Microbial Levan

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I. Introduction

Industry uses large quantities of natural polysaccharides, and new sources of polysaccharides are being sought. In recent years, attention has been directed toward producing extracellular polysaccharides by microbial fermentation. Examples are dextran and xanthan gum produced by *Leuconostoc mesenteroides* and *Xanthomonas campestris*, respectively. Recently, the sugar industry has faced intense competition from high-fructose corn syrup, which is used as a low-cost alternative sweetener. This chapter investigates the possibility of producing microbial levan from sucrose for industrial applications.

Levans are fructans, natural polymers of fructose. The two main types of fructans are the levans, with mostly $\beta(2 \to 6)$ linkages, and the inulins, with $\beta(2 \to 1)$ linkages. Branched fructans with both types of
linkages also exist. Levan is a common name for a fructan in which most fructose has $\beta(2 \rightarrow 6)$ linkages. A more descriptive name would be $\beta(2 \rightarrow 6)$-$\alpha$-fructan. Levans and inulins of low molecular weight ($M_\text{r} < 5000$) are abundant reserve carbohydrates in many plant tissues (Vandamme and Derycke, 1983). Many microorganisms, when grown on sucrose, produce extracellular levans of high molecular weight.

Microbial levan, like dextran, is often an undesirable by-product of sugar juice processing because it increases the viscosity of the processing liquor (Avidad, 1968; Fuchs, 1959). It was reported by Lippmann as early as 1881, and the name “levulan” was proposed for the compound. Greig-Smith (1901) showed that a strain of Bacillus, when grown on sucrose, produced fructans, and the name “levulan” was introduced as analogous to “dextran.” The term levulan now denotes partially degraded levan fractions. Early reports about levan are confusing because microbial nomenclature was unsystematic and materials were inadequately described. Levans have never had extensive industrial use.

The few existing review articles on levan either are obsolete or are part of reviews of other microbial polysaccharides (Avidad, 1968; Evans and Hlibert, 1946; Hohr, 1955; Pontis and Del Campillo, 1985). In this chapter, we present some of our recent work on microbial production of levan and review the earlier work of others.

### II. Occurrence

A variety of microorganisms produce extracellular polysaccharides as a capsule attached to the cell wall or as a slime secreted into the growth medium. These materials are formed as a defense mechanism or as a food reservoir. Some soil microorganisms produce levan, especially *Bacillus* sp. Oral bacteria such as *Rothia dentocariosa*, *Streptococcus salivarius*, and *Odontomyces viscosus* produce and accumulate levan in human dental plaque (Higuchi et al., 1970; Manly and Richardson, 1968; Newbun, 1969). Several species of yeast (Fuchs et al., 1985; Loewenburg and Reese, 1957) and fungi (Loewenburg and Reese, 1957) also produce levan. Table 1 lists some of the microorganisms that produce levan.

All species of bacilli belonging to group I in Smith et al. (1952) (B. subtilis, B. megaterium, B. cereus, and B. pumilus) produce levan from sucrose (Fuchs, 1959). Many strains of *B. polymyxa* that belong to group II also produce polysaccharides—mostly heteropolysaccharides, which consist of $\alpha$-glucose, $\alpha$-mannose, $\alpha$-galactose, $\alpha$-fructose, glucuronic acid, and pyruvate (McNeely and Kang, 1973; Glukhova et al., 1985; Mitsuda et al., 1981; Madden et al., 1986; Fukui et al., 1985; Ninomiya and Kizaki, 1970). The ratios of various sugars in the polysaccharides depend on the composition of the growth medium and the microbial strain used. Polysaccharide yield increases with sucrose but decreases with glucose and arabinose: mannose has the lowest polysaccharide

### Table 1

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>References</th>
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</thead>
<tbody>
<tr>
<td><em>Acetobacter pasteurianus</em></td>
<td>Loewenburg and Reese (1957)</td>
</tr>
<tr>
<td><em>Actionomyces viscosus</em></td>
<td>Palist (1972); Warner and Miller (1978)</td>
</tr>
<tr>
<td><em>Achromobacter sp.</em></td>
<td>Lindeg (1957)</td>
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<tr>
<td><em>Arenobacter aerogenes</em></td>
<td>Srinivasan and Quastel (1958)</td>
</tr>
<tr>
<td><em>Arachnoderma levicentum</em></td>
<td>Evans and Hibbert (1946); Feingold and Galatia (1957); Takeshita (1973)</td>
</tr>
<tr>
<td><em>Arthrobacter ureafaciens</em></td>
<td>Tanaka et al. (1985)</td>
</tr>
<tr>
<td><em>Azotobacter chroococcum</em></td>
<td>Hestrin and Goldblum (1953); Schubach and Berndt (1964)</td>
</tr>
<tr>
<td><em>Bacillus amylophilus</em></td>
<td>Mantsala and Puntila (1982)</td>
</tr>
<tr>
<td><em>Bacillus megaterium</em></td>
<td>Evans and Hibbert (1946)</td>
</tr>
<tr>
<td><em>Bacillus mesentericus</em></td>
<td>Hehre (1951)</td>
</tr>
<tr>
<td><em>Bacillus polymyxa</em></td>
<td>Hestren et al. (1943); Ninomiya and Kizaki (1970); Han (1989); Murphy (1952)</td>
</tr>
<tr>
<td><em>Bacillus subtilis</em></td>
<td>Dodonov (1960); Tanaka et al. (1981); Hestren et al. (1943); Mantsala and Puntila (1982); Perlot and Monson (1984); Yamamoto et al. (1985)</td>
</tr>
<tr>
<td><em>Corynebacterium levifavormans</em></td>
<td>Dias and Bhat (1962)</td>
</tr>
<tr>
<td><em>Corynebacterium beticol</em>*</td>
<td>Abdou (1960)</td>
</tr>
<tr>
<td><em>Glucosinobacter oxydans</em></td>
<td>Eliaashviili (1961)</td>
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<tr>
<td><em>Leuconostoc mesenteroides</em></td>
<td>Corrigan and Roby (1979)</td>
</tr>
<tr>
<td><em>Mycobacterium levaniformans</em></td>
<td>Fuchs et al. (1985)</td>
</tr>
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<td><em>Odontomyces viscosus</em></td>
<td>Krishkovsky et al. (1969)</td>
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<td><em>Pseudomonas pruni</em></td>
<td>Evans and Hibbert (1946)</td>
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<tr>
<td><em>Pseudomonas sp.</em></td>
<td>Fuchs (1956); Evans and Hibbert (1946); Gross and Rudolph (1987)</td>
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<tr>
<td><em>Rothia dentocariosa</em></td>
<td>Lesher (1976)</td>
</tr>
<tr>
<td><em>Streptococcus sp.</em></td>
<td>Corrigan and Roby (1979); Shimamura et al. (1987)</td>
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<tr>
<td><em>Streptococcus salivarius</em></td>
<td>Kleczkowski and Wierzchowski (1940); Fuchs (1956); Jung et al. (1987); Lipeiro et al. (1986); Davie et al. (1966); Park et al. (1983); Lynes and Doelle (1983); Long et al. (1975)</td>
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<td><em>Xanthomonas sp.</em></td>
<td>Dedonov and Peaud-Lenoel (1957); Fuchs (1959)</td>
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<td><em>Zymomonas mobilis</em></td>
<td>Ribeiro et al. (1988); Davie et al. (1966); Lynes and Doelle (1983); Viikari (1984)</td>
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<td><em>Yeasts</em></td>
<td>Fuchs et al. (1985); Loewenburg and Reese (1957)</td>
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<tr>
<td><em>Aspergillus sydowii</em></td>
<td>Loewenburg and Reese (1957)</td>
</tr>
<tr>
<td><em>Aspergillus versicolor</em></td>
<td>Loewenburg and Reese (1957)</td>
</tr>
</tbody>
</table>
yield (Glukhova et al., 1985). *Bacillus polymyxa* grown on sucrose produces homopolysaccharides, consisting of 90–100% fructose, depending on the analytical method used (Murphy, 1952; Han, 1989).

Levans are also found in plants, mainly in monocotyledons, whereas inulin is found in dicotyledons. Levans, a fructan, are closely related to inulins, but have different structure and properties. Plant levans, also called phleins, have a much lower molecular weight than bacterial levans (Pontis and Del Campillo, 1985). Levans are distributed throughout plants: leaves contain small amounts, but large quantities are found in roots, bulbs, tubers, rhizomes, and sometimes in mature fruits, where they serve as storage carbohydrates and may increase resistance to cold and drought (Meier and Reid, 1982; Shioda et al., 1976). The presence of levans (and fructans in general) in plants has no apparent correlation with the presence or absence of starch. No correlation has been found between the presence of levans (fructans) and the occurrence of C₃ and C₄ photosynthetic pathways (Bender and Smith, 1973). The water-soaked appearance of plants is often caused by levans formed by infected bacteria (Gross and Rudolph, 1988). Levans may help retain moisture and aggregate soil at the plant rhizosphere (Webley et al., 1965).

III. Biosynthesis

A. LEVANSUCCASE

The biosynthesis of levan requires the extracellular enzyme levansucrase (sucrose 6-fructosyltransferase, EC 2.4.1.10), which shows specificity for sucrose. Most research on the biosynthesis of levan has been conducted using the enzymes from *B. subtilis*, *Aerobacter levanicum*, and *S. salivarius*. These enzymes have been extensively purified and the mode of action explored (Bauer and Brisk, 1979; Elisashvili, 1984; Feingold et al., 1956; Fouchet et al., 1984; LeBrun and Van Rapanbusch, 1980; Matsuya and Punata, 1982; Pascal and Dedonder, 1972; Reese and Avidad, 1966). Levansucrase of *B. subtilis* is inducible and extracellular, whereas that of *A. levanicum* is constitutive and endocellular (Dedonder, 1966). It is uncertain whether levansucrase is one enzyme or a complex of multiple enzymes that synthesize the main chain β(2→6) and the branch β(2→1) linkages.

Chains of levan, like dextran and starch, grow in steps by repeated transfer of a hexosyl group from a donor to a growing acceptor molecule (Hestrin et al., 1956; Hehre, 1955; Sato et al., 1984).

The action of levansucrase is a step-by-step addition of single fructofuranosyl units at the C₆ hydroxyl of the nonreducing fructose terminal unit of a growing levan chain (Fig. 1). The exact nature of this initiation step of the polymer growth from sucrose is not clearly understood. The levansucrase may catalyze the reactions of a readily reversible primary step and a subsequent irreversible step as follows:

\[ \text{fru-R + enz} \rightleftharpoons \text{fru-enz + R} \]  \hspace{1cm} (1)

\[ \text{fru-enz + acceptor} \rightarrow \text{fru-acceptor + enz} \]  \hspace{1cm} (2)

where fru is fructose, enz is levansucrase, and R represents a carbonyl of aldose. Possibly, the aldose part of the substrate molecule is replaced by an enzyme-linked group, and partial decomposition of this levan precursor to aldose and ketose furnishes the energy necessary for levan synthesis. All levan-forming systems, either cell-free or whole-cell, produce fructose and a series of oligosaccharides as products of transfructosylation in addition to glucose and the polymer itself. The yield of

![Diagram of levan molecule synthesis](image_url)
levan, therefore, is −20−30% of sucrose utilized or 40−60% of fructose available (Avigad, 1968).

The enzyme utilizes sucrose as "donor substrate" with the Michaelis constant from 0.02 to 0.06 M. The optimal pH for levansucrase on sucrose is 5.0−6.0, and the activity at pH 4.4 and 7.5 was half the rate at pH 6.0 (Doddondor, 1966; Kiss, 1968). The enzyme activity is stable for several days at pH 5.0. The enzyme gradually loses its activity at 37°C and complete, almost immediate inactivation occurs at 100°C. The addition of metals (e.g., Fe²⁺, Al³⁺, Zn²⁺) increases the stability of the enzyme in relation to temperature. Levansucrase prepared from B. subtilis has a sedimentation coefficient of 2.7, a diffusion coefficient of 6.0 × 10⁻⁷, and a Mₚ of ~40,000 (Doddondor, 1966). An enzyme freeze-dried or kept at −20°C shows no loss of activity for long periods.

Because hydrolytic breakdown of sucrose and polymerization of fructose occur simultaneously, the exact position of equilibrium in levansucrose synthesis is difficult to define. The ratio of levansucrose to sucrose at equilibrium is generally thought to be >1, because a reversal of the reaction has not been detected. A yield of levansucrose as high as 62% of the theoretical maximum has been reported (Hesbr, 1955). The presence of levansucrose is not essential for levansucrase formation, but adding preformed primer (levansucrase) to a levansucrase-synthesizing system accelerates the rate of polymerization, increases final yield, and affects the production of homogeneous, high molecular weight levansucrase (Mattoon et al., 1955; Hestrin, 1956). However, these effects occur only under conditions of low ionic strength (Tanaka et al., 1980). The degree of polymerization of levansucrase is generally regulated by ionic strength, and the enzymatic synthesis of levansucrase could occur in the absence of an "adaptor" (preformed primer). Bacillus subtilis levansucrase synthesized levansucrase far more effectively at lower than ambient temperatures (Tanaka et al., 1979).

B. SPECIFICITY

The specificity of levansucrase depends not only on the D-fructosyl but also on the aldose residue of the substrate (Hestrin et al., 1956). Levansucrase of A. levanicus acts on sucrose, raffinose, and invert sugar (a mixture of glucose and fructose). Terminal fructose is generally believed to be necessary for the enzyme activity. However, some substrates with terminal fructose are not suitable for levansucrase production. The enzyme does not utilize any other common sugars or other substrates having a terminal fructose group (e.g., fructose phosphate, methylfructoside, and inulin). Levan yield from raffinose is about one-third of that from sucrose. The amounts of sucrose consumed are accounted for entirely as levansucrase and reducing sugars. When raffinose (gal-glu-fru) is used as a substrate, the products are levansucrase, melibiose (gal-glu), and fructose, but not sucrose and galactose. A series of glucose-ended, 2,6-linked polysaccharides can be used in synthesis of the levansucrase molecule (Hesbr, 1951). However, neither levansucrase, levansucrase, nor levantetraose is found to serve as an acceptor substrate. In addition, levansucrase from B. subtilis appeared to have some affinity for glucose but not for fructose.

C. INHIBITION AND ACTIVATION

The formation of levansucrase by levansucrase is inhibited by various sugars and sugar alcohols (Table II). Inhibition was caused by both sugars and glycosides, for example, by glucose, methyl-α-glycoside, and others that have a configuration about carbon atom 2 similar to that of D-glucose, such as D-galactose, maltose, and lactose (Hestrin and Avineri-Shapiro, 1944). However, no appreciable inhibition occurred for products like D-mannose, D-fructose, or D-mannitol, for which configuration or structure at carbon atom 2 differs from that of D-glucose. The inhibition of levansucrase activity by glucose is a function of glucose concentration, and complete inhibition of levansucrase production from 1% sucrose occurs at 16% glucose concentration. The inhibitory effect diminished as the glucose concentration fell relative to that of sucrose. Thus, it appears that glucose inhibition is caused

<table>
<thead>
<tr>
<th>Sugar</th>
<th>(% Inhibition)</th>
</tr>
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<tbody>
<tr>
<td>Lactose</td>
<td>76</td>
</tr>
<tr>
<td>D-Xylose</td>
<td>76</td>
</tr>
<tr>
<td>L-Arabinose</td>
<td>76</td>
</tr>
<tr>
<td>D-Glucose</td>
<td>62</td>
</tr>
<tr>
<td>L-Sorbose</td>
<td>62</td>
</tr>
<tr>
<td>Mehtyl-D-glucoside</td>
<td>49</td>
</tr>
<tr>
<td>D-Galactose</td>
<td>42</td>
</tr>
<tr>
<td>Maltose</td>
<td>37</td>
</tr>
<tr>
<td>L-Sorbitol</td>
<td>10</td>
</tr>
<tr>
<td>D-Fructose</td>
<td>4</td>
</tr>
<tr>
<td>D-Mannitol</td>
<td>3</td>
</tr>
<tr>
<td>D-Mannose</td>
<td>2</td>
</tr>
</tbody>
</table>

*Adapted from Hestrin and Avineri-Shapiro (1944).
by competition with the glucose moiety of sucrose for the enzyme (Tkachenko and Loityanskaya, 1976). Polyethylene glycol of M, 4000 has an activating effect, increasing the maximum velocity by a factor of three (Dedonder, 1966).

D. HYDROLYSIS

Many levan-forming microorganisms also produce hydrolytic enzymes, levanases, that degrade the levan (Hestirin and Goldblum, 1953; Avigad, 1965). Certain strains of *Pseudomonas*, *Azotobacter*, *Aerobacter*, *Serratia*, *Bacillus*, *Clostridium* produce exocellular levanase (Fuchs, 1959). Levanucrase itself is suspected to cause the hydrolysis under certain conditions (Rapoport and Dedonder, 1963). Although indirect evidence of the reversal of enzymatic synthesis of levan can be observed, little is known about the nature of such enzyme degradation. Smith (1976) showed that β-fructofuranosidase present in tall fescue degraded levan by removing one fructose residue at a time until a molecule of sucrose remained. Levanucrase of *B. subtilis* has a hydrolytic effect on small levans (Dedonder, 1966). This hydrolytic action stops at branchpoints. Neither inulin, inulobiose, inulotriose, nor methyl β-fructofuranoside is hydrolyzed, although these substrates are hydrolyzed by inulinase and yeast invertase. This hydrolytic activity may be responsible for the appearance of heterogeneous short-chain polysaccharides, rather than uniform high molecular weight polymers, in the final product of many levan preparations.

IV. Chemical Structure and Properties

A. STRUCTURE

Levans are polymers of β-fructose attached by β(2 → 6) linkages that carry a β-glucosyl residue at the end of the chain. They constitute a series of homologous oligosaccharides and polysaccharides, which can be considered derivatives of sucrose (Hirst, 1957; Tanaka et al., 1981). Figure 2 shows the chemical structures of levan and inulin. They are usually represented by the formula G-F(F)n, where G-F denotes a sucrosyl group, F the fructose, and n the number of fructose units present in the molecule. Although the structure of levan is represented by a straight chain of β(2 → 6), linkages of many bacterial levans are branched through β(2 → 1) bonds (Feingold and Canatia, 1957; Avigad and Feingold, 1965; Dawes et al., 1966; Khorrmanian and Stivala, 1982; Lindberg et al., 1973; Marshall and Weigel, 1980). The branch chains are usually short and sometimes consist of one fructose residue.

The structures of levans synthesized in cell-free enzyme systems are similar to those produced by whole-cell systems, but they differ in length. The average chain length of levans produced by the enzyme system was reported to be 10–12 monomeric units, whereas that by whole cell was much longer; sometimes the molecular weight exceeded several million (Evans and Hibbert, 1946; Marshall and Weigel, 1980; Stivala and Zweig, 1981; Tanaka et al., 1983; Pontin and Del Campo, 1985). In general, levans produced by different organisms have similar structures. The difference may be a varying degree of polymerization and branching of the repeating unit.

Levans are one of the few natural polymers in which the carbohydrate exists in the furanose form. This structural feature plays an important role in the final conformation of the molecules in solution (Marchessault et al., 1980). Moreover, the enhanced flexibility of the furanose ring in comparison with the relatively rigid pyranose ring of the majority of reserve polysaccharide gives additional flexibility to the whole fructan molecule. The linear 2 → 6-linked levan molecules are flexible.
and tend to be left-hand twist, whereas the twist of inulin is right-handed (French, 1989).

B. Properties

The composition and properties of levan depend greatly on the environmental factors in which the microorganisms are grown (Stivala and Khormanian, 1982). The general properties of levans resemble those of dextrins. Levans are levorotatory, amorphous or microcrystalline, of varying solubility in cold water, very soluble in hot water, and insoluble in absolute ethyl alcohol. Levans are generally more soluble than inulin, which is almost insoluble (<0.5%) in water at room temperature (Phelps, 1965). The high solubility of levan may be a characteristic of \( \beta(2 \rightarrow 6) \) linkage compared to \( \beta(2 \rightarrow 1) \) linkage. Branching may be only a support factor. Levans are nonreducing, not hydrolyzed by yeast invertase and amylase action, but very susceptible to hydrolysis by acid. They are not colored by iodine, but hydrogen chloride imparts a purple color that distinguishes levan from other polysaccharides not containing fructose (Pontis and Del Campillo, 1985). The molecular weight and viscosity of levans vary depending on the organisms used. Generally, their viscosity in aqueous solution increases sharply when various salts are present, but it decreases drastically with a small increase in temperature (Kang and Cottrell, 1979).

Certain biological properties of levan, such as promotion of infection and necrosis (Feingold and Gehatia, 1957; Shilo, 1959), tumor inhibition and stimulation (Leibovici and Stark, 1984; Yavetz et al., 1985; Keibovici et al., 1986; Stark and Leibovici, 1986), and increase in cell permeability for a cytotoxic agent (Leibovici and Stark, 1985) have attracted attention. These effects are partly caused by suppression of normal inflammatory response. Only levan with \( M > 10^7 \) promotes infection; the effect is lost when the polymer degrades. Levan given intravenously to mice greatly increases the virulence of intraperitoneally injected bacteria. This is partly caused by the intravenous levan sealing the vascular lining, thus affecting its permeability and preventing escape of blood constituents into the peritoneal cavity. The endothelial sealing of levan may have practical importance. Natural levans are serologically active and elicit antibody production, but purified levan preparations are not antigenic.

C. Composition and Properties of Bacillus polymyxa Levan

The levan produced by a strain of \( B. \) polymyxa (NRRL B-18475) consisted of \( \sim 98\% \) fructose when analyzed by high-performance liquid chromatography (HPLC) of the acid hydrolysate (Han and Clarke, 1990). The product was readily soluble in water (up to \( \sim 5\% \)) at room temperature, but further increase in levan concentration produced a colloid. The product was very susceptible to hydrolysis when heated for 15 minutes in 0.5% oxalic acid. The easy hydrolysis is typical of the fructofuranose structure (Feingold and Gehatia, 1957). Because the initial molecule in levan formation is sucrose, a terminal glucose should be present in levan chains. However, because of the small portion of terminal groups in high molecular weight levan, no significant amount of glucose was observed on hydrolysis.

A 5\% aqueous solution of crude levan, after dialysis through a membrane with 12,000 Da cut off, gave one sharp, clean peak just below \( 2 \times 10^6 \) Da on Sephacryl S-500 (Clarke et al., 1988). This peak was sharper (representing a narrower range of molecular mass) than those of the commercially available dextrins used as gel permeation chromatography (GPC) standards. The uniformity of the product was perhaps caused by long fermentation (=10 days) and absence of hydrolytic activity in the enzyme system. The compound was stable in aqueous solution at pH 4.5 for \( \sim 36 \) hours. The \( B. \) polymyxa (NRRL B-18475) levan has a specific optical rotation \([\alpha]_D^{24}\) of \(-42.6^\circ\), whereas most other levan preparations were reported to be \(-44 \pm 4^\circ\) (Schubach and Berndt, 1964; Murphy, 1952; Dedonder and Paud-Lenoel, 1957; Barker et al., 1955). The \( B. \) polymyxa levan was nonhygroscopic; lyophilized sheets of levan have been maintained under atmospheric conditions for \( \leq 6 \) months.

In Fig. 3, carbon-13 nuclear magnetic resonance (\(^{13}\text{C-NMR}\) peaks

![Fig. 3. \(^{13}\text{C-NMR spectrum of (a) inulin and (b) levan (spectra by S. Elizey).}](image)
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 to the peak positions for levan previously identified by Shimamura et al. (1987). The peak positions vary slightly from values in the literature, but the relative spacings between the values obtained from experimental levan and those from the literature are similar. The anomeric peak (C-2) is at $\sim$104 ppm for both. The primary carbons (C-1 and C-6) are more closely grouped in inulin, and the ring carbons (C-3, C-4, and C-5) are more closely grouped in levan; this is characteristic of the differences between inulin and levan (Seymour et al., 1979). Data clearly show the polysaccharide produced by the B. polymyxa (NRRL B-18475) to be levan type with the linkage of $\beta(2 \rightarrow 6)$-fructofuranoside (Table III). Figure 4 shows the infrared spectra of bacterial levan and inulin. The infrared spectra of the bacterial levan were similar to inulin but the bacterial levan showed a characteristically weaker peak at 984 (cm$^{-1}$) (Barker and Stephens, 1954). Lesher (1976) reported that levan had infrared absorption peaks (cm$^{-1}$) at 919, 860, and 803, whereas inulin had peaks at 933, 872, and 813.

Methylation analysis revealed that the B. polymyxa levan was made of 71% $\beta(2 \rightarrow 6)$ linkage (1,3,4-trimethyl-D-fructose), 13% terminal group at 1 or 2 position (1,3,4,6-or 2,3,4,6-tetramethyl-D-fructose), 12% branching at 1,2, or 6 position (3:4 dimethyl-D-fructose), and 4% free fructose (Clarke et al., 1988). The branches occurred in the C-1 position with a $\beta(1 \rightarrow 2)$ linkage, with side chains of $\beta(2 \rightarrow 6)$-linked residues. Murphy (1952) also reported a similar composition for B. polymyxa levan. The degree of branching in other microbial levans is reported to be 5–20% (Lindberg et al., 1973).

The B. polymyxa polysaccharides exhibit relatively high viscosity and high pseudoplasticity (Kang et al., 1983). The viscosity of the product is highest when grown on sucrose or glucose and lowest on xylose. Dea and Madden (1985) report that B. polymyxa (NCIB 11429) polysac-

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**TABLE III**

<table>
<thead>
<tr>
<th>Chemical shifts for $^{13}$C-NMR spectra of inulin, levan, and the polysaccharide produced by Bacillus polymyxa (NRRL B-18475)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Carbon atom</strong></td>
</tr>
<tr>
<td>C-1</td>
</tr>
<tr>
<td>C-2</td>
</tr>
<tr>
<td>C-3</td>
</tr>
<tr>
<td>C-4</td>
</tr>
<tr>
<td>C-5</td>
</tr>
<tr>
<td>C-6</td>
</tr>
</tbody>
</table>

* Assignment cited from Shimamura et al. (1987).
charides contain two components, and the polysaccharides when dissolved in water, exhibit temperature-dependent yield-stress solution and gel-forming properties, depending on the concentration.

V. Analytical Methods

A. Levan

Levan may be determined by using any of the colorimetric methods specific for ketose and expressed as their fructose content (Lee et al., 1979). The thiobarbituric acid method (Percheron, 1962) or the acid resorcinol color reagent method (Roe et al., 1949; Yaph and Arsenault, 1965) may be used. Changes in reducing power brought about by acid hydrolysis (e.g., 0.5% oxalic acid, 100°C, 15 minutes) may also be used. The amount of fructose in the hydrolysate can be determined by the degree of optical rotation. The free sugars and levan in the fermentation mixture can be separated by passing the mixture through a column of cellulose gel filtration medium (e.g., Aminex Matrix Cellulase, G2-25 medium, Amicon Corp., Danvers, Massachusetts). A small amount of a mixture of levan and dextran was analyzed by passing the sample through a polarimeter and using an automated resorcinol-thiouria test (Manly and Cormier, 1970).

A direct method of measuring levan is by HPLC. Levan and free sugars were easily separated on an anion exchange column (e.g., Aminex HPX-87C, Biorad, Richmond, California) (Han and Clarke, 1989). Paper and thin-layer chromatography (TLC) can be used for determination of short-chain components in levan-producing systems or levan hydrolysate. Small quantities of the monomeric levan can be separated from other keto sugars by TLC. The position of levan can be identified by spraying reagents specific for levan, such as resorcinol HCl and naphtoresorcinol HCl (Forsyth, 1950; Yaph and Arsenault, 1965), urea HCl and urea metaphosphoric acid (Dedonder, 1966), and dimedon [(5,5-dimethyl-1,3-cyclohexanedione)phosphoric acid] (Adachi, 1964). Levan in fermentation broth can be approximated by weighing the precipitate formed by adding ethanol or isopropanol. The precipitate can be purified in advance by redissolving in water, dialyzing, and freeze drying.

The structure of levan can be studied by instrumental analysis. Infrared spectroscopy is widely used to investigate polymer structure and to analyze functional groups. The infrared absorption spectrum of levan has typical bands, which can help in its analysis and differentiate it from inulin and other polysaccharides (Hestrin et al., 1954; Lesher, 1976).

Because p-fructofuranosides are heat-labile and partly decompose during derivatization to forms suitable for analysis, $^{13}$C-NMR spectroscopy is a useful method to determine the linkage type of polyfructan (Shimamura et al., 1987; Seymour et al., 1979). The $^{13}$C-NMR spectrum of levan and inulin produces six main resonances, and each has its own distinguishing characteristic. These methods are much less involved than classical methylation analysis, in which polysaccharides are exhaustively methylated, hydrolyzed, derivatized with alditol acetate, and the methyl ether obtained is analyzed by gas-liquid chromatography (Hakomori, 1964). These studies provide information on the linkage type and extent of branching, but cannot show the length of side chains that may be of great importance in studying immunochemical properties (Lindberg et al., 1973).

Immunoassay methods utilizing antifructan activity of some myeloma immunoglobulins have been proposed (Gludemans, 1975). Some are specific for levan (Cisar et al., 1974), whereas others are specific for inulin (Streefkerm and Glaudemans, 1977). The precipitate formed with concanavalin A differentiates levan and inulin (Goldstein and So, 1965). Although no attempts have been made to quantify levan by this method, the method may make it possible to determine levan in a highly specific way.

B. Levanusurase

Levanusurase is an enzyme responsible for forming levan from sucrose and accumulating glucose in the menstruum. Therefore, levanusurase assay is generally based on the determination of free glucose in the reaction mixture (Chambert et al., 1974; Tanaka et al., 1979). The reaction conditions are set so that the velocity of glucose formation depends only on the amount of enzyme (zero-order reaction). The reaction is stopped by dilution in boiling buffer; the free glucose is determined by the glucose oxidase method or the Somogy-Nelson method (Coska, 1971). This method can be used for levanusurase in cultural broth, cell extract, or intact cells. However, this method cannot be used to test for activity in cultures grown on glucose as a substrate. The addition of levan primer to the reaction mixture provides more consistent conditions for the assay during purification. Because conditions of the reaction produce significant variations in levan yields, any methods based on levan determination cannot be used to assay the enzyme.
activity. Such a determination may be useful for the study of the reaction conditions.

VI. Production of Levan

All known production of exopolysaccharides is by aerobic submerged fermentation. Microbial production of polysaccharides requires fermentation and handling of a highly viscous solution, which is not involved in many nonviscous fermentation processes. Unique fermentation parameters of aeration, agitation, and pH and temperature controls should be established for each polysaccharide fermentation. The conditions for producing levan by growing cultures of bacteria vary according to the microorganisms used. Figure 5 shows the flow diagram of levan production by *B. polymyxa*.

*Bacillus polymyxa* (Strain NRRL B-18475) produces a large quantity of extracellular polysaccharides when grown on 4–16% sucrose solution. Table IV lists the composition of the medium for levan production. Yeast extract enhances the levan yield. The highest level of levan was obtained in media containing 8–15% sucrose. The organism converted sucrose (S) to levan (L) and accumulated a small amount of glucose (G) in the growth medium (Fig. 6). During fermentation, the sucrose levels dropped and levan started to appear in 2 days; thereafter, sucrose levels gradually decreased as levan increased. Glucose was the major by-product. A small amount of fructose (F) and unidentified fermentation products smaller in molecular weight were also observed. The pH of the growth medium fell from 7.0 to 4.7 because of acid

![Flow diagram of levan production by *Bacillus polymyxa* strain NRRL B-18475.](image)

**Fig. 5.** Flow diagram of levan production by *Bacillus polymyxa* strain NRRL B-18475.

<table>
<thead>
<tr>
<th>Component</th>
<th>Percentage of total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sucrose</td>
<td>15.0</td>
</tr>
<tr>
<td>Peptone</td>
<td>2.0</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>2.0</td>
</tr>
<tr>
<td>K$_2$HPO$_4$</td>
<td>2.0</td>
</tr>
<tr>
<td>(NH$_4$)$_2$SO$_4$</td>
<td>2.0</td>
</tr>
<tr>
<td>MgSO$_4$·H$_2$O</td>
<td>0.2</td>
</tr>
</tbody>
</table>

**Fig. 6.** Levan formation during fermentation of sucrose by *Bacillus polymyxa* strain NRRL B-18475.
production. In other levant production, maintaining pH above 5.5 was important because the optimum pH for levansucrase was 5.5–7.0 and levans may be hydrolyzed at a lower pH (Avigad, 1968). Optimum temperature for growth and levant production was 30°C.

Aeration is important in biosynthesis of levansucrase (Tkachenko and Loitsyzanskaya, 1979). The polysaccharide production was especially pronounced when the culture was gently shaken during cultivation, but vigorous agitation and aeration inhibited levant production (Han, 1989). A small amount of microbial polysaccharide (alcohol precipitate) was also produced when the organism was grown on lactose, maltose, or raffinose, but not on glucose or fructose. The organism produced polysaccharide from sugarcane juice, but the yield was much less than that from sucrose. High sucrose concentration lowered the average molecular weight of the levans synthesized (Dedonder and Peud-Lenoel, 1957).

Levan was harvested by precipitation from the culture broth by adding ethanol or isopropanol. Acetone and methanol can also be used. 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 1:1.2, and the yield peaked at 1:1.5. Further increase in the ratio hardened the levan and made the product less fluid. Slightly less isopropanol than ethanol was needed to precipitate levan. Although most of the bacterial cells, unfermented sugars, and other solubles remained in the aqueous alcohol phase, pre-removal of microbial cells by centrifuging was needed to obtain a pure form of levan. The product was further purified by repeated precipitation and dissolution in water, followed by dialysis or ultrafiltration. The final product was an off-white, gummy material that could be freeze- or vacuum-dried. Alternate methods of harvesting would be drying by trituration and pulverization with absolute alcohol in a high-speed blender. In a typical fermentation, B. polymyxa produced ~3.6 g of levan in 100 ml of 15% sucrose in 10 days (~50% yield on available fructose). Methods of preparing levan by A. levunicum and from a cell-free enzyme—sucrose system have been reported (Hestrin et al., 1956; Avigad, 1965).

VII. Utilization of Levan

A. INDUSTRIAL GUMS

Industry consumes natural polymers in tremendous quantities. They contribute important properties to products, even when used in low concentration, and do this at low cost. Useful properties of poly-
saccharides include providing viscosity; solubility in water and oil; suspending properties; rheological properties; compatibility with salts and surfactants; stability when exposed to heat, acid, and alkali; film formation; holding capacity for water and chemicals; and biological activities. Commercial polysaccharides are water-soluble or water-dispersible hydrocolloids. Their aqueous dispersion usually possesses suspending, dispersing, and stabilizing properties, and can be used to emulsify and stabilize foods.

Natural polysaccharides can be modified to alter their chemical and physical properties. Modification of a low-cost polysaccharide can make it a suitable replacement for a more expensive gum. In general, the properties of neutral polysaccharides can be remarkably altered by introducing a very small amount of substituent groups of either neutral or ionic types.

Several factors are important in selecting a polysaccharide for industrial use. Among these are physical and chemical properties, production cost, consistency in composition and supply, possibility of eventual replacement by another, and acceptability by government agencies if the product is intended for food use (Whistler, 1973). A host of microbial polymers with widely differing properties is available; fermentation technology is expected eventually to develop others to meet many industrial needs. Because it is similar in physical properties and mode of production to dextran, levan could be used in similar ways. Levan should be considered for applications regarding low viscosity, high water solubility, and susceptibility to acid hydrolysis.

B. Blood Plasma Extender

Bacterial dextran has been extensively used as a plasma substitute. The extender is prepared by partial hydrolysis of the high molecular weight polysaccharides and subsequent methanol fractionation of the hydrolyzed product to obtain a material ranging in $M_w$ from 25,000 to 200,000 (Hines et al., 1953). The necessity for hydrolysis, however, is one of the major disadvantages of clinical dextran preparation.

Because levan is similar in physicochemical properties to bacterial dextran, using levan as a blood volume extender has been studied (Mattoon et al., 1955; Schecter and Hestrin, 1963). Native levan was hydrolyzed at pH 3.2 to an extent of 1–2% hydrolysis and $M_w$ between 30,000 and 100,000. After neutralization, fractions of polysaccharide were collected by precipitation with increasing ethanol concentration (Hestrin et al., 1956). A partial acid hydrolysis of levan produced a series of oligosaccharides and levulans (degraded levans) (Feingold and
C. Sweeteners

Because levan is a polymer of fructose, its hydrolytic products may be used as sweetener or precursors of sweetener. Industrial production of ultrahigh-fructose glucose syrups (UHFGS) from fructose has been reported. Jerusalem artichoke was hydrolyzed by acid or enzyme (inulinase), and the resulting fructose was precipitated and purified. The fructose syrup thus obtained was adjusted to the desired fructose content by concentration or blending. Enzymatic hydrolysis was preferred over acid hydrolysis, because the formed products had a higher fructose and also yielded a product with better flavor and less color. Acid hydrolysis of a microbial levan produced a series of β(2 → 6)-fructofuranosyl oligosaccharides (di-, tri-, and tetramers) (Kennedy et al., 1989). Although their sweetness and nutritional values have not been studied, their characteristics may resemble those of Neosugar, a fructooligosaccharide, nonnutritive sweetener (Hidaka, 1983; Adachi, 1983). Neosugar is prepared from sucrose by the action of fructosyltransferase of fungi such as Aspergillus sp. (Hidaka, 1983; Pazur, 1952). Fusarium sp. (Gupta and Bhalla, 1982), and Aureobasidium pullulans (Smith et al., 1982; Jung et al., 1987). Neosugar has a pleasant taste with half the sweetness of sucrose but is not utilized by the body (Oku et al., 1984).

D. Other Applications

Levans could possibly be used as an emulsifier, formulation aid, stabilizer and thickener, surface-finishing agent, encapsulating agent, and carrier for flavor and fragrances. Incorporation of levans in photographic emulsion to improve silver granularity has been suggested (Chambers and Overman, 1964). Cross-linked levan preparations may be used for molecular sieves for gel filtration. The low viscosity and high solubility of levan may make it a suitable substitute for gum arabic in a variety of uses in foods, pharmaceuticals, medicine, cosmetics, adhesives, paints, inks, lithography, textiles, and others. The high solubility of gum arabic is responsible for its excellent stabilizing and emulsifying properties when incorporated with large amounts of insoluble materials (Glicksman, 1973).

VIII. Summary

Levans are natural polymers of the sugar fructose found in many plants and microbial products. Like dextrins, they are formed as an undesirable by-product of sugar juice processing. On the other hand, levans, which can only be produced from sucrose, have potential industrial applications as thickeners and encapsulating agents and could provide additional, valuable products from sugarcane juice. A strain of B. polymyxa (NRRL B-18475) produced a high yield of polysaccharide when grown on sucrose solution. Hydrolysis and subsequent analyses showed the product to consist entirely of α-fructose. 13C-NMR and methylation analyses indicated the products to be a β(2 → 6)-linked polymer of fructose, with 12% branching. The polysaccharide has a Mₘ of ~2 million and is readily soluble in water. Levan has not been utilized, but if developed, could be useful in food and other industrial applications.

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References