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Isolation and characterization of a novel polysaccharide from *Bacillus licheniformis* NCIB 11634

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SUMMARY

A polysaccharide producing strain of *Bacillus licheniformis* was isolated from exudate of raffia palm, *Raffia vinifera*. The optimum conditions for growth and polysaccharide production have been investigated and established. No appreciable polysaccharide was formed on glucose. It grew best in Czapek-Dox media with sucrose as the carbon source. The polysaccharide has been characterized as a heteropolymer containing D-glucose, D-mannose and D-xylose.

INTRODUCTION

Several products of industrial significance have been derived from microbial metabolism [4]. Some of these include extracellular microbial polysaccharide such as xanthan gum, dextran and polytran. Xanthan gum which is now used commercially is produced by *Xanthomonas campestris* [17,28]. For several years, some of these products have been successfully employed for food, petroleum, textile, and numerous other industrial uses [7,10,11]. The unique characteristics of these polymers guarantee their commercial exploitation. These unique attributes include consistent physical and chemical properties, diverse source, regularity of supply and better functional properties [27,35].

Polysaccharide producing bacteria have received considerable attention both from the point of view of the bacteria and the nature of the polysaccharide produced [32]. The antibiotic producing potential of *Bacillus licheniformis* has occupied the attention of many researchers [16,36,37]. Understandably, little or no attention has been directed to the intrinsic potentials and properties of the polysaccharide produced by this species. The present investigation was designed to establish the optimal fermentation conditions for production of an extracellular polysaccharide by *Bacillus licheniformis*.

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Furthermore, preliminary experiments on the rheological, suspending, and emulsifying properties of the polysaccharide were carried out using the biosynthesized extracellular polysaccharide (BLMG 135), and a purified natural polysaccharide gum (BLNG 137) obtained from the natural source of the bacteria. For these studies, methylcellulose, acacia, and tragacanth were used as the basis for comparison. At a concentration of 3–4%, the microbiologically synthesized polysaccharide (BLMG 135) showed some promise for use as a suspending agent [6]. The suspension of sulphadimidine was more stable than the suspension of zinc oxide prepared with the same concentration of the polysaccharide. This was attributed to the density differential of the suspended particles. Thus, the microbiologically produced gum may be used for formulating stable suspensions of light rather than heavy powders.

However, the natural gum (BLNG 137) at low concentrations of 1–1.5% showed better emulsifying and suspending properties than tragacanth and methylcellulose, respectively [6].

MATERIALS AND METHODS

Materials. The test organism was a new strain of *Bacillus licheniformis* NCIB 11634 initially isolated by Udeala from exudate of the raffia palm. The various biochemicals used and their respective sources include: sucrose, *n*-butanol (BDH, England); D-glucose, potassium di-hydrogen phosphate, di-sodium hydrogen phosphate, D-xylose, Folin-Ciocalteau reagent (Merck,

Darmstadt, F.R.G.); anhydrous bovine serum albumin (RHS, Hannover); sodium hydroxide, ethanol, acetone (May & Baker, England), and silica gel (Fluka, Buchs, Switzerland). The Czapek-Dox and semi-synthetic media used were prepared in our laboratory.

Media and cultures. The bacterial colony originally isolated on a plate of Czapek-Dox (CD) agar was purified and routinely subcultured on plates of the same medium [34]. The medium was comprised of: 0.3% $NaNO_3$, 0.1% K_2HPO_4, 0.06% $MgSO_4$, 0.05% KCl, and 0.001% $FeSO_4$ and 3.0% sucrose as the carbon source. Solid medium contained 1.5% agar. For the polysaccharide production, a routinely used semi-synthetic medium was adapted. It had the following composition: 0.6% $NaHPO_4$, 0.4% KH_2PO_4, 0.2% $(NH_4)_2SO_4$, 0.02% $MgSO_4$, 0.001% boric acid, 0.001% $CaCl_2$, 0.001% $FeSO_4$, and 5% sucrose as the carbon source. Preliminary experiments indicated that optimal growth and polysaccharide production occurred with the initial pH of the culture media being 7.0. Thus all media were adjusted to pH 7.0 and sterilized by autoclaving at 121 °C for 15 min. Liquid cultures were grown in 100-ml aliquots in 250-ml Erlenmeyer flasks on a reciprocated shaker at 28 ± 0.5 °C.

General inoculation procedure. Preceding each experiment the bacteria was subcultured three successive times in Czapek-Dox broth until sufficient inoculum was obtained. One-ml aliquots were used to inoculate 100 ml of the production medium in 250-ml Erlenmeyer flasks. The flasks were incubated at 28 ± 0.5 °C for 96 h at 125 rpm on a reciprocated water bath shaker.

Isolation and purification of polysaccharide. Cultures were harvested, pooled and diluted with three volumes of distilled water to reduce viscosity. The diluted culture was mechanically stirred for 30 min to detach any polysaccharide adhering to the walls of the bacterial cells. The culture was centrifuged at 25000 × *g* for 30 min to sediment the bacterial cells. The supernatant was concentrated to one-tenth of its volume under vacuum at room temperature and a pressure of 4–6 mmHg. Enough KCl was added to the concentrate to bring the salt concentration to 1.0%. The pH was adjusted to 4.0 using 1 N HCl [26]. This was then deproteinized twice by the Sevag method [30]. The polysaccharide was recovered by fractional precipitation using three volumes of 70% ethanol. Each fraction was washed twice with ethanol and once with acetone and finally dried under vacuum. The dried polysaccharide was stored in vials in a desiccator at room temperature and designated BLMG 135.

Biochemical tests. The biochemical tests for catalase and oxidase were carried out using the official procedures [3,9]. Other tests included Vogues Proskauer, methyl red, Indian ink, starch and gelatin hydrolysis, hydrogen sulfide production, and indole tests [9].

Experimental protocols. All investigations were carried out using the semi-synthetic medium of pH 7.0 except for pH, and temperature optimization studies in which the Czapek-Dox broth was used. For the latter, 100 ml of the broth culture at pH 7.0 were incubated at different temperature conditions and varied between 20–35 °C. Similarly, for the pH studies phosphate buffer was used as the diluent to vary the pH of the broth between 5.0 and 8.0, and incubated at 28 ± 0.5 °C for 96 h on a reciprocated water bath shaker.

The effects of carbon source, and electrolyte composition of the fermentation medium on growth and polymer biosynthesis were similarly tested. The composition of the medium was varied to contain 5% of either glucose, maltose, galactose, fructose, xylose or lactose as carbon source. Similarly, the electrolyte composition of the medium was varied respectively; phosphate ion (0–1.2%), nitrate ion (0–0.6%), and magnesium ion (0–0.4%). Yeast extract in concentrations between 0 and 0.15% as supplement of the fermentation medium was also evaluated. All experiments were conducted in replicates and incubated at 28 ± 0.5 °C for 96 h at 125 rpm on a reciprocated water bath shaker (Gallenkamp, England). At various time intervals, samples were withdrawn from each flask and measured for pH, reducing sugar level, viscosity, protein content, dry weight per ml of culture medium, and absorbance at 530 nm.

Analytical procedures. The polysaccharide content of the culture samples was determined at varying times by estimating the dry weight of the polysaccharide per ml of the bacterial suspension. The reducing sugar content was measured by adapting Summer and Somers' method [20,31]. Protein content of samples were estimated by method of Lowry et al. [15]. The relative number of bacteria was determined by measuring the absorbance at 530 nm using sterile medium as blank [1]. The colony forming unit per ml was determined by method of Miles and Misra [14].

Acid hydrolysis of the polysaccharide. A 1% solution (2.0 ml) of the polysaccharide in 4 N H_2SO_4 was hydrolysed by autoclaving at 121 °C for 1 h and neutralized with $BaCO_3$. This was centrifuged to separate the sulphate while the precipitate was washed twice with fresh distilled water. The combined supernatant was concentrated on a water bath to 0.5 ml and stored at 5 °C.

Paper chromatography. Samples of the hydrolysate were spotted on Whatman paper No. 1 (21 × 11 cm) and the chromatogram developed three times respectively in methanol : acetic acid : water (16 : 1 : 3) and *n*-butanol: acetic acid : water (4 : 1 : 5) using the one way ascending technique [29]. The sugar was detected by first spraying the dried chromatogram with aniline hydrogen phthalate reagent and then heated at 105 °C for 5 min.

Thin layer chromatography. Chromatograms were run on 0.25 mm silica gel plates impregnated with 0.3 M NaH_2PO_4 [22]. The one way ascending technique was used. The chromatogram was developed thrice consecutively in each of the solvent systems used: *n*-butanol : acetone : water (4 : 5 : 1) and *n*-butanol : 2-propanol : water (3 : 5 : 2). All sugar spots were visualized on dried plates using aniline hydrogen phthalate reagent.

Infrared analysis. Samples for infrared analysis were prepared by mixing approximately 1 mg of polysaccharide with 300 mg of KBr and pressing the mixture into pellet at 20 000 psi, for 2–3 min [8]. Infrared spectra of the pellets were obtained using model SP 1000 infrared spectrophotometer (Pye Unicam, England).

Osazone test. The composition of the polysaccharide was further confirmed by osazone test using the official method reported by Pasto and Johnson [23].

Viscometry. A suspended level viscometer (Size A, Gallenkamp, England) was used for the viscosity measurements.

RESULTS

Characteristics of the bacterial isolate

The bacteria were very motile Gram-variable rods with ellipsoidal spores. The culture was catalase and oxidase positive but negative for indole. The organism was positive for gelatin, starch hydrolysis, hydrogen sulphide production, Voges-Proskauer, and methyl red tests (Table 1). Negative stain with Indian ink procedure did not reveal any capsule, but a copious amount of loose slime was observed, and a similar result has been reported by Duguid [5].

On Czapek-Dox agar with sucrose as the carbon source, milky flat circular colonies of about 1–2 mm diameter having the odor of "aged palm wine" were observed. The broth culture revealed moderate but uniform growth with few floccules, a variation from the characteristic clear turbidity produced by the *Bacillus licheniformis* species [2].

TABLE 1

Biochemical characteristics of *Bacillus licheniformis* NCIB 11634

Biochemical characteristics	Results
Nitrate reductase	+
Catalase enzyme	+
Hydrogen sulfide production	+
Gelatine hydrolysis	+
Starch hydrolysis	+
Voges-Proskauer	+
Methyl red	+
Indole production	–

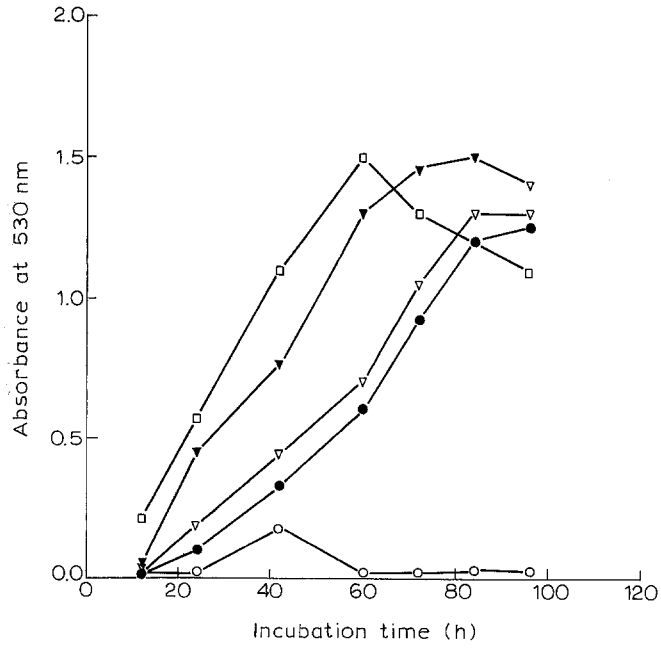

Fig. 1. Effect of temperature on growth of the bacteria. ○, 20 °C; ●, 25 °C; ▽, 28 °C; ▼, 30 °C; □, 35 °C.

Optimum conditions for growth and polysaccharide biosynthesis: effects of pH and temperature

Optimum conditions for growth and polysaccharide production were affected by temperature, pH, aeration, presence of agitation and the size of inoculum [25]. The temperature of 30 °C was found to be optimal for growth and polymer biosynthesis at neutral pH (Fig. 1).

Effect of medium composition

The nature of carbon source, and electrolyte composition of the fermentation medium showed some influence on growth and polymer biosynthesis by the bacteria. A production medium containing yeast extract as a supplement with sucrose as the carbon source also exhibited similar results. The effect of carbon source on growth and polymer production is expressed as dry weight of polysaccharide per ml of suspension obtained using a given carbon substrate (Table 2). The replacement of sucrose with either glucose or galactose resulted in good growth but no appreciable polysaccharide production, which is analogous to results reported by Sutherland and Williamson in a study of strains of bacterium with unusual characteristics for polysaccharide biosynthesis [34]. No growth was recorded when xylose or fructose was used as carbon source. Similarly, replacement of ammonia with nitrate ions, when sucrose was the carbon source, resulted

TABLE 2

Dry weight of the polysaccharide obtained using different carbon sources

Carbon source	Total dry weight (mg)[a]	Percentage dry weight (mg %)
Galactose	355	710
Sucrose	880	1760
Xylose	–	–
Lactose	198.5	397
Glucose	398.5	797
Fructose	–	–
Maltose	260	520

[a] mg polysaccharide recovered from 50 ml culture.

in good growth and polymer production. However, higher concentration of the nitrate ions (0.06% $NaNO_3$) was required for maximum growth and polymer production whereas medium composition of 0.2% $(NH_4)_2SO_4$ as the source of nitrogen gave maximum results (Fig. 2).

The effects of phosphate ions, as sodium or potassium salts, as well as magnesium ions on growth and polymer biosynthesis were similarly examined. Phosphate ions did not exert remarkable influence on growth but did enhance polysaccharide synthesis by the organism. Exclusion of

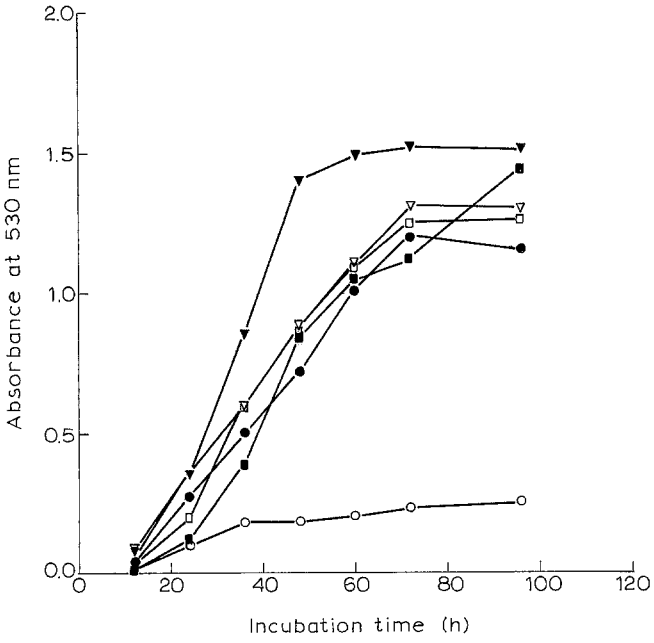

Fig. 2. Effect of ammonium sulfate concentration in culture media on growth of the bacteria. ○, 0%, ●, 0.1%; ▽, 0.15%; ▼, 0.2%; □, 0.3%; ■, 0.4%.

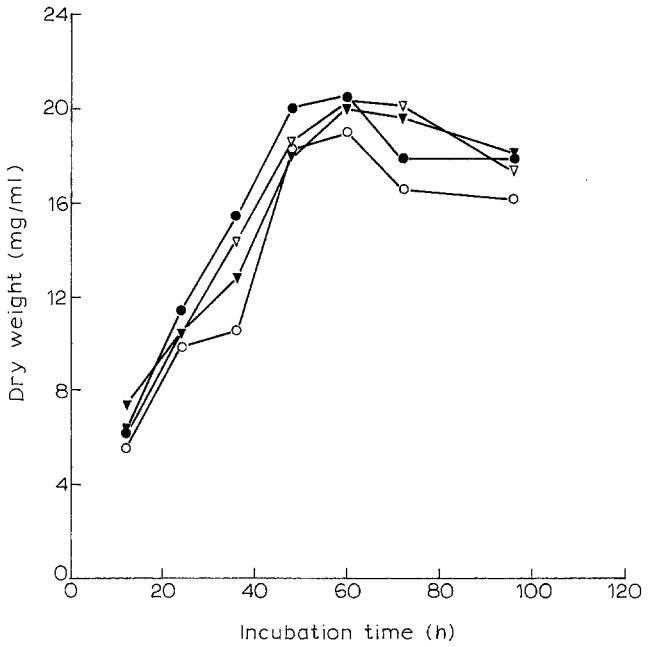

Fig. 3. Effect of magnesium sulfate concentration in culture media on polysaccharide biosynthesis. ○, 0%; ●, 0.01%; ▽, 0.02%; ▼, 0.04.

magnesium ions from the fermentation medium did not affect growth of the bacteria (Fig. 3). However, concentrations of Mg^{2+} ions above 0.01% did not favor polysaccharide production by the bacteria.

Yeast hydrolysate often contains growth factors such as vitamins and amino acids that may be favorable for growth of certain bacteria [25]. Yeast extract at a concentration as low as 0.05% exhibited considerable influence on growth and polymer production (Fig. 4).

Variations in the reducing sugar level of the fermentation medium also showed some influence on growth and polymer production. The reducing sugar level of the fermentation medium increased up to 48 h under optimum fermentation conditions. Thus, it appears that sucrose is broken down into glucose and fructose through enzymatic activities. These intermediate products are consequently utilized by the organism for its metabolism.

Fig. 5 shows the relationship between reducing sugar level, viscosity, and dry weight of the polysaccharide per ml of the culture medium containing 0.01% $MgSO_4$. The reducing sugar level was maximum after 36 h which corresponds with the mid-logarithmic phase preceding the time (48–60 h) when the polysaccharide production was maximum. Also, concurrent increase in viscosity and dry weight of the polysaccharide per ml of the culture medium were observed. The exclusion of boric acid from the fermentation medium did not affect microbial growth or polysaccharide production.

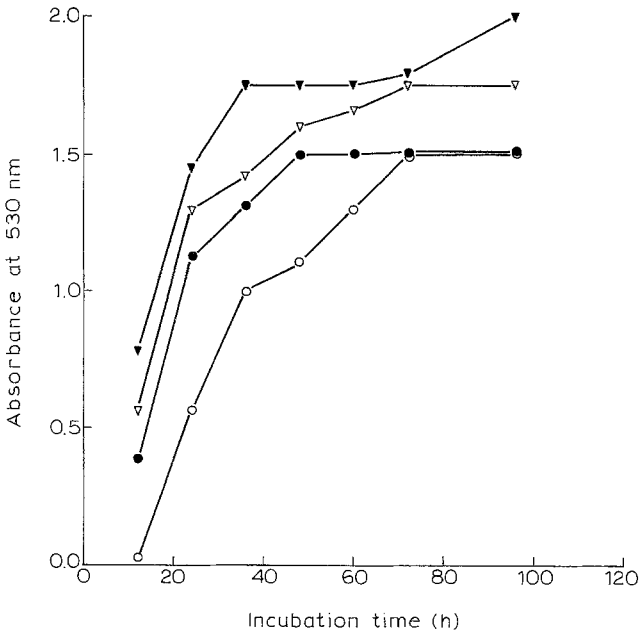

Fig. 4. Effect of yeast extract concentration as a supplement in the culture media on growth of the bacteria. ○, 0%; ●, 0.05%; ▽, 0.1%; ▼, 0.15%.

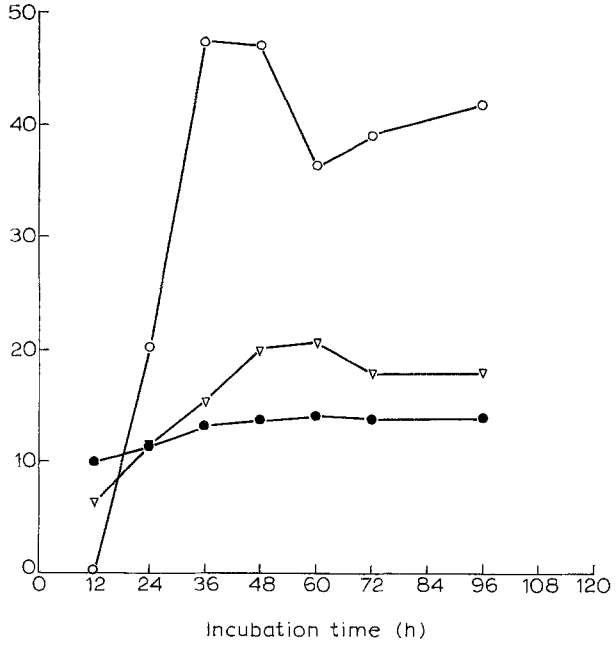

Fig. 5. Relationship between viscosity (○) (× 10 centistokes), dry weight of polysaccharide (▽) (mg/ml), and reducing sugar content (○) (mg/ml) of culture medium containing 0.01% magnesium sulfate.

Characterization of the polysaccharide

Paper chromatography of the polysaccharide hydrolysate revealed only one spot equidistant with glucose and galactose. Mucic acid test of the polysaccharide sample was negative indicating the possible absence of galactose. Thin layer chromatography of the hydrolysate showed three distinct spots which co-chromatographed with D-glucose, D-mannose and D-xylose. Thus, the polysaccharide was identified as a heteropolymer comprising D-glucose, D-mannose, and D-xylose. The composition of the polysaccharide was further confirmed by Osazone test and infrared spectral analysis. The infrared spectrum of the polysaccharide sample revealed the presence of the following functional groups: O-H at 3400 cm^{-1}, C-H at 1660 cm^{-1}, and C-C-O-C at 1287 cm^{-1}. These are characteristic functional groups found in a polysaccharide molecule.

DISCUSSION

The results described here demonstrated the polysaccharide producing potentials of the *Bacillus licheniformis* strain. The production of extracellular polysaccharide is facilitated by intrinsic biochemical characteristics of this strain of Bacillus. This includes its ability to metabolize specific carbon substrate for polymer production, i.e., the organism metabolizes sucrose into glucose and fructose. Preliminary investigations indicated that the bacteria did not produce polysaccharide when sucrose was substituted with glucose as the carbon source. Similarly, the bacteria did not grow in the fermentation medium with either fructose or xylose as the carbon source. Sutherland and Williamson [34], while screening strains of a bacterium with unusual characteristics for polymer biosynthesis, have demonstrated that the bacterium used glucose for growth whereas fructose played a major role in the polymer synthesis. Also, a species of bacteria that uses both glucose and galactose for polysaccharide production has been reported by Sutherland [33]. This author indicated that the organism uses one sugar as substrate for polysaccharide synthesis whereas the other provides carbon for the cell.

It is envisaged that a high yield of the polymer can be recorded when fructose is incorporated into the fermentation medium at the mid-logarithmic phase. This phase precedes the time for optimum polysaccharide production between the late exponential and stationary phases [24].

Examination of results obtained from these investigations has facilitated construction of a model biosynthetic pathway for the polysaccharide biosynthesis. Thus, the polysaccharide is synthesized inside the cell by the activities of exocellular enzymes and consequently excreted into the exterior. Markovitz and Dorfman [18]

have demonstrated that the enzymes involved in the synthesis of exopolysaccharide are associated with the membrane underlying the cell wall as particulate complexes. The sequence of reactions for transfer of the monosaccharide from the sugar nucleotide to the carrier lipid isoprenoid phosphate has been established.

Though the optimum temperature range for polysaccharide production has been established to be between 30–35 °C, a temperature of 28 °C also gave good results at pH 7. Since the latter temperature is close to the ambient temperature of 28 ± 1 °C it became convenient to carry out the remaining studies at a temperature of 28 ± 0.5 °C, for temperature regulation under the working laboratory conditions was feasible. This again falls within the optimal temperature range of 28–50 °C specified for the *Bacillus* species for growth and metabolism [2].

Thus, at a temperature of 28 ± 0.5 °C, using optimum production medium maintained at pH 7, a good yield of the polysaccharide has been obtained. More than 10% of the substrate is converted into polysaccharide. Ammonium ions could be substituted for nitrate ions as the source of nitrogen required for microbial growth and metabolism. A minimum amount of Mg^{2+} ions, not less than 0.01% w/v is required for cellular activity by the organism. Yeast extract as a supplement of the fermentation medium could reduce the time required for polysaccharide biosynthesis. A notable set-back, however, is that pigmentation is enhanced in the presence of yeast extract. This may affect the color of the polysaccharide produced. However, a cheap and efficient procedure for the recovery of the polysaccharide from the fermentation broth is desired. The low recovery of the polysaccharide with the Sevag technique appeared to be due mainly to its loss into the emulsion layer from which a considerable amount of polysaccharide can be recovered [34].

The isolated microbial polysaccharide contains D-glucose, D-mannose and D-xylose and it is the first report of the polysaccharide of this composition. Xanthan gum, produced by *Xanthamonas phaseoli* is reported to consist of D-glucose, D-mannose, and D-glucuronic acid, and also some trace sugars [12–14,21]. However, the composition of the latter has been challenged since xanthan-like gum does not contain either rhamnose or xylose [12].

This strain of *Bacillus licheniformis* also produced an antibiotic agent as indicated in the preliminary studies. The cell-free filtrate of a 96 h culture of the organism showed appreciable antimicrobial activity against *E. coli*, *Staphylococcus aureus*, *Bacillus subtilis* and *Klebsiella* species. Therefore, it is pertinent to further investigate the antibiotic production by this organism. Also, preliminary experiments indicated that the polysaccharide gum shows some promise for use as an emulsifying and suspending agent. It may be necessary, as the next step, to further characterize the polysaccharide and subsequently improve on its recovery techniques from the fermentation broth.

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