffinose, but no polysaccharide was

Table 1

Comparative production of levan by different organisms

| Organism | Levan[a] (g/100ml) |
|---|---|
| *Acetobacter pasteurianus* | |
| ATCC 11142 | 0 |
| *B. polymyxa* | |
| NRRL B-68 | 0 |
| NRRL B-130 | 0 |
| NRRL B-510 | 1.2 |
| NRRL B-4317 | 1.4 |
| Isolate | 3.6 |
| *B. subtilis* | |
| NRRL B-447 | 1.0 |
| NRRL B-577 | 0 |
| NRRL B-644 | 0 |
| NRRL B-675 | 1.0 |
| NRRL B-744a | 1.5 |
| NRRL B-2612 | 0 |
| *Enterobacter levanicum* | |
| NRRL B-1678 | 0.7 |
| *Microbacterium laevaniformans* | |
| ATCC 15953 | 1.2 |

[a] Alcohol precipitate, air dried (about 50% moisture), average of triplicate samples.

produced on glucose and fructose. The organism produced polysaccharide from unfortified sugar cane juice, but the yield was much less than that obtained from the basal medium containing the same concentration of sucrose.

Table 2

Composition of the spent fermentation broth of *B. polymyxa* grown under various cultural conditions

| Cultivation | Levan[a] (%) | sucrose[a] (%) | glucose[a] (%) | fructose[a] (%) |
|---|---|---|---|---|
| Shake flask | 2.21 | 1.82 | 0.41 | 0.10 |
| Still flask | 0.99 | 2.12 | 0.33 | 0.07 |
| Shake flask, dark[b] | 2.12 | 1.89 | 0.42 | 0.09 |
| Fermentor[c] | 1.11 | – | – | – |
| Unfermented medium | 0 | 7.50 | 0.15 | 0.10 |

[a] Carbohydrate determined by HPLC after 10-day fermentation at 30°C.

[b] Shaken culture under dark environment.

[c] A 10-liter culture in a baffled fermentor, agitated (200 rpm) with a motor driven impeller and aerated (2 l/min) for 10 days.

Table 3

Effect of sugar on levan production by *B. polymyxa*

| Sugar (%) | Levan[a] (g/l) |
|---|---|
| Control (no sugar) | 0 |
| Sucrose, 2 | 7.1 |
| Sucrose, 4 | 29.1 |
| Sucrose, 6 | 50.0 |
| Sucrose, 8 | 57.3 |
| Sucrose, 10 | 40.2 |
| Sucrose, 12 | 36.0 |
| Sucrose, 14 | 38.0 |
| Sucrose, 16 | 32.0 |
| Glucose, 6 | 0 |
| Fructose, 6 | 0 |
| Lactose, 6 | 5.1 |
| Maltose, 6 | 5.5 |
| Raffinose, 6 | 8.1 |
| Sugar cane juice[b] | 11.9 |

[a] Alcohol precipitate, air dried (about 50% moisture), average of triplicate samples.

[b] Unfortified sugar cane juice containing about 15% sucrose.

Levan was harvested by precipitation from the culture broth by addition of ethanol or isopropanol. The yield and consistency of the product varied depending on the amount of alcohol added. The levan started to precipitate at the medium/alcohol ratio of 1:1 and the yield peaked at the ratio of about 1:1.5. Further increase in the ratio resulted in hardening of the levan which made the product less fluid. Slightly less isopropanol than ethanol was needed to precipitate an equal amount of 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 desirable to obtain a pure form of levan. The final product was a brownish-white, gummy material which could be freeze-dried or vacuum-dried.

*Product identification*

The acid hydrolysate of the polysaccharide produced by the isolate was exclusively fructose as revealed by HPLC. The microbial gum was laevorotatory, amorphous or microcrystalline, soluble in

cold water and very soluble in hot water, and insoluble in 75% alcohol. In contrast to low solubility of inulin (B-(2→1) linkage) the high solubility of the product may be characteristic of B-(2→6) linked levans. It was nonreducing and resistant to amylase action, but very susceptible to hydrolysis by acid (e.g. 0.5% oxalic acid). Most of the fructans are reported to contain a terminal glucose unit [5], but because of glucose being only a small proportion of the total polymer, it was difficult to confirm the presence of a glucose unit in the polymer. Gel permeation chromatography (on Sephacryl S-500) revealed that the molecular weight of the product was in the range of 1–3 million daltons.

Because D-fructofuranosides are heat-labile and partly decomposed during the process of derivatization to suitable forms for analysis by gas–liquid chromatography $^{13}$C-n.m.r. spectroscopy is reported to be a good method to determine the linkage type of a polyfructan [9]. The $^{13}$C-n.m.r. spectrum of the product showed six main resonances at 104.2, 80.5, 77.0, 75.7, 63.6 and 60.7 ppm. (Table 4). These chemical shifts were similar to those respectively assigned to C-2, C-5, C-3, C-4, C-6, and C-1 of levan rather than those for inulin. Therefore, the polysaccharide produced by the isolate is of the levan type with the linkage of B-(2→6) fructofuranoside.

Table 4

Chemical shifts for $^{13}$C-n.m.r spectra of inulin, levan, and the polysaccharide produced by *B. polymyxa*

| Carbon atom | Chemical shift (ppm) Inulin | levan[a] | Isolate[b] |
|---|---|---|---|
| C-1 | 60.9 | 59.9 | 60.7 |
| C-2 | 103.3 | 104.2 | 104.4 |
| C-3 | 77.0 | 76.3 | 77.0 |
| C-4 | 74.3 | 75.2 | 75.7 |
| C-5 | 81.1 | 80.3 | 80.5 |
| C-6 | 62.2 | 63.4 | 63.6 |

[a] Assignment cited from Shimamura et al, 1987.

[b] Polysaccharide of the isolate (*B. polymyxa*) dissolved in $D_2O$ at 60°C.

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 and the residues linked by B-(2→6) fructofuranoside linkage. Once the properties are fully ellucidated, levan may be useful in food and industrial applications.

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