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Production of Levan by *Bacillus licheniformis* for Use as a Soil Sealant in Earthen Manure Storage Structures

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Abstract: Manure application is not permitted on frozen land in Canada and therefore, manure management and storage are the primary issues facing the agri-food industry. Low-cost, effective and environmentally safe earthen manure storage (EMS) facilities will lower costs and help make the livestock industry more competitive and efficient. The goal of this study was to develop a biological sealing technology for earthen manure storages. The results showed that it is feasible to use a growing culture of *Bacillus licheniformis* to produce a non viscous water insoluble levan. Levan can only be produced by *Bacillus licheniformis* during the growth mode. No levan was produced during the death phase. About 0. 36 g of levan was produced per gram of sucrose which is 91. 1% of theoretical yield. The polymer can be used as a plugging agent to plug the pores of high permeability soils. From the biological and biochemical characteristics of the *Bacillus licheniformis,* it appears that the organism is capable of producing levan from sucrose under most field and soil conditions. As a soil organism, *Bacillus licheniformis* should be able to compete with most common soil species such as *Arthrobacter* and *Bacillus*. The bacteria could be grown either in the non-polysaccharide producing mode or in the polysaccharide producing mode. The first would permit distribution of the bacteria to the lower soil layers but would delay the production of the polysaccharide due to the lag period required to produce the enzyme (levansucrase). Upon production of levan, pore spaces would close and hence, the hydraulic conductivity would be substantially reduced.

Keywords: Mnure, soil, biological sealing, bacteria, polysaccharide, plugging agent, levan

INTRODUCTION

Approximately 1000 million tonnes of manure (wet weight) are produced annually in Canada[1], With greater livestock demand, the trend in manure production is expected to increase and therefore, health and environmental concerns over livestock manure collection and storage are also growing. Manure can not be applied on frozen land in Canada and it must be stored for 6-8 months[2]. Without proper storage of manure, ground water contamination becomes a major concern[3]. Therefore, Agriculture and Agri-Food Canada deemed the need for an effective, low cost storage of manure with minimal environmental risks as a priority[4].

Although earthen manure storage structures are considered an economical means of storing animal waste, they cause contamination of the groundwater from the stored liquid manure. Earthen manure storage structures are typically engineered to limit seepage by considering the hydraulic properties of the soil when designing the basin[5]. Environmental authorities in Canada seem to believe that such a site selection criterion will guarantee groundwater protection because of the sealing of soils by manure. However, several field studies showed an "initial flush" behaviour after a fresh loading of manure into these reservoirs. De Tar[6] observed substantial seepage during the time required for the seal to develop, which resulted in groundwater pollution. Reese and Lauden[7] reported that some of the manure constituents percolated through the soil during the initial period resulting in high levels of pollutants in groundwater.

There are three different types of soil sealing: physical, chemical and biological. Physical soil sealing is the clogging and plugging of soil pores by particles which work to reduce the hydraulic conductivity of the soil. Chemical soil sealing results from changes in the soil structure caused by chemical reactions within the soil. Biological soil sealing is the clogging of soil pores with microbial by-products such as enzymes and polysaccharides. Rice[8] demonstrated that clogging of

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the soil occurred within the first 5 cm of the soil due to hydraulic head which forced fine organic material into the soil pores near the surface and concluded that the efficiency of this sealing will be reduced substantially after emptying the manure storage systems. Maule[9] reported that the reduced hydraulic conductivity caused by the formation of manure seal occurs just in the upper layer (3-5cm) of the soil. Therefore, an enhanced biological sealing, deep in the soil layers, is of paramount importance.

The oil industry uses induced biological sealing for enhancing oil recovery. Microorganisms are used to produce exopolymers which plug soil pores[10,11]. Bacteria have been used as a plugging agent for enhanced oil recovery since 1946[12,13]. Atlas and Bartha[14] stated that the most prominent soil microorganisms are of genera *Arthrobacter* and *Bacillus*. Ramsay[13] stated that in order for these bacteria to successfully produce exopolymers in soil, the microorganisms must be capable of surviving the competition of native soil microorganisms and the polymers produced must be water-insoluble so that they will not be removed from the soil pores by water flow. Keeping these required characteristics in mind (as well as many other necessary traits including: control mechanism, pathogenicity, size and habitat properties), it has been suggested that suitable soil-plugging agents would be the polysaccharides dextran[11]and levan[15].

The aim of this study was to culture a biological species whose metabolic end product (polymer) can be used as a selective plugging agent for sealing the soil pores in earthen manure storage structures.

MATERIALS AND METHODS

Experimental apparatus: The experimental apparatus is shown in Fig. 1. Four 5 L batch bioreactors, each constructed from Plexiglas cylinder of 5 mm thickness, were used. The dimensions of the fermenter are shown in Fig. 2. Four vertical baffles positioned at 90^o apart) made from Plexiglas were used in the fermenter to improve the bottom turnover and to reduce the vortex. Provisions were made on the cover mounting for the temperature probe, pH probe, dissolved oxygen probe, mixing shaft and sample collection.

The agitation was facilitated by the mixing system, which consisted of an electric motor (Model 4Z142, Dayton Electric MFG Co., Chicago, Illinois, USA) with a controller and a mixing shaft. The mixing shaft has two flat-bladed impellers of 75 mm diameter, mounted 148 mm apart (the bottom impeller being 30 mm from the fermenter floor).

The fermentation temperature was controlled using a specially designed well insulated water bath. Water circulation within the water bath was facilitated by a pump (Model No. 1-M AT, Tecumseh Products Co., Oklahoma City, Oklahoma, USA)was inserted in the water bath. A uniform distribution of the water to the heating unit was facilitated by the holes around the steel tube, inside which a 2. 0 KW heating element (Cat. No. MT 30201250, Chromalox Canada Inc., Rexdale, Ontario, Canada) was inserted. A temperature sensor (Model No. T675A2100, Honeywell, North York, Ontario, Canada), inserted in the water bath, was used

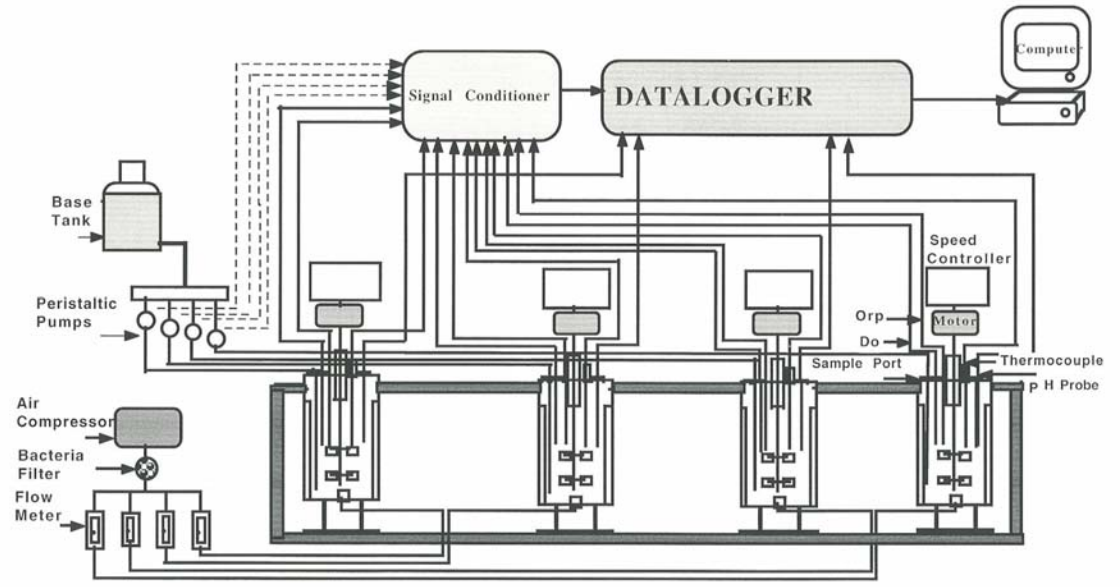


Fig. 1: Experimental apparatus.

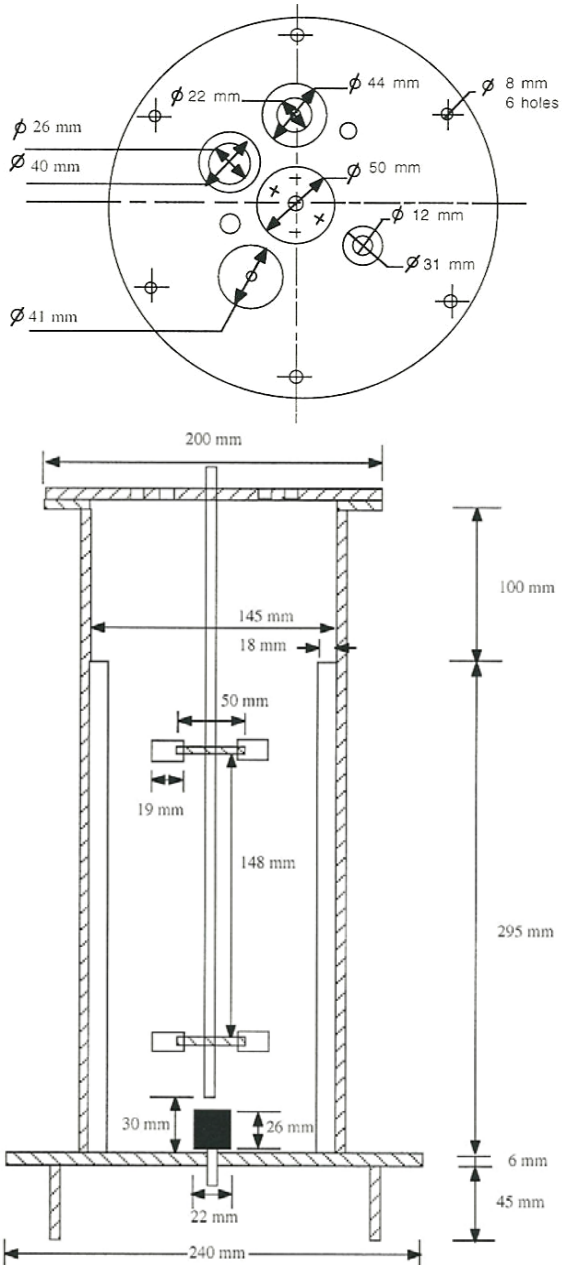


Fig. 2: Batch reactor

to monitor the water temperature and the temperature control unit regulated the water temperature at a preset value.

An air compressor (Model No. LGH-210-H02, Pnuemotive Air Power Products, Los Angeles, California, USA) was used to supply air to the bioreactor. The air was passed through a bacterial filter (Cat. No. 4210, Bacteria air vent-Gelman Sciences, Montreal, Quebec, Canada).

Table 1: Levan-producing microorganisms

Microorganism	Yield	Reference
Acetobacter acetigenum		Loewenberg and Reese[16]
Acetobacter pasteurianus		Loewenberg and Reese[16]
Actinomyces viscosus	0.0245**	Pabst[17]
Achromobacter sp.		Han[18]
Aerobacter aerogenes		Srinivasan and Quastel[19]
Aerobacter levanicum	0.051	Evans and Hibbert[20]
Arthrobacter ureafaciens		Han[18]
Azotobacter chroococum		Hestrin and Goldblum[21]
Bacillus asterosporus		Hestrin and Goldblum[21]
Bacillus amyloliquefaciens		Han[18]
Bacillus megaterium		Evans and Hibbert[20]
Bacillus mesentericus		Han[18]
Bacillus polymyxa	0.45	Han[22]
Bacillus subtilis	0.285*	Shih[23]
Corynebacterium levaniformans		Han[18]
Corynebacterium beticola		Han[18]
Gluconobacter oxydans		Han[18]
Leuconostoc mesenteroides		Han[18]
Micbacterium laevaniformans		Han[18]
Odontomyces viscosus		Han[18]
Phytomonas pruni		Han[18]
Pseudonomas aureofaciens	83%	Fuchs[24]
Pseudonomas chlororaphis		Fuchs[24]
Pseudonomas fluorescens		Fuchs[24]
Rothis dentocariosa		Han[18]
Streptococcus sp.		Han[18]
Streptocuccus salivarius		Fuchs[24]
Xanthomonas sp.		Han[18]
Zymomonas mobilis	0.0063	Dawes, Ribbons and Rees[25]
Yeast		Leowenberg and Reese[16]
Aspergillus sydawi		Leowenberg and Reese[16]
Aspergillus versicolor		Leowenberg and Reese[16]

*Note that *Bacillus licheniformis* is a type of *Bacillus subtilis*
**This yield is for cell-free production of levan

The pH control system consisted of a base tank (30 L), four peristaltic pumps (Model 70 16-52, Cole-Parmer, Chicago, Illinois, USA) and associated tubing connections and the pump control units. Throughout the fermentation process, the pH of the broth was maintained at the desired pH by automatic addition of controlled amounts of 1 N NaOH or 1 N HCl.

The data acquisition system consisted of a data logger, pH probes, thermocouples, dissolved oxygen probes, signal conditioner unit and a personal computer. The data logger (Model No. 525, SYSCON International Inc., Los Angeles, California, USA) was connected to the signal-conditioning unit and to a personal computer through a serial communication port. Four thermocouples (Cat. No. L-08530-74, Cole Parmer, Chicago, Illinois, USA) were connected to the data logger, whereas four pH probes (Model No. 25643-04, Cole Parmer, Chicago, Illinois, USA) were connected to the data logger through the signal conditioning unit. A Quick Basic environment was used to develop the software and to operate the data acquisition system.

Selection of the microorganism and polysaccharide: Selection of microorganisms used in the study was based on four criteria: pathogenicity, cell size, type of

Table 2: The biological and biochemical characteristics of *Bacillus licheniformis*

Parameter	Description	Reference
Habitat	Mainly soils, water and food	Harwood[26]
Motility	By Peritrichous flagella	Sneath et al.[27]
Staining	Gram positive	Sneath et al.[27]
Shape	Rod - often in chains	Sneath et al.[27]
Size	0.6-0.8 μm by 1.5-3.0μm	Sneath et al.[27]
Weight	$2*10^{-12}$ gram/cell	Sneath et al.[27]
Spore shape	Elliptoidal to cylindrical	Sneath et al.[27]
	Does not swell	Sonenshein et al.[28]
	Only produced aerobically	Yakimov et al.[29]
Spore size	0.6- 0.9 μm by 1.0-3.0 μm	Yakimov et al.[29]
Oxygen	Aerobe	Sneath et al.[27]
	Facultative Anaerobe (in the presence of nitrate)	Sonenshein et al.[28]
Temperature	Mesophilic (30-55˚C)	Sneath et al.[27]
	Spores form at 37˚C	Breed et al.[30]
pH	5.4-9.0	Ramsay[13]
Pressure	Known to withstand up to 60 atm	Ramsay[13]
Salt	<12% NaCl tolerable (<6% NaCl best growth)	Yakimov et al.[29] and Ramsay[13]
Growth on Agar	Opaque colonies with rough surface and hair-like outgrowths, firmly attached to the agar surface, becomes brown with age.	Stetzenbach and Yates[31]
Reactions	Produces Levan in presence of sucrose	Ramsay [13],
	Produces protease from protein	Sneath et al.[27] and
	Produces amylase from starch	Stetzenbach andYates[31]
	Produces acetic acid from lactose	
	Produces citric acid from glucose	
	Produces bacitracin (an antibiotic for treatment of infected wound, and upper respiratory tract)	
	Produces gases while nitrate is reduced to nitrite	

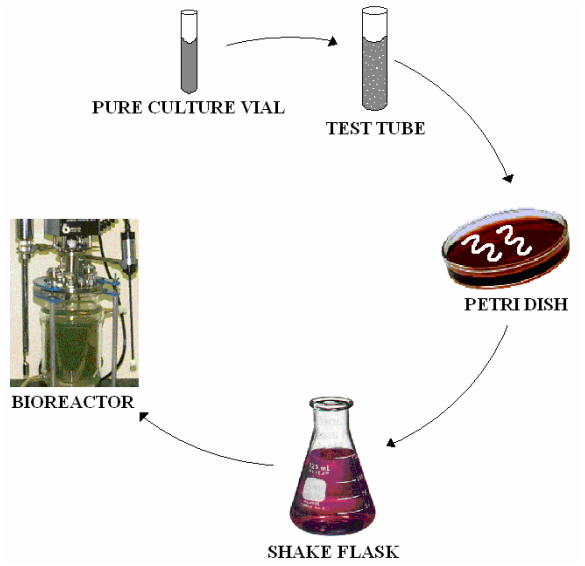


Fig. 3: Preparation of *Bacillus licheniformis* culture

polymer produced and competition with other soil microorganisms. As manure will be spread on agricultural land after the storage period, contaminated manure could spread the disease to healthy animals and thus, the selected microorganism must be non-pathogenic. Cells smaller than the average pore diameter of the soil (the average pore diameter of agricultural soils is 7-15 μm) are desirable. Non-soluble polysaccharide is required in order to form stable sealing. *Arthrobacter* and *Bacillus* species are the most dominant soil microorganisms[14]. Thus, selected microorganisms must be able to compete with these as well as other microorganisms such as yeast and fungi. Levan was selected as the polysaccharide and the literature was reviewed for levan producing bacteria (Table 1). The biological and biochemical characteristics of *Bacillus licheniformis* (Table 2) indicated that the microorganisms would be suitable for the production of β-D Fructofuranose (levan) from sucrose.

Preparation of the microbial cultures: Pure cultures of *Bacillus licheniformis* ATCC 9945 were obtained from the American Type Culture Collection (ATTC, Rockville, Maryland, USA) and were grown in the laboratory. Fig. 3 shows the various steps of culture preparation of the bacteria. The freeze-dried cultures of *Bacillus licheniformis* were first revitalized in screw-top test tubes in Bacto® Nutrient Broth (Difco Laboratories, Detroit, Michigan, USA) following the ATCC protocol. The nutrient broth was made of 5 g Bacto Yeast Extract, 10 g Bacto Tryptone and 2 g Bacto Dextrose, all dissolved in 1 L of deionized

distilled water.

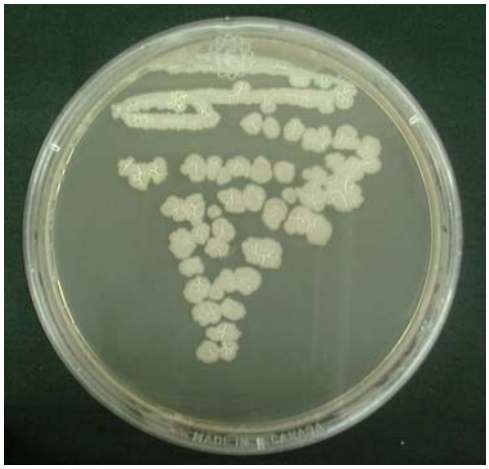
The reactivated culture of *Bacillus licheniformis* was transferred aseptically to nutrient broth (Difco Laboratories, Detroit, Michigan, USA) and plate count agar (Difco Laboratories, Detroit, Michigan, USA) and incubated at 30°C for 48 hrs to increase cell numbers and confirm purity and identity. The Nutrient Broth contained 5 g Bacto Yeast Extract, 10 g Bacto Tryptone and 2 g Bacto Dextrose, all dissolved in 1 L of deionised distilled water. The plate count agar was made of 5 g Bacto Tryptone, 2. 5 g Bacto Yeast Extract, 1 g Bacto Dextrose and 20 g Bacto Agar, all dissolved in 1 L of deionised distilled water. Cultures were then transferred to 250 mL Erlenmeyer flasks, each containing 150 mL Nutrient Broth (Difco Laboratories, Detroit, Michigan, USA) and placed on a controlled environment rotary shaker (Incubator Shaker, Series 25, New Brunswick Scientific, Edison, NJ, USA) for 24 hrs at 200 rpm and 30 °C to increase cell number and serve as inoculants for the batch reactors.

All of the media containers (test tubes and Erlenmeyer flasks) were first sterilized in an autoclave for 15 min at 121°C and then cooled before the bacterial cultures were hydrated and propagated.

Polymer production: The prepared cultures were transferred from the shake flasks to 4 bioreactors (stirred tank reactors) having 4. 8 L each of sterilized liquid media. Each reactor was injected with 10% (v/v) of homogeneous mixture from the shake flask bacterial culture. The reactors were operated at 30˚C with constant aeration (2 volumes of air per volume of media) and mixing (200 rpm) for five days under batch condition.

Samples were drawn from the reactor for biomass, sucrose and polysaccharide determination. Sampling was frequent (every 4 h) during the first 24 h, since most of the changes were expected to take place. Sampling was done every 6 h during the period of 24 - 72 h. and every 12 h thereafter until the end of the experiment.

Polymer measurement: Measurements of the polymers were performed according to the procedures described by Ramsay[13]. After the growth, the culture was centrifuged at 2400xg to remove bacterial cells and dialyzed to remove unfermented sugars and any fermentation products with smaller molecular weight. Levan was precipitated by adding 3 volumes of methanol to the cell-free supernatant. The precipitated polysaccharide was then isolated after vortexing the mixture for 10-15 sec and then centrifuging it at 2400xg for 10 min. To correct for precipitated salts, an uninoculated control was used. Once the pH was adjusted to 2. 0, the samples were heated in boiling water. After having been cooled, the samples were analyzed using High Performance Liquid Chromatography (Agilent 1100 HPLC, Agilent Technologies Inc., Santa Clara, CA, USA)



(a) Grown without sucrose

(b) Grown with sucrose (the mucoid slimy appearance indicates the presence of levan)

Fig. 4: Colonies of *Bacillus licheniformis*

RESULTS

Culture propagation: Once the freeze- dried culture of *Bacillus licheniformis* was revitalized in the test tubes, it was grown on plate count agar with and without sucrose. When the culture was grown on agar with sucrose, the colonies had a mucoid slimy appearance which indicated the production of the polysaccharide from sucrose (Fig. 4b). This indicated that the bacteria produce the enzyme levansucrase which converted the soluble sucrose into the polysaccharide ß-D fructoside

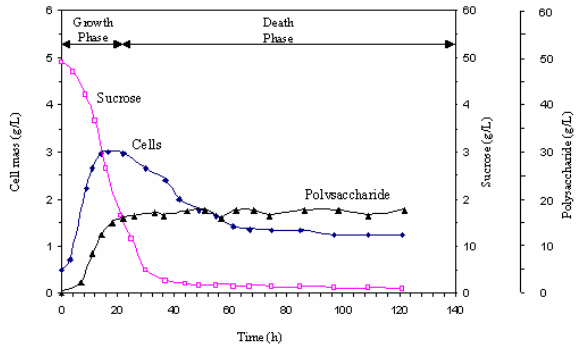


Fig. 5: Sucrose, cells and polysaccharide concentrations

(levan) and glucose. The plate count test performed on the media obtained from the shake flasks (which was used to prepare the inoculum for the bioreactor) revealed that there was a count of approximately 8. 24 x 10^8 microbial cells per mL.

Batch culture operation: The results of batch culture propagation of *Bacillus licheniformis* in the bio-reactors are presented in Fig. 5. The bacterial culture grew exponentially reaching a maximum biomass concentration of 3 g L^{-1} after 16 h. There was no apparent lag period since the culture in the inoculum was grown on a similar medium. The concentration of sucrose decreased, exponentially reaching about 1. 5 g L^{-1} after 56 h. With the depletion of sucrose, the bacterial cell mass decreased with time reaching 0. 14 g L^{-1} after 68 h. The production of levan took place during the period of exponential growth reaching a maximum of 18 g L^{-1}. No polysaccharide was produced during the death phase.

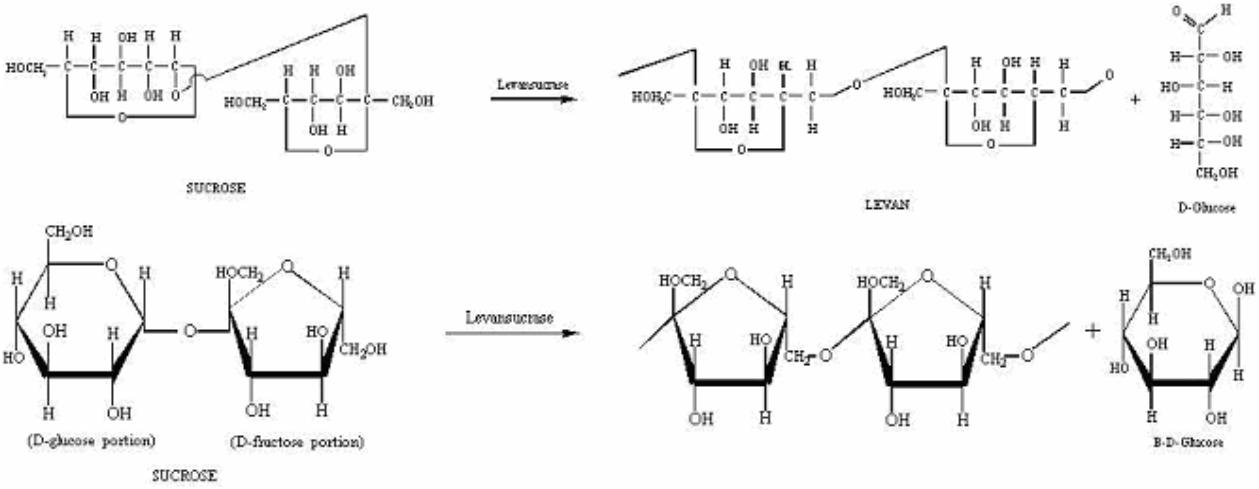


Fig. 6: Chemical equation and structure of levan.

DISCUSSION

Microbial levans contain up to 3 million residues compared to plant levans which contain about 100 residues[22]. The polysaccharide levan $(C_6H_{10}O_5)_n$ consists of fructose monomers linked mainly by β(2→6) linkages[18]. The chemical equation and structure are shown in Fig. 6. The polysaccharide (levan) produced was non-viscous and water insoluble. The viscosity of the culture broth of *Bacillus licheniformis* was the same as that of water even at a concentration of 18 g L^{-1}. The polymer was a non-transparent suspension and was found to deflect visible light. It was possible to correlate the concentration with turbidity as shown in Fig. 7.

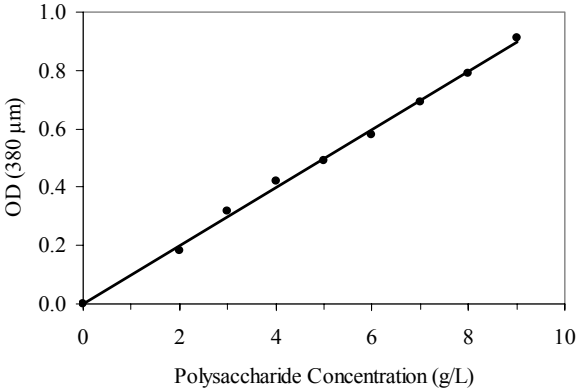


Fig. 7: Optical density of various concentrations of the polysaccharide

Table 3: Levan applications

Application	Usage	Reference
Industrial	Provide viscosity	Shih et al.[23]
	Holding capacity for water and chemicals	Ramsay[13]
	Selective plugging agent	Gabitto et al.[32]
Medical/Pharmaceutical	Blood plasma extender	Shih et al.[23] and Kirk[33]
	Hypocholesterolemic agent	Ben Ammar[34]
	Modification of cell membrane (tumor cells)	Shih et al.[23] and Ahmed[35]
	Tablet binder	Ben Ammar[34], Shih et al.[23] and Ahmed[35]
Foods	Add sweetness to foods	Han[22], Shih[23] and Ahmed[35]
	Fillers	Kirk[33]
	Bulking agent	Kirk[33]
	Subtitue for gum arabic	Kirk[33]
Others	Emulsifier	Shih[23]
	Formulation aid	Shih[23]
	Stabilizer and thickener	Shih[23]
	Surface-finishing agent	Shih[23]
	Encapsulating agent	Shih[23]
	Carrier for flavour and fragrances	Shih[23]
	Cosmetic	Han[36] and Ben Ammar [34]

Levans are viewed as possible substitutes for dextrans in cases where low viscosity, high water-soluble and susceptibility to acid hydrolysis is required. Some uses of levan are shown in Table 3.

When the bacteria were grown on a medium containing sucrose, the production of an extracellular enzyme (levansucrase) was induced and sucrose was converted to levan and glucose. During the fermentation process, the bacteria also utilize sucrose for maintenance and growth. The following equations describe product formation, respiration and energy production and growth and reproduction.

Respiration and energy production

$$\underset{\text{sucrose}}{(C_{12}H_{22}O_{11})} + 12O_2 \xrightarrow{\text{Cells}} 12CO_2 + 11H_2O + \Delta \quad (1)$$

Growth and reproduction

$$5\underset{\text{sucrose}}{(C_{12}H_{22}O_{11})} + 12NH_4^+ \xrightarrow{\text{Cells}} 12\underset{\text{cells}}{C_5H_7O_2N} + 31H_2O + 12H^+ \quad (2)$$

Product formation

$$30\underset{\text{sucrose}}{(C_{12}H_{22}O_{11})} \xrightarrow{\text{levansucrase}} \underset{\text{levan}}{(C_{66}H_{10}O_5)} + 30\underset{\text{glucose}}{(C_6H_{12}O_6)} \quad (3)$$

Equation (1), (2) and (3) can be combined to yield the following equation:

$$36(C_{12}H_{22}O_{11}) + 12O_2 + 12NH_4^+ \rightarrow (C_6H_{10}O_5)_{30} + 30(C_6H_{12}O_6) + 12C_5H_7O_2N + 42H_2O + 12CO_2 + 12H^+ + \Delta \quad (4)$$

From equation (4), it appears that the theoretical yield is 0. 395 gram of levan per gram of sucrose and the cell yield is 0. 130 gram of cells per gram of sucrose. In this study, the levan yield was 0. 360 gram per gram of sucrose (91. 1% of theoretical yield) and the cell yield was 0. 06 gram cell per 1 gram sucrose (46. 2 % of theoretical yield).

CONCLUSION

The results showed that it is feasible to use a growing culture of *Bacillus licheniformis* to produce a non viscose water insoluble levan. About 0. 36 g of levan can be produced per gram of sucrose which is 91. 1% of the theoretical yield. The polymer can be used as a plugging agent to plug the pores of high permeability soils. From the biological and biochemical characteristics of the *Bacillus licheniformis,* it appears that the organism is capable of producing levan from sucrose under most field and soil conditions. As a soil organism, *Bacillus licheniformis* should be able to compete with most common soil species such as *Arthrobacter* and *Bacillus*. The bacteria could be grown in the laboratory either in the non-polysaccharide producing mode or in the polysaccharide producing mode. The first would permit distribution of the bacteria to the lower soil layers but would delay the production of the polysaccharide due to the lag period required to produce the enzyme (levansucrase). Upon production of levan, pore spaces would close and hence, the hydraulic conductivity would be substantially reduced.

ACKNOWLEDGMENT

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