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Extracellular Polysaccharide Produced from Glucose by *Cryptococcus laurentii* var. *flavescens* NRRL Y-1401: Chemical and Physical Characterization*

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Synopsis

The polysaccharide that occurs freely dispersed in culture fluids in which the nonpathogen, *Cryptococcus laurentii* var. *flavescens* NRRL Y-1401, has been grown on the carbohydrate substrate, glucose, has been isolated, purified, and characterized. This macromolecular polysaccharide is composed of D-mannose, D-xylose, and D-glucuronic acid (as the potassium salt) and a small proportion of *O*-acetyl groups. Factors favorable to practical application of this hydrocolloid are its adaptability to large-scale production, its stability in storage, and the properties of its homogeneous dispersions. Dispersions in water or aqueous alcohol are highly viscous and tend to soft gelation. Dispersions in water show plastic rheological characteristics and thixotropy with rapid regain of viscosity after shear. Moderate decreases in viscosity result from the presence of electrolytes or from heating, and only small differences in viscosity occur in the pH range 4–11. Indications have been presented that variation in properties of the polysaccharide product may depend upon fermentation conditions.

INTRODUCTION

The extracellular polysaccharide from the nonpathogen, *Cryptococcus laurentii* var. *flavescens* NRRL Y-1401, is the third of a series of hydrophillic polysaccharides produced at this laboratory through microbial action on a medium containing starch-derived sugars and considered suitable for practical use. Unlike our previous yeast product, a phosphomannan,[1] this is a heteropolysaccharide composed of D-mannose, D-xylose, and D-glucuronic acid (as the potassium salt). Present also are about 7% *O*-acetyl groups. Unlike the heteropolysaccharide from the bacterium *Xanthomonas campestris* NRRL B-1459,[2] this yeast product contains a pentose sugar, its gum is dense and cohesive, and its dispersions are thixotropic and show rapid regain of viscosity after shear.

The Y-1401 polysaccharide that is the subject of this investigation occurs freely dispersed in the culture fluids, apparently unattached to the yeast cells. Simple physical procedures suffice for its recovery. However, con-

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siderable amounts of other polysaccharide materials are either bound to the cells or retained within them and can be removed only by chemical treatment or cell disruption.[3] From our point of view, the accumulation of these bound polysaccharides entails an unprofitable diversion of the glucose substrate. However, from the medical standpoint, capsular and endocellular polysaccharides from yeasts closely related to *C. laurentii* var. *flavescens* have positive significance.[4]

EXPERIMENTAL

Production and Laboratory Purification of Native Polysaccharide Y-1401

This polysaccharide is produced by culturing strain NRRL Y-1401 on an aerated medium containing commercial glucose, yeast autolyzate, and a trace of manganous sulfate for 5 days at 25°C. For the purification and isolation work reported here, we started with culture fluid from a 12-liter fermentation for which the temperature was 21–23°C. and the initial and final pH values were 5.0 and 3.0.[5] The culture fluid appeared homogeneous, showed short flow characteristics, and had a viscosity of about 11000 cpoise.

The purification procedure described resulted in a product suitable for compositional analysis; more practical procedures employed by others for large-scale operations[6] have been similar to those for polysaccharide B-1459.[7]

Culture fluid (2500 ml.) was diluted with an equal volume of water, and potassium chloride, ethanol, and chloroform were added with mechanical stirring to give concentrations of 1% (w/v), 35% (v/v), and 1% (v/v), respectively, based on total water volume. The water, salt, and alcohol thinned the culture somewhat and the chloroform inactivated the cells. After the mixture stood 1 hr., pH was adjusted to about 6, and viscosity was reduced to about 100 cpoise by adding an aqueous solution containing ethanol (35% concentration, v/v) and potassium chloride (1% concentration, w/water volume). The resultant solution, totaling about 9000 ml., was passed slowly four successive times through a continuous Sharples supercentrifuge to remove the voluminous cell residue. Then ethanol was stirred into the supercentrifugate until precipitation of the polysaccharide was complete at about 60% ethanol concentration. The precipitate settled rapidly to a sticky, cohesive mass. Most of the supernatant was decanted; the remainder was separated from the precipitated gum by a brief period of centrifugation at about 2000 rpm.

The dense, rubbery residue dispersed very slowly in water, especially if it had been compacted by centrifugation. Dense gelatinous masses were avoided by gradual addition of water to achieve very viscous, homogeneous dispersions before further dilution was made. By an alternate procedure, loosely compacted polysaccharide gum was dispersed more readily by vigorous mechanical stirring in 60.5% ethanol than in water. Further dilu-

tion of this viscous dispersion was accomplished easily by adding water and stirring.

Two successive reprecipitations of the polysaccharide from aqueous solution served to free it from entrained and adhering water-soluble constituents from the medium. To an approximately 0.5% aqueous dispersion of the polysaccharide was added ethanol to give a concentration of about 50% (v/v) and potassium chloride (as a filtered 30% aqueous solution) to give a concentration of 1% (based on water volume). Further increase of ethanol concentration to 60.2% (v/v) precipitated the polysaccharide.

Part of the twice-reprecipitated product was dialyzed in Visking tubing against distilled water for 54 hr. at 25°C. Initially this solution had polysaccharide concentration 0.25%, pH 7.5, and viscosity 270 cpoise; the presence of toluene insured asepsis. The retentate[8] was adjusted from pH 4.6 to 6.1 with potassium hydroxide solution and filtered through sintered glass to remove extraneous matter. The filtrate was concentrated *in vacuo* and lyophilized.

Another part of the product was dehydrated in methanol. Twice-reprecipitated gum (containing about 12 g. polysaccharide on a dry weight basis) was transferred from a 250-ml. centrifuge bottle into 1 liter of vigorously stirred 85% aqueous methanol. The precipitate was finely divided, soft, and dry-looking, in contrast to coarse, tough particles which would have resulted if absolute methanol had been used. After washing with absolute methanol, the precipitate was dried *in vacuo* in the presence of anhydrous calcium chloride.

The total yield recovered, based on the weight of glucose ($C_6H_{12}O_6$) in the medium, was about 13.5%. Examination of the initial precipitation supernatant showed that polysaccharide removal had been complete. Similarly, it was shown that supernatants from purification and dehydration contained essentially no polysaccharide.

Analyses for the dialyzed, lyophilized product were: chloride, 0.085%; sulfated ash (corrected for potassium equivalent to the chloride content), 8.24%; and nitrogen, 0.13%. Corresponding values for the methanol-dehydrated products were: 0.05, 9.28, and 0.06%. A value of 9.27% for sulfated ash was calculated from the uronic acid content based on carbazole analysis and assuming potassium to be the only cation present.

Polysaccharide Materials

All the materials used in our work were products of experimental fermentations. For observations on composition and chemical characterization, we used the products having fermentation and purification histories described in the preceding section.

For observations involving measurement of viscosity and factors influencing viscosity, we used products isolated and purified on a pilot-plant scale.[6] One of these, designated preparation A, was produced under fermentation conditions established through preliminary investigations in which yield was the major criterion.[5] A second preparation, designated

B, was produced under fermentation conditions that differed from those for preparation A in some details of medium composition, in temperature, and in initial and final pH of the culture fluid.[6] Pertinent data on these preparations are shown in Table I.

TABLE I
Production and Analytical Data on Polysaccharide Y-1401, Preparations A and B

	Preparation	
	A	B
Fermentation temperature, °C.	24.5–25.5	28–29
Initial and final pH of culture fluid	5.0–3.0	6.7–4.8
Final viscosity of culture fluid, cpoise[a]	7300	1550
Yield recovered, %[a,b]	16	9
Ash (sulfated), %	10.60	8.60
Nitrogen, %	0.15	0.23
Phosphorus, %	0.38	0.12
Acetyl, %	6.0	5.0
Glucuronic acid, %	18.5	20.0

[a] Data of Rogovin.[6]
[b] Expressed as percentage of anhydrous glucose supplied.

The significant differences found in some physical properties of these preparations illustrate variations that might arise from apparently minor differences in composition or in fermentation-dependent factors related to constitution or molecular size.

Analytical Methods

Dry polysaccharide products, as obtained by methanol dehydration or lyophilization techniques, were equilibrated with atmospheric moisture under constant conditions of temperature (20°C.) and relative humidity (50%). The products were stored and all samples for analyses were weighed under these same conditions. For determination of moisture, approximately 0.3-g. samples were held *in vacuo* in an unheated Abderhalden drier in the presence of phosphorus pentoxide desiccant for about 16 hr. and then heated at 100°C. and 2 mm. pressure to constant weight (about 5 hr.). Moisture contents were in the range 14–16%. All other analytical data are calculated to the dry basis.

Uronic acid was determined by a modification of the carbazole method,[9] and acetyl, by an hydroxamate procedure.[10] Acetyl was identified as described previously.[11] To decrease viscosity and thus improve pipetting accuracy, polysaccharide solutions used in these analyses either were autoclaved (20 min. at 15 lb. psig) or were made to about pH 3 with dilute sulfuric acid and then neutralized.

Constituent sugars were determined by qualitative and quantitative paper chromatographic analysis. Polysaccharide (0.5% concentration) was hydrolyzed in a sealed tube in 2*N* hydrochloric acid for 2 hr. at 100°C.

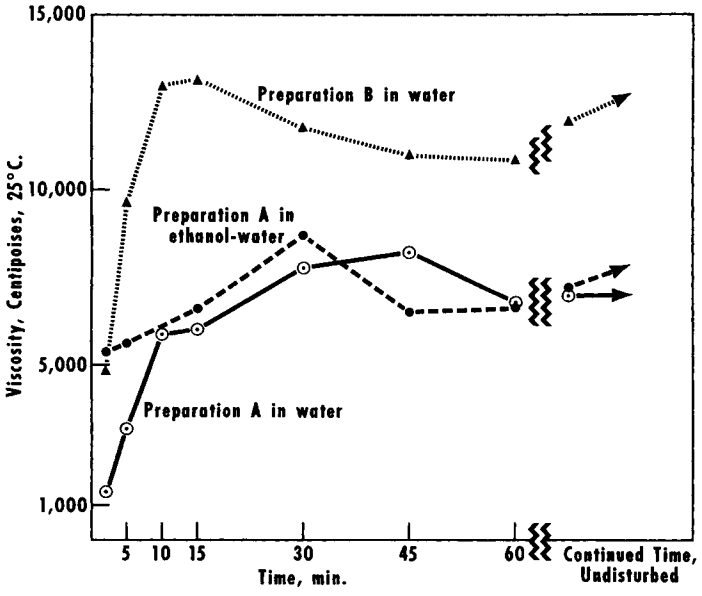

Fig. 1. Rate of solvation and dispersion of polysaccharide Y-1401 in water and in ethanol-water (1:9 v/v). Polysaccharide concentration, 1%.

Chloride was removed with silver carbonate, silver ions were precipitated by hydrogen sulfide, and the clarified solution was concentrated *in vacuo* (30°C. bath temperature). Solvent systems for paper chromatography were the upper phase of ethyl acetate, acetic acid, and water (3:1:3 v/v)[12] and *n*-butyl alcohol, pyridine, and water (3:2:1.5 v/v).[13] Spray reagents used were ammoniacal silver nitrate,[14] and *o*-aminobiphenyl phosphate in glacial acetic acid.[15] Whatman No. 1 paper was used. The phenol-sulfuric acid assay[16] was used for determination of total carbohydrate (when calibrated on purified polysaccharide) and of eluted sugars. For eluted acidic oligosaccharides, the arbitrarily chosen calibration standard was the barium salt of 2-*O*-(β-D-glucopyranosyluronic acid)-D-mannose.[11]

To determine neutral equivalent values, an aqueous dispersion was decationized on a column of Dowex 50 × 4 resin, 20–50 mesh, and then titrated potentiometrically with 0.1*N* potassium hydroxide solution (inflection point, pH 7.5). Viscosity determined the concentration of dispersions suitable for decationization. The polysaccharide concentration normally used was 0.05%, but if the dispersion had been autoclaved (20 min.), 0.125% could be used. The neutral equivalent values obtained were 1280 and 1230, respectively.

Viscosity measurements were made with Brookfield viscometers types LVF and LVT at 25°C. and 30 rpm unless otherwise indicated. All values are based on equilibrium readings.

Homogeneous dispersions free from agglomerated masses usually were prepared for viscosity measurements and other physical tests by alter-

nately layering part of the water and finely divided polysaccharide solid into a beaker and allowing the mixture to stand overnight at 4°C. Individual particles hydrated uniformly, swelled, and dispersed slowly. The remaining water then was added, and the mixture was stirred gently until homogeneous and allowed to stand 15–20 min. before viscosity measurements were made. For measurement of the rate of solvation (Fig. 1), all the water was added at once to the polysaccharide or polysaccharide plus ethanol, and vigorous mechanical stirring was applied continuously except momentarily when viscosity measurements were being made.

Dispersions for viscosity–concentration curves were prepared by serial dilution, and the effect of salt on viscosity was determined by incremental addition of small amounts of solid salt to homogeneous dispersions of the polysaccharide. For observation of effect of heating, a polysaccharide dispersion was heated rapidly to a selected temperature, cooled rapidly to 25°C. for measurement of viscosity, and then heated to the next higher temperature. Changes in volume by evaporation were prevented. Gentle mechanical stirring was used to achieve uniform mixing after adding salt and when heating and cooling. The dispersions were left undisturbed 5–10 min. before measuring viscosity.

RESULTS AND DISCUSSION

Composition and Chemical Constitution

The extracellular polysaccharide from strain Y-1401, when produced and purified as described, is composed of D-mannose, D-xylose, and D-glucuronic acid(as the potassium salt) in the approximate molar ratio of 4:1:1. When produced under different conditions by others for structural and metabolic studies,[17,18] both an acidic polysaccharide and a neutral polysaccharide were obtained, and the proportion of constituents in the acidic polysaccharide differed somewhat from ours. Our product has a higher proportion of D-mannose and a higher percentage of D-glucuronic acid (totaling about 18.5%); in addition, it contains *O*-acetyl substituents (about 7%) not reported previously. Traces of D-glucose and D-galactose, observed in hydrolyzates of some of our preparations, appeared to come from endocellular or capsular material.

The weight of polysaccharide per uronic acid group, calculated from carbazole analysis, was 940 in contrast to the neutral equivalent value, 1230. This discrepancy is believed to result from lactone structure that develops during decationization and is not hydrolyzed in determination of the neutral equivalent. Lactonization is believed to occur also during dialysis and is not reversed when the retentate is neutralized. This hypothesis correlates with the lower sulfated ash content of a dialyzed product (8.24%), as compared with the value calculated (9.27%) from the uronic acid content based on carbazole analysis.

The presence of α-linkages in the polysaccharide is indicated by the

specific optical rotation, $[\alpha]_D^{25}$ + 38°, in either water or 0.1*M* potassium chloride solution.

The rapid liberation of D-xylose upon acid hydrolysis indicates that the D-xylose units probably are present as side chains. It has been reported previously that both the D-xylose and D-glucuronic acid units occur as nonreducing endgroups.[17]

When treated with sodium metaperiodate at room temperature, the polysaccharide undergoes extensive overoxidation. However, at 4°C. the oxidation appears to be controlled, and half of the sugar units are oxidized with liberation of formic acid.

The polysaccharide is deacetylated readily by dilute alkali at pH 11.5. The resultant product, as precipitated from neutral solution by alcohol, is fibrous. The deacetylated polysaccharide shows essentially the same viscosity as the native, but its viscosity is decreased to a greater extent in the presence of salt.

Stability and Solubility

Polysaccharide Y-1401, as dehydrated by methanol, is a white powder that absorbs about 14% moisture when equilibrated under conditions of 20°C. and 50% R.H. A humidified sample has been stored 2 yr. under these conditions without any decrease in viscosity or acetyl content. Another sample containing 5–10% moisture has been stored at 20–25°C. for 5 yr. without detectable change. Aqueous solutions of the polysaccharide have remained unchanged in homogeneity and viscosity when held under aseptic conditions for many months at 4°C.

Solid particles solvate rapidly in water to form a cohesive gum that disperses slowly. The rate of solvation of the dry powder (preparation A) in water is increased significantly by the presence of about 10% ethanol, and the rate of dispersion is increased by mechanical stirring. As shown in Figure 1, after vigorous mechanical stirring for 2 min., solvation and dispersion in ethanol–water was about fourfold greater than in water alone, and complete solvation and dispersion appeared to have been reached within 30 min. in ethanol-water, as compared with about 45 min. in water alone. Under the same conditions of stirring, preparation B appeared to have solvated completely in 10–15 min. in water alone, to give a viscosity more than 50% greater than the highest shown by A. In each case, continued stirring resulted in a decrease in viscosity followed in two of the cases by an increase upon standing undisturbed (Fig. 1).

Unless stirred or heated, aqueous dispersions of methanol-dehydrated products such as preparation B have a grainy, discontinuous aspect due to small gel masses. Such a gel state, when encountered in purification procedures as a dense, cohesive mass, can be dispersed advantageously in about 60% ethanol and then diluted as desired by addition of water. The solvation and dispersion characteristics described might be related to combined effects of the number and distribution of *O*-acetyl, uronic acid, and

xylose side-chain groups. All these are factors which might vary with fermentation conditions.

When essentially free of yeast cells, aqueous dispersions show only slight opalescence. The pH is in the range 6.0–7.0 for concentrations of 0.1–1.5%.

Factors Affecting Viscosity

As shown in Figure 2, the viscosity is higher at all concentrations observed than that for our bacterial polysaccharide B-1459, as well as for two high-grade plant gums of commerce, gum guar and sodium alginate. Polysaccharide Y-1401 preparation B showed about 25–50% greater viscosity than A throughout the curve.

The dispersions have plastic rheological characteristics (Fig. 3), and plots of rate of shear versus torque show yield values.[19] Dispersions are thixotropic,[19] and regain of viscosity after shear is rapid, especially by preparation B. Thus a 1% dispersion of B which decreased 25% in viscosity by vigorous mechnical agitation, regained its original viscosity by standing undisturbed 10 min. Dispersions having 1.5–2.0% concentration are gels that thicken perceptibly when allowed to stand after stirring, especially those of preparation B. Such gels have sufficient structure not to adhere to glass.

The effect of salt addition on the viscosity of aqueous dispersions of polysaccharide Y-1401 (preparation A) is influenced by polysaccharide concentration, as seen in Figure 4. Preparation B showed a comparable

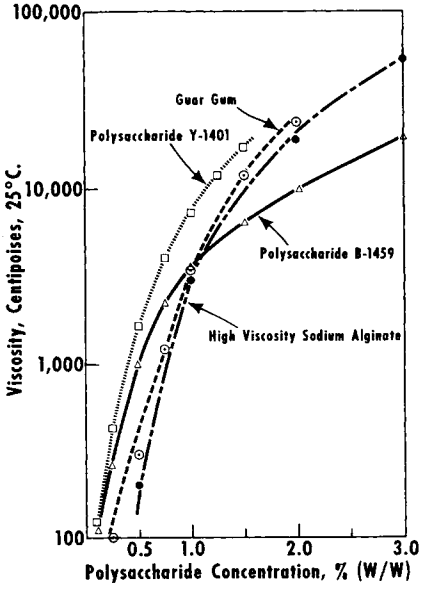

Fig. 2. Viscosity-concentration relationships of polysaccharide Y-1401 (preparation A) compared with those of other representative hydrocolloids.

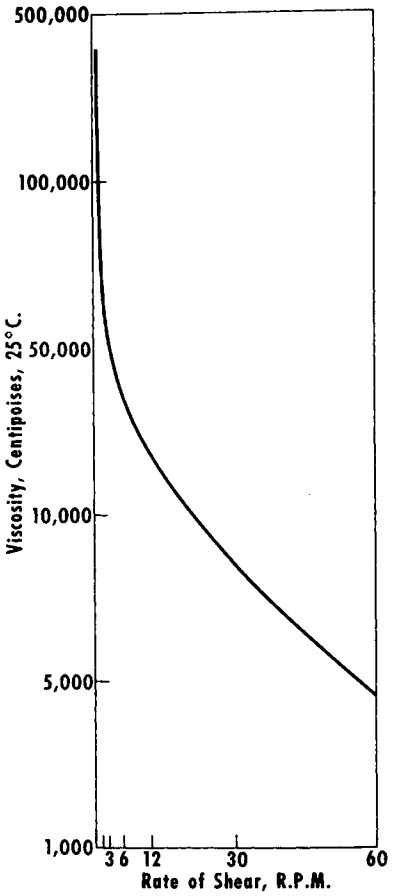

Fig. 3. Viscosity vs. rate of shear for polysaccharide Y-1401 (preparation A). Concentration of aqueous dispersion, 1%.

gradational effect of polysaccharide concentration, and comparable viscosity changes in 0.1% dispersion, but differed in that the viscosity of its more concentrated dispersions decreased upon addition of salt. Thus, for 1% concentration of preparation B, the decrease in viscosity observed upon direct (not incremental) addition of potassium chloride was about 15% for 0.1% salt and 25% for 1.0% salt. Calcium chloride and borax gave essentially the same results as potassium chloride. The gradational concentration effect of both polysaccharide preparations emphasizes the influence of colloidal structure on the behavior of these dispersions. However, the reason for the partial suppression of electroviscous response of preparation A when at higher concentrations is not evident. The approximately 1% greater direct ash content of A (Table I) seems insufficient to cause the differences observed, although a critical effect of some specific ion is not excluded.

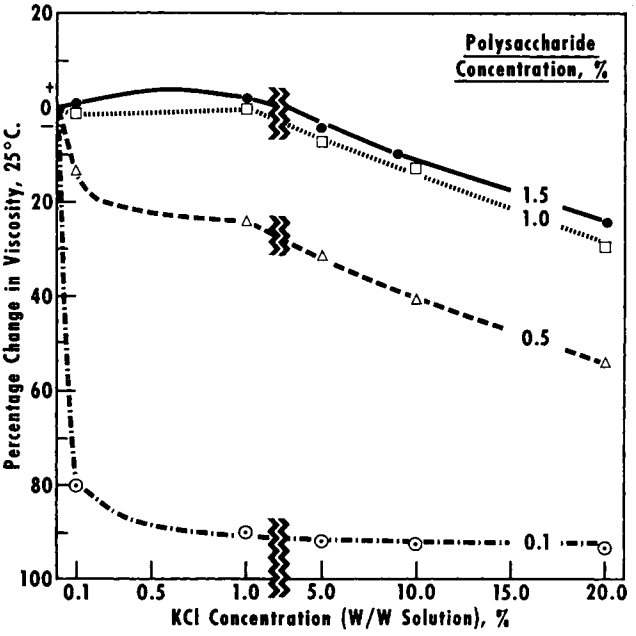

Fig. 4. Percentage change in viscosity of aqueous dispersions of polysaccharide Y-1401 (preparation A) upon addition of KCl.

For comparison with other polyanionic polysaccharides, the percentage changes in viscosity upon adding potassium chloride (1%) to 1% dispersions under our established conditions are as follows: our bacterial polysaccharide B-1459, +55; high viscosity sodium alginate, −30; gum tragacanth, −50; and gum karaya, −90.

Solubility does not decrease at acidic or basic pH values. Viscosity of 1% dispersions is essentially constant between pH of about 5 and 8, and changes little between about pH 5 and 11.5. The pH of unbuffered dispersions adjusted to initial pH values in the range of 7–12.2 decreased slowly with time (Table II), probably due to saponification of *O*-acetyl

TABLE II

Relation of Initial pH of Unbuffered Dispersions of Polysaccharide Y-1401 (Preparation A), 0.5% Concentration, to Changes in pH and Viscosity with Time at 25°C.[a]

Sample number	pH			Viscosity, cpoise		
	Initial	24 hr.	72 hr.	Initial	24 hr.	72 hr.
1	2.9	2.9	2.9	840	840	700
2	4.2	4.2	4.2	1520	1480	1400
3	7.0	6.5	6.3	1780	1680	1680
4	10.9	7.7	6.7	1500	1720	1740
5	11.5	7.9	6.7	1400	1740	1740
6	12.2	11.9	11.9	1200	900	740

[a] Throughout this test, dispersions were protected from oxygen and carbon dioxide.

TABLE III

Effect of Heating with and without KCl on Viscosity of Polysaccharides Y-1401 (Preparation A), B-1459, and Some Commercial Gums[a]

Heated to temperature, °C.	Change in viscosity (25°C.), % of original							
	Polysaccharide Y-1401		Polysaccharide B-1459		Gum Karaya		Gum tragacanth	
	No KCl	1% KCl	No KCl	1% KCl	No KCl	1% KCl	No KCl	1% KCl
40	−7.5	−9	−3	0				
50	−13	−15	−11	0				
60	−21	−24	−33	0				
70	−27	−30	−50	+1				
80	−31	−34	−56	+2.5				
90	−33	−34.5	−57	+8				
99	−35	−35.5	−62	+8				
120	−43.5[b]	−44[b]	−75[c]	−2[c]	−95[c]	−90[c]	−75[c]	−53[c]

[a] Uniform aqueous dispersions, 1% concentration.

[b] Held at 120° at natural pH for 10 min.

[c] Held at 120° at natural pH for 30 min.

groups. Corresponding viscosity values increased somewhat with time when the initial pH was basic through 11.5 but decreased when it was acidic. The decrease in viscosity with time at pH 4.2 and lower suggests that hydrolysis of some chain linkages or of the acid-sensitive xylose side-chain units occurs under these conditions.

Unbuffered aqueous solutions of polysaccharide Y-1401 decrease moderately in viscosity (measured at 25°C.) after heating, preparation B less than A. Decrease in viscosity with heat is only slightly greater in the presence of potassium chloride. Decreases are partially reversible with time at 25°C. A complete series of values for preparation A are shown in Table III and are compared with corresponding observations on polysaccharide B-1459 and on gums karaya and tragacanth.

Film Formation

Dispersions of polysaccharide Y-1401, which had been stirred and heated to about 80°C. and then cooled to 25°C., were cast on plates and dried in accordance with our usual procedure for making experimental, unsupported films.[20] Preliminary tests on the transparent films indicated good tensile strength and elongation values, especially when glycerol plasticizer was present.

We are pleased to acknowledge our indebtedness to Mr. M. C. Cadmus for experimental culture fluids, to Mr. S. P. Rogovin for providing us pilot-plant-grade polysaccharide, and to Mrs. Clara McGrew for the inorganic microanalyses.

Mention of trade names should not be construed as a recommendation or endorsement by the Department over those not mentioned.

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Résumé

Le polysaccharide qui apparait dispersé librement dans les mileux liquides de cultur dans lesquels on a cultivé le *Cryptococcus laurentii* non-pathogène, variété *flavescens* NRRL Y-1401, sur un substrat d'hydrate de carbone, le glucose, a été isolé, purifié et caractérisé. Ce polysaccharide macromoléculaire est composé de mannose-D, xylose-D et c'acide glucuronique-D (sous forme de son sel potassique) et d'une faible teneur en groupements *O*-acétyl. Les facteurs, qui militent en faveur des applications pratiques de cette hydrocolloïde, sont une adaptibilité à être produit sur grande échelle, sa stabilité à la conservation, et les propriétés de ses dispersions homogènes. Les dispersions dans l'eau ou dans l'alcool aqueux sont hautement visqueuses et ont tendance à former des gels légers. Les dispersions dans l'eau révèlent des caractéristiques rhéologiques de plasticité et une thixotropie avec réaugmentation rapide de la viscosité après cisaillement. La présence d'électrolytes ou le chauffage provoquent une diminution modérée de la viscosité et seules de petites variations de viscosités ont lieu dans la gamme de pH allant de 4 à 11. On signale l'influence que peuvent provoquer des conditions de fermentation sur les variations de propriétés du polysaccharide.

Zusammenfassung

Das in Kulturflüssigkeiten, in denen der nicht pathogene *Cryptococcus laurentii* var. *flavescens* NRRL Y-1401 auf Kohlenhydratsubstrat, Glucose, gezüchtet Worden war, freidispergierte Polysaccharid wurde isoliert, gereinigt und charakterisiert. Dieses makromolekulare Polysaccharid besteht aus D-Manose, D-Xylose und D-Glucuronsäure (als Kalisalz) und einer geringen Menge an *O*-Acetylgruppen. Was dieses Hydrokolloid zur praktischen Verwendung geeignet macht, ist seine Eignung zur Produktion in grossem Massstab, seine Stabilität bei Lagerung und die Eigenschaften seiner homogenen Dispersion. Dispersionen in Wasser oder wässrigem Alkohol sind hochviskos und neigen zur Bildung weicher Gele. Dispersionen in Wasser zeigen plastische rheologische Charakteristika und Thixotropie mit schneller Wiederherstellung der Viskosität nach Scherung. Die Anwesenheit von Elektrolyten oder Erhitzen verursacht eine geringe Abnahme der Viskosität; im pH-Bereich 4–11 treten nur geringe Viskositätsunterschiede auf. Es wurde Befunde vorgelegt, dass die Änderung der Eigenschaften des Polysaccharidproduktes von den Fermentierungsbedingungen abhängen kann.

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