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Flocculant and Chemical Properties of a Polysaccharide from *Pullularia pullulans*

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An extracellular polysaccharide, PP-floc, was synthesized from glucose by *Pullularia pullulans* (or *Aureobasidium pullulans*) in a pilot plant batch fermentor containing 175 liters of culture medium. At 58 h of fermentation, the concentration of PP-floc was 1.03 g/100 ml, giving a 25.8% conversion of initial glucose to polysaccharide. The flocculant activity of the culture medium increased during the fermentation process and reached its maximum at 50 h of culture age. Less PP-floc (0.33 lb/ton of slimes [approximately 149.7 g/0.907 t]) was required to give the same flocculant activity as a synthetic polymer of acrylamide, Separan NP-10 (0.5 lb/ton of slimes [approximately 226.8 g/0.907 t]), at all temperatures from 25 to 100 C. The degree of inactivation of PP-floc and Separan NP-10 at elevated temperatures was almost identical, and they were completely inactivated at about the same temperature (80 C). PP-floc also gave better compaction of slimes than Separan NP-10 at all temperatures tested. PP-floc was soluble in water and its specific optical rotation was $[\alpha]_D^{25} + 194°$ in water (c, 0.4). PP-floc contained 83.3% carbohydrate, 3.2% protein, and 8.1% water. Glucose was found to be the principal sugar monomer with traces (>5%) of galactose and mannose present. Structural studies on the fractions of purified polysaccharide by methylation and by periodate oxidation techniques prove that PP-floc is linear and composed of α-(1 → 4) and α-(1 → 6) glucopyranosyl units in the approximate ratio of 2:1. The action of pullulanase on crude PP-floc suggested the ordered arrangement of two consecutive α-(1 → 4)-linked glucopyranosyl units flanked by α-(1 → 6)-linked glucopyranose residues.

Pullulan, an extracellular polysaccharide produced by the yeast-like fungus *Pullularia pullulans* (or *Aureobasidium pullulans*), has been studied earlier by Bernier (2). The chemical structures of the polysaccharides sythesized from various carbohydrate sources such as glucose (1, 14, 15), sucrose (3), maltose (4), and xylose (5) have been also investigated. Recently, the utilization of single mono- and disaccharides by *P. pullulans* (6) and the effects of pH and nitrogen limitation (7) on the elaboration of polysaccharide have also been conducted in laboratory-scale experiments.

The extracellular polysaccharide from *P. pullulans* has been found to be an efficient flocculating agent (J. E. Zajic, U.S. patent no. 3320136, 1967). Flocculating agents find use in a large number of applications in industry, such as in the flocculation of clay slimes from aqueous solutions resulting from the beneficiation of uranium potash or aluminium and from other hydrometallurgical processes.

The production of PP-floc (an extracellular polysaccharide used as flocculating agent) by *Pullularia pullalans* in pilot plant-scale equipment using a medium containing glucose is investigated. Studies also included evaluation of the flocculating activity and give some data on the chemical structure of the polymer. Because of its flocculating properties, the polymer is termed PP-floc herein.

MATERIALS AND METHODS

Culture maintenance and inoculum. *P. pullulans* was isolated from soil on a mineral salts medium with methane gas used as a source of energy (J. E. Zajic, U.S. patent no. 3320136, 1967). The cultivation medium in all experiments consisted of 4.4% dextrose monohydrate, 0.5% K_2HPO_4, 0.1% NaCl, 0.02% $MgSO_4 \cdot 7H_2O$, 0.06% $(NH_4)_2SO_4$, 0.04% yeast extract (BBL) 0.0002% thiamine hydrochloride, 0.004% $MnSO_4 \cdot H_2O$, and 0.01% Antifoam (UCON). Percentages are expressed on a weight/volume basis. The pH was adjusted to 5.4 before autoclaving. Incubation was at 28 C.

The culture of *P. pullulans* was maintained on agar slants and transferred weekly. Inocula for shake flasks, carboys, or fermentor experiments were prepared by inoculating broth cultures from slants and building up equal volumes of inocula to give 2.0% (vol/vol). The age of inocula for all experiments was 48 h.

Batch production of Pullularia pullulans. Batch fermentations were conducted in the pilot plant fermentor containing 175 liters of culture medium. The composition of the culture medium is the same as that described above. The medium ingredients were added to water in a presteamed pilot plant fermentor, and the volume of the fermentor was adjusted to 152 liters. The culture medium was adjusted to pH 5.4 before sterilization with concentrated HCl. The medium was sterilized under a pressure of 15 psi (approximately 10,546 k/m^2) at 121 C for 30 min by direct steam injection. The volume of the medium after sterilization and cooling was 175 liters. After cooling of the medium, 3.5 liters of inoculum (2.0% of the nominal volume) of *P. pullulans* was added to the fermentor. Aeration, agitation, tank pressure, and temperature conditions were set at 6.28 cfm (approximately 0.0030 m^3/s), 100 rpm, 10 psi (approximately 7,031 k/m^2), and 25 to 28 C, respectively, immediately after inoculation. These conditions were controlled at the initial set points until the fermentation was 58-h old. From 58 to 69 h the agitation was stopped, and the temperature was controlled to 21 C. The contents of the tank measured 160 liters after 69 h of fermentation. This loss was attributed to sampling and to evaporation.

During the course of the fermentation, samples were taken at time intervals of 3 or 4 h and analyzed for the following: pH, viscosity, percent packed cell volume (PCV), polysaccharide concentration, flocculant activity, and residual glucose. The viscosity of the culture medium was measured with a Brookfield model HAT viscometer with spindle no. 1 at 1.0 rpm and 25 C. PCV was determined in calibrated conical tubes by measuring the volume of packed cells from a specific volume of broth after centrifugation at 1,500 rpm for 5 min. Polysaccharide was determined by first removing cells by centrifugation and then precipitating the polysaccharide from the broth, with one volume of methanol, to one volume of broth. The methanol was stripped off under vacuum, and the polymer was dried at 50 C for 12 h. The anthrone reagent test for total carbohydrate (13) was used to determine the residual glucose in the broth sample after the polysaccharide was removed from the broth by methanol precipitation. Methanol did not interfere with the anthrone test.

Measurement of flocculant activity. The flocculating activity determined during the progress of the fermentation was measured by the 50% flocculation settling time for 2.5 ml of a 1:40 dilution of the culture medium for 1% potash cold slimes (clay) at room temperature.

Studies on the flocculant activity of PP-floc at elevated temperatures were conducted on 0.1% potash clay slimes in saturated potash brines. The aqueous flocculant solutions at 0.1% concentration were prepared. Brines and slimes were prepared at ambient temperature. The desired amount of clay slime for testing at each temperature was added to a 1-liter graduated cylinder and placed in a constant-temperature bath. After the temperature of the slime had equilibrated, 300 ml of the brine and slime was syphoned off. An appropriate amount of flocculant was added to the 300 ml, and the mixture was returned to the test cylinder in three 100-ml increments. After each addition of flocculant, the cylinder was stirred by vertical stroking. Temperatures tested were 25, 40, 50, 60, 70, 80, 90, and 100 C. All flocculant tests were conducted in duplicate on successive days, alternating the items of test equipment used for evaluating PP-floc and Separan NP-10 to eliminate variation attributed to equipment construction. Separan NP-10 is a synthetic polymer of acrylamide produced by Dow Chemical Co., which was specifically selected for its ability to flocculate clay slimes. Activities of PP-floc and Separan NP-10 were determined daily at ambient temperatures, and the amount of PP-floc equivalent to known amounts of Separan NP-10 was calculated. On the average, only 0.33 lb (approximately 149.7 g) of PP-floc per ton (approximately 0.907 t) of slime was needed to equal 0.5 lb (approximately 226.8 g) of Separan NP-10 in flocculant activity.

Clay compaction was determined by measuring the volume of compacted clay present in the cylinders at the end of 24 h. The percent increase compaction or volume reduction of compacted clay given by PP-floc as compared with Separan NP-10 is calculated by

$$\begin{pmatrix}\%\text{ increase}\\ \text{compaction}\end{pmatrix} = \frac{\begin{pmatrix}\text{compaction}\\ \text{with NP-10}\end{pmatrix} - \begin{pmatrix}\text{compaction}\\ \text{with PP-floc}\end{pmatrix}}{(\text{compaction with NP-10})} \times 100$$

Chemical properties of PP-floc. The specific optical rotation of PP-floc samples was determined on a Bendix ETL-NPL automatic polarimeter type 143A.

For studies on the chemical composition of PP-floc, samples were dried in vacuo in an Abderhalden dryer after being refluxed in water for 6 h. Protein was determined by the Miller modification of the Folin-Lowry method (12) with bovine plasma albumin used as a reference standard. Total carbohydrate was determined by the phenol-sulfuric acid procedure with glucose as the reference standard (8). The moisture content was determined by drying PP-floc in vacuo at 90 C.

To determine the carbohydrate monomers of PP-floc, paper chromatography was performed on Whatman no. 1 paper by the descending method with the following solvent systems: (A) *n*-butanol-acetic acid-water (2:1:1), (B) pyridine-ethyl acetate-water (2:5:7). Sugars were observed by spraying the chromatograms with ammoniacal silver nitrate (equal volumes of concentrated ammonium hydroxide and 2 N aqueous silver nitrate) or *p*-anisidine-trichloroacetate (0.4 g of *p*-anisidine and 2.0 g of trichloroacetic acid in 100 ml of water) followed by heating the paper at 120 C until spots appeared. PP-floc (0.10 g) was hydrolyzed in 1 N sulfuric acid (4 ml) at 100 C in a sealed tube for 5 h. The solution was neutralized with barium carbonate and filtered, and the filtrate was concentrated to dryness in vacuo. The residue was dissolved in 50% ethanol (4 ml), filtered, and deion-

ized on a column of Amberlite IR 120 (H^+) resin. The column eluate was concentrated to a syrup. Paper chromatography of the syrupy residue on solvents A and B was performed.

Fractionation of PP-floc. The fractionation of PP-floc was conducted in the following manner. (i) Complex formation with Fehling's solution: to a 0.5% aqueous solution of PP-floc (7 ml) was added 10 ml each of Fehling's A and B. No insoluble complex formed. (ii) Ethanol fractionation: PP-floc (2 g) was dissolved in water (250 ml). Ethanol (150 ml of 95%) was added gradually with stirring. The polysaccharide (A) precipitated sharply with the adding of ethanol, and was removed by centrifugation. Ethanol (150 ml of 95%) was added to the supernatant fluid with the consequent formation of a negligible amount of precipitate. Polysaccharide A was again dissolved in water and reprecipitated with ethanol in the same manner. (iii) Fractionation with cetyl pyridinium chloride: polysaccharide A, isolated from the ethanol fractionation, was dissolved in 300 ml of water. A 1-ml sample of this solution was treated with a 0.25 N solution of cetyl pyridinium chloride. No insoluble complex was formed. Chloroform (60 ml), *n*-butanol (6 ml), and 0.25 N cetyl pyridinium chloride (1 ml) were added to the aqueous solution of A, and the mixture was stirred and shaken for 30 min. The layers were allowed to separate, and the upper, aqueous layer was decanted. The grey, turbid emulsion which was formed at the interface and the clear chloroform layer were separated. The aqueous layer was again treated with chloroform, butanol, and cetyl pyridinium chloride. Upon separation of the aqueous layer after this second treatment, ethanol was added to precipitate the polysaccharide. The purified polysaccharide was washed three times with ethanol and two times with petroleum ether and dried in vacuo to give fraction AI (yield 1.3 g, 65%); $[\alpha]_D^{27}$ + 219° in water (c, 0.64). Ethanol was added to the combined chloroform layers containing the interface emulsion. The resulting precipitate (AII) was washed with ethanol and petroleum ether and dried to give a black, horny mass (yield 0.3 g, 15%). A portion of AI (60 mg) was hydrolyzed with 1 N sulfuric acid for 7 h at 100 C. the solution was neutralized with barium carbonate, filtered, and concentrated. Glucose was the only sugar detected in the hydrolysate by paper chromatography on solvent B. The syrupy hydrolysate was passed through a column of Duolite A-4 (OH^-) resin, and the column was washed thoroughly with water and then eluted with 2 N sodium hydroxide (2 ml). The column was washed with water, and the combined alkaline eluate and washings were passed through a column of Amberlite IR 120 (H^+). The eluate was concentrated to dryness, and the residue was dissolved in a few drops of water and subjected to paper chromatography on solvent A. No uronic acids were detected. (iv) Fractionation with chloroform-butanol-cetyl pyridinium chloride: the fractionation previously found to be effective on a small scale was repeated on a larger scale. PP-floc (9.19 g) was dissolved in water (1.5 liters). Chloroform (300 ml), *n*-butanol (30 ml), and 0.25 N cetyl pyridinium chloride solution (5 ml) were added and the mixture was stirred for 15 min. The layers were allowed to separate, and the aqueous layer was decanted again treated with chloroform, *n*-butanol, and cetyl pyridinium. After the second treatment the polysaccharide was precipitated with ethanol and dried, as described previously, to give fraction BI (yield ~4.1 g, ~45%). Hydrolysis of a portion of BI and paper chromatographic analysis of the hydrolysate showed glucose to be the major sugar, with a trace of mannose also present. Ethanol was added to the chloroform layer from the isolation of BI to precipitate 1.5 g of brown-colored polysaccharide (BII) which was dried with solvent in the usual way (yield 1.5 g, 16%), $[\alpha]_D^{27}$ + 179° in water (c, 0.7). (v) Fractionation with chloroform-pentyl alcohol: fraction BI (4.0 g) was dissolved in water (11) and shaken with chloroform (200 ml) and pentyl alcohol (20 ml) for 30 min. The chloroform layer was separated and retreated in the same manner. Addition of ethanol to the aqueous layer precipitated the polysaccharide (fraction BIII) which was dried by solvent exchange in the usual manner (yield 3.7 g, 93%), $[\alpha]_D^{27}$ + 212° in water (c, 0.5). Fraction BIII on hydrolysis yielded glucose on paper chromatograms. From the chloroform layer left from the isolation of BIII, there was obtained 0.25 g (2.7%) of fraction BIV, $[\alpha]_D^{27}$ + 161° in water.

Acetylation of polysaccharide. Attempts to acetylate PP-floc directly by using pyridine and acetic anhydride in formamide resulted in an incompletely acetylated product which was difficult to solubilize in organic solvents. Because of solubility problems, this particular procedure was abandoned. Fraction BIII (1 g) was dissolved in dimethylformamide (75 ml). Pyridine (40 ml) and acetic anhydride (25 ml) were added, and the reaction mixture was stirred overnight. The reaction mixture was poured into cold water, and the precipitated acetate was collected (centrifuge), washed with water, and dried. The dried acetate was dissolved in chloroform, and petroleum ether was added slowly with stirring. The acetate precipitated sharply over a very narrow range of petroleum ether concentration. The precipitated acetate was washed with petroleum ether and dried, yield 1.3 g, $[\alpha]_D^{25}$ + 180° in chloroform (c, 1.1).

Methylation study on purified polysaccharide. The purified polysaccharide is methylated in two stages, first by the methyl sulfate-alkali procedure of Haworth (9) to provide solubility in dimethylformamide and then by the Kuhn methylation (10) with silver oxide and methyl iodide.

Polysaccharide acetate (1.2 g) was dissolved in acetone (40 ml) and stirred vigorously while 40% potassium hydroxide (100 ml) and methyl sulfate (50 ml) were added slowly over a period of 1 h at 40 to 50 C. The reaction mixture was heated at 80 C for 1 h, cooled, and neutralized with acetic acid after which the solution was dialyzed.

The dialyzed solution was concentrated to a small volume (50 ml), and potassium hydroxide (20 g) was added. Methylation was again effected by adding methyl sulfate (25 ml) slowly over a period of 0.5 h at 40 to 60 C with vigorous stirring of the reaction mixture. After this, 40% potassium hydroxide (100 ml) and methyl sulfate (50 ml) were added over a 1-h

period. The methylated product which agglomerated was filtered and dissolved in 1,4-dioxane (30 ml) and remethylated for 2 h by the Haworth procedure by 40% potassium hydroxide (200 ml) and methyl sulfate (100 ml). At the end of the methylation, the reaction mixture was heated to 80 C, cooled, and neutralized with acetic acid, and the methylated polysaccharide was extracted with chloroform. Petroleum ether was added to the chloroform extract, precipitating the product which was washed with petroleum ether and dried (yield 0.53 g). The methylated product contained 41.07% OCH_3.

The incompletely methylated product (0.50 g) was dissolved in dimethylformamide (13 ml) and treated with methyl iodide (1.51 ml) and freshly prepared silver oxide (1.3 g) with stirring in the dark for 18 h. Chloroform was added, the mixture was filtered, and the filtrate was concentrated.

The methylation was repeated two times in the same manner except that the dimethylformamide was omitted. At the end of the third Kuhn methylation, chloroform was added, the reaction was filtered, and the filtrate was concentrated first on the water pump and then in vacuo at 2 mm Hg and 50 C to remove all dimethylformamide. The residue was dissolved in chloroform, and the methylated product was precipitated with petroleum ether. The product was washed with petroleum ether and dried; yield, 0.470 g.

The methylated product (0.40 g) was dissolved in methyl iodide (10 ml) and stirred with silver oxide (2 g added in four equal portions) for 4 days at 30 C. The reaction mixture was filtered, and the product was precipitated with petroleum ether. The methylated polysaccharide had 45.01% OCH_3.

Methylated fraction BIII (55.2 mg) was dissolved in cold 72% sulfuric acid (0.2 ml) and kept at 5 C for 1 h. Cold water was added to a volume of 5 ml, and the solution was refluxed for 6 h. The solution was neutralized with barium carbonate and was filtered; the filtrate was concentrated to a syrup with a yield of 56.7 mg. Paper chromatographic analysis of the hydrolysate with solvent C (butanone-water-azeotrope) revealed a component indistinguishable from 2,3,4-; 2,3,6-; and 3,4,6-tri-*o*-methyl-D-glucoses. No component corresponding to 2,4,6-tri-*o*-methyl-D-glucose or 2,3,4,6-tetra-*o*-methyl-D-glucose was visible. Only a trace of a di-*o*-methyl-glucose was visible, and the amount of this suggested that it had arisen through incomplete methylation or demethylations.

Paper electrophoresis of the hydrolysate in 0.1 M borate buffer at 600 V for 1.5 h revealed that 3,4,6-tri-*o*-methyl-D-glucose was not present in detectable quantity.

Characterization and quantitative analysis of tri-o-methyl-D-glucoses by periodate oxidation. The hydrolysate of the methylated BIII (56.7 mg) was dissolved in water (10 ml). A 5-ml sample of this solution was treated with sodium borohydride (25 mg) for 16 h and was neutralized with 25% acetic acid (1 ml). After 2 h, 0.12 M periodic acid (4 ml) was added, and the reaction was kept at 5 C for 24 h. Samples (1 ml) were transferred to flasks containing 10 ml of water. Lead acetate solution (1 ml of 38%) was added, the volume was adjusted to 25 ml, and the mixture was filtered. Samples (1 ml) were analyzed with chromotropic acid. The amount of formaldehyde liberated corresponded to 8.46 mg of 2,3,4-tri-*o*-methyl-D-glucose or 30% of the mixture.

The remainder of the periodate oxidation reaction mixture was neutralized with barium hydroxide, filtered, deionized on Amberlite IR 120 (H^+) resin, and concentrated. The dry residue was dissolved in methanol and again concentrated. Paper chromatography of the residue on solvent D *n*-butanol-ethanol-water (2:1:1) showed two components corresponding to 2,3-di-*o*-methyl-threose (derived from 2,3,6-tri-*o*-methyl-D-glucose) and 2,3,4-tri-*o*-methyl-xylose (derived from 2,3,4-tri-*o*-methyl-D-glucose).

Periodate oxidation of PP-floc fractions. The periodate oxidation of PP-floc fractions was conducted in the following manner. (i) Determination of periodate consumption: fractions of PP-floc (50–65 mg) were dissolved in water (25 ml) and treated with 0.2 N sodium periodate (25 ml) at +5 C in the dark. Samples (2 ml) were removed at timed intervals for titration. Standard iodine solution (0.05275 N) was used. A blank control was carried throughout. (ii) Determination of formic acid: a sample (10 ml) of the periodate oxidation reaction mixture of PP-floc fractions was titrated with 0.00972 N sodium hydroxide. (iii) Analysis of the polyalcohol derived from PP-floc fraction by periodate oxidation: the solution containing the periodate-oxidized polysaccharide (polyaldehyde) was dialyzed and then treated with sodium borohydride (0.20 g) for 6 h. Excess borohydride was decomposed with acetic acid, and the solution was again dialyzed. The dialyzed solution was concentrated, and the polyalcohol was hydrolyzed in 0.2 N sulfuric acid (10 ml) at 25 C for 12 h. Barium carbonate was added to neutralize the acid, the mixture was filtered, and the filtrate was concentrated.

Paper chromatography of the residue on solvent B showed the presence of glycerol and erythritol only. The remainder of the hydrolysate of the polyalcohol was treated with 1 N sulfuric acid at 100 C for 3 h. The solution was neutralized as previously described and concentrated. Chromatography again revealed that glycerol and erythritol were the only components. No glucose was detected. Glycerol and erythritol were separated by chromatography of a portion of the hydrolysate on Whatman no. 3 paper in solvent B. The zones of the paper containing the glycerol and erythritol were eluted with water (50 ml for glycerol and 100 ml for erythritol), and samples were analyzed by the method of Lambert and Neish (11).

Fractions (2 ml) of the glycerol and of the erythritol eluates were treated with 0.2 N sodium periodate (2 ml) for 15 min. A saturated solution of lead acetate (1 ml) and water (5 ml) was added and the mixtures were centrifuged. Samples (1 ml) of the supernatant fluids were treated with chromotropic acid reagent (10 ml), centrifuged, and heated in a boiling-water bath for 30 min. Absorbances were read at 570 nm, and the mole ratio of glycerol and erythritol was calculated.

RESULTS AND DISCUSSION

Batch production of PP-floc. The results of pilot plant batch fermentation are shown in Fig.

1. The pH decreased from 5.0 after inoculation to below 3.0 at the 12-h sample, which was the pattern observed in the shake flasks and laboratory-scale batch fermentation. The percent PCV increased towards its maximal value after 15 h and leveled off afterwards. The percent PCV can be directly related to the change in pH of the culture medium. The yield of polysaccharide per 100 ml of broth was 1.03 g at 58 h of fermentation, giving a 25.8% conversion of glucose to polysaccharide (based on 4 g glucose per 100 ml of broth in the original media). The lag time between cell growth (expressed by percent PCV) and the production of polysaccharide is approximately 10 to 12 h, which is the same as for the shake flasks and laboratory-scale batch fermentation. The viscosity of the culture medium increased to very high value (~2,700 centipoises at 58 h) and appeared to be a reflection of the increase in polysaccharide produced. A concentration of 1.47% glucose remained at the end of 48 h. The increase in glucose above the initial level of 4.0 g/100 ml from 3 to 6 h of fermentation age was unexpected. The phenomenon might be due to the hydrolysis of polysaccharides present in the inoculum prior to exponential growth and polysaccharide synthesis.

Flocculant activity of PP-floc. The evolution of the flocculant activity of the culture medium is shown in Fig. 1. After 50 h of fermentation, 2.5 ml of a 1:40 dilution of the fermentation broth gave a flocculation settling time for 1% potash cold slimes of 2 min and 43 s. The linear rate of fall of the upper clay-liquid interface was determined at each temperature (Fig. 2). As the temperature increased from 25 to 100 C, both PP-floc and Separan NP-10 lost their effectiveness as flocculants. This decrease in flocculant activity with increased temperature from 40 to 70 C was nearly linear for both flocculants. The inactivation curves were almost identical. At 25 C the linear settling rates of the clay upon addition of PP-floc and Sepa-

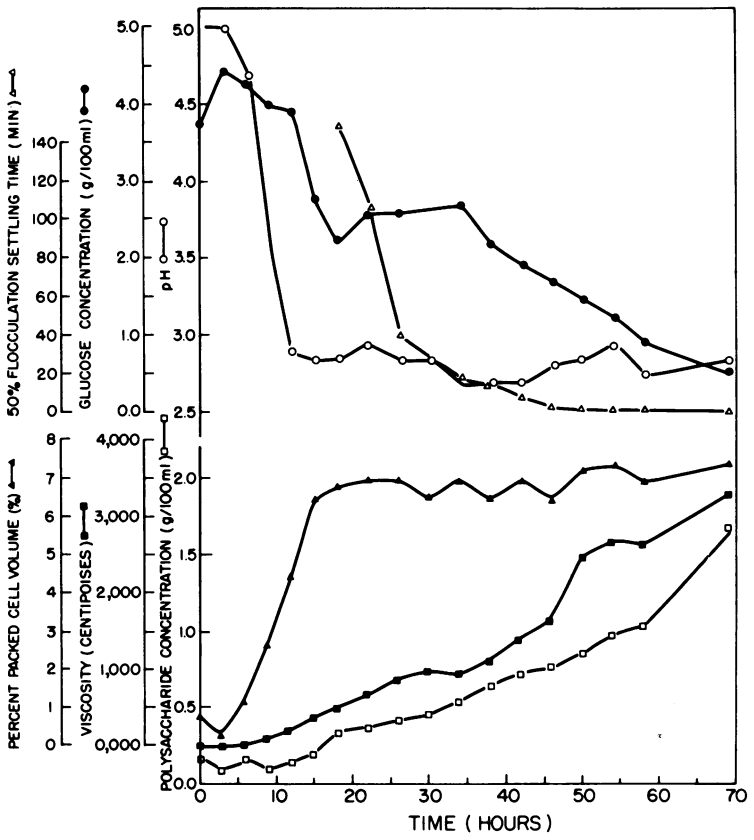

FIG. 1. *Progress of batch production of PP-floc by Pullularia pullulans on glucose medium. Symbols:* ○, *extracellular pH;* ■, *viscosity (centipoises);* △, *50% flocculation settling time (minutes);* ▲, *percent packed cell volume (%);* □, *polysaccharide concentration (g/100 ml);* ●, *residual glucose concentration (g/100 ml).*

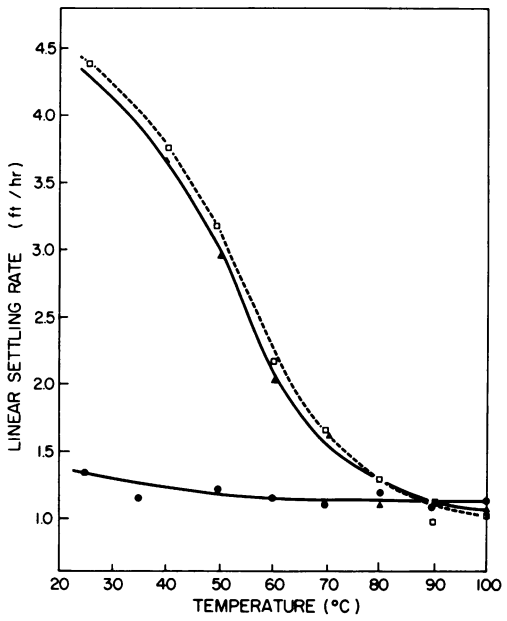

FIG. 2. *Flocculant activity of PP-floc and equivalent amounts of Separan NP-10 at elevated temperatures on 0.7% potash clay slimes in brine. Symbols: ▲, PP-floc; □, Separan NP-10; ●, no flocculant (control).*

ran NP-10 were, respectively, 4.08 and 4.38 ft/h (approximately 1.24 and 1.33 m/h); at 60 C the corresponding rates decreased to 2.02 and 1.84 ft/h (approximately 0.61 and 0.56 m/h). At 80 C and above, the rate of fall of clay through the cylinders containing flocculant approximated the controls.

PP-floc appears to possess a special property which increases the compaction of clay slime. At all temperatures tested, greater compaction of flocculated clay was noted in cylinders containing PP-floc (Fig. 3). This increase was about 12% at 25 C, reached a maximum of 30% at 50 C, and then fell off again to an increase of 10% or less above 90 C. The compaction characteristic was far superior to Separan.

Physical properties of PP-floc. PP-floc was soluble in water. The specific optical rotation was $[\alpha]_D^{25}$ + 194° in water (c, 0.4).

Chemistry of PP-floc. PP-floc analyzed chemically as a crude polysaccharide contained carbohydrate (83.3%), protein (3.2%), and water (8.1%). Glucose was found to be the principal sugar monomer, with traces only (<5%) of galactose and mannose present also. No uronic acids could be detected.

The polysaccharide was purified before structural studies were initiated. Fractional precipitation of the polysaccharide from aqueous solution with ethanol yielded only one fraction. After two reprecipitations with ethanol, the product was subjected to a Sevag fractionation wherein the aqueous solution of the polysaccharide was shaken with a mixture of chloroform, *n*-butanol, and cetyl pyridinium chloride. The purified polysaccharide (AI) was precipitated from the aqueous layer with ethanol and recovered in 65% yield. AI gave glucose exclusively on hydrolysis and, on direct analysis of the polymer by the phenol-sulfuric acid method, 100% carbohydrate was indicated. A small amount of protein (0.76%), however, still appeared to be present. A significant quantity of polysaccharide, along with the majority of the protein, was held in the interface between the chloroform and aqueous layers, and this fraction (AII) was recovered in 15% yield. This polysaccharide material was not readily recovered from the interface, even by repeated washing with water, suggesting the possibility of a chemical association between the polysaccharide and either the protein or possibly a lipid.

The Sevag fractionation process was reproducible; however, when applied on a larger scale, two treatments with the chloroform mixture

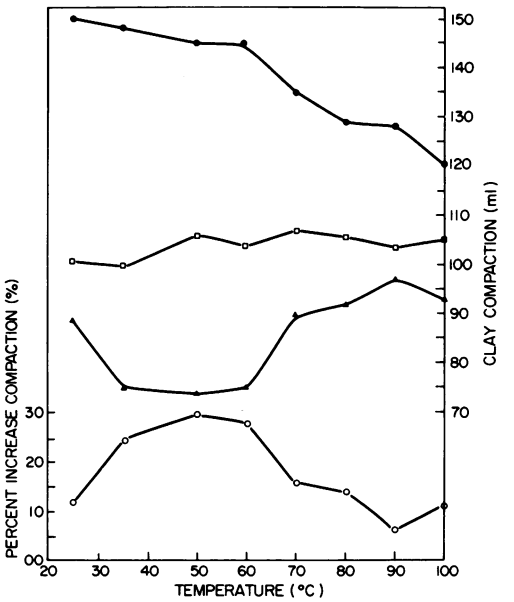

FIG. 3. *Effect of elevated temperatures upon compaction of 0.7% potash clay slimes in brine flocculated with PP-floc and equivalent amounts of Separan NP-10. Symbols: ▲, PP-floc; □, Separan NP-10; ●, no flocculant (control); O, percent increase compaction.*

were required to give the purified polysaccharide (BIII). Polysaccharide-protein material (BII and BIV) was isolated from the interface both times. The composition and physical properties of the various fractions are summarized in Table 1.

It is apparent from Table 1 that fractions AI and BIII are comparable and represent a polymer composed of glucose monomer units glycosidically linked by α linkages as deduced from the high, positive specific rotation.

Fraction BIII was converted into the acetylated polysaccharide, $[\alpha]_D^{25}$ + 180° in chloroform. Attempts to further fractionate this acetate by fractional solvent precipitation were unsuccessful, suggesting that the polysaccharide is relatively homogeneous.

Structural studies by methylation and by periodate oxidation techniques prove that the polysaccharide is linear and composed of α-1 → 4 and α-1 → 6 glucopyranosyl units in the approximate ratio of 2:1.

Thus, methylation of the polysaccharide by the Haworth technique afforded the methylated polymer (OCH_3; actual, 45.01%; calculated, 45.54%), which on complete acid hydrolysis afforded 2,3,6-tri-*o*-methyl-D-glucose (arising from 1 → 4-linked glucose residues) and 2,3,4-tri-*o*-methyl-D-glucose (arising from 1 → 6-linked glucose residues). The occurrence of 1 → 2 and 1 → 3 linkages in the polysaccharide are excluded since neither 3,4,6-tri-*o*-methyl-D-glucose or 2,4,6-tri-*o*-methyl-D-glucose were detected in the hydrolysate of the methylated polysaccharide. Similarly, the absence of a significant proportion of di-*o*-methyl- and 2,3,4,6-tetra-*o*-methyl-D-glucoses establishes that the polysaccharide is linear and of relatively high molecular weight.

The molar proportions of 2,3,4- and 2,3,6-tri-*o*-methyl-D-glucose in the hydrolysate were determined by a method involving reduction, periodate oxidation, and colorimetric analysis of the formaldehyde liberated from carbon six of the 2,3,4-tri-*o*-methyl-D-glucose (under these conditions the 2,3,6-isomer does not give formaldehyde). The mole ratio of 2,3,6- to 2,3,4-tri-*o*-methyl-D-glucose and hence the ratio of 1 → 4 to 1 → 6 linkages was found to be 2.30 to 1.

Analyses of these two sugars are difficult in that the sugars are not readily separated and that 2,3,4-tri-*o*-methyl-D-glucose readily forms the 1,6-anhydro derivative. Since this 1,6-anhydro derivative does not yield formaldehyde under the conditions employed for quantitation of 2,3,4-tri-*o*-methyl-D-glucose, it is possible that the apparent content of 1 → 4 linkages, determined by this method, is somewhat high.

Periodate ion oxidatively cleaves the carbon-carbon bond of 1,2-glycol systems which renders it applicable to carbohydrate systems. A 1,2-diol reduces 1 mol of periodate and each carbon is oxidized to an aldehyde; whereas a 1,2,3-triol reduces 2 mol of periodate, with the liberation of 1 mol of formic acid derived from the central carbon and the transformation of the two terminal carbons into aldehydes. It is apparent then that a 4-substitued glucose residue will consume 1 mol of periodate, whereas a 6-substituted glucose residue will consume 2 mol of periodate and liberate 1 mol of formic acid.

Fractions AI and BIII were each oxidized with sodium periodate; the reaction was complete in 24 h and unchanged after 5 days (Table 2).

These data correspond to a repeating unit in the polysaccharide consisting of two 1 → 4-linked glucopyranose residues and one 1 → 6-linked glucopyranose residue (theoretical: 0.33 mol of formic acid reduced per glucose residue and 1.33 mol of periodate reduced per glucose residue).

If the polysaccharide is subjected to the

TABLE 1. *Composition and physical properties of PP-floc fractions*

Fraction	Yield (%)	$[\alpha]_D$[a]	Sugar composition	Carbohydrate[b] (%)	Protein (%)
Original		+194	Glucose, mannose (trace), galactose (trace)	83.3	3.2
AI	65	+219	Glucose	100.0	0.76
AII	15				
BI	~45		Glucose, mannose (trace)		
BII	16	+179		80.3	>1.68
BIII	93	+212	Glucose	97.6	0.82
BIV	2.7	+161		70.7	>1.56

[a] Degree of angle.
[b] Accuracy was ± 2%.

TABLE 2. *Periodate oxidation of PP-floc fractions*

Fraction	IO_4 reduction (mol/glucose residue)	Formic acid (mol/glucose)	Molar ratio (erythritol/glycerol)
AI	1.32	0.32	1.93
BIII	1.33	0.32	
BII	1.36	0.33	

sequence of reactions—periodate oxidation, sodium borohydride reduction, and complete acid hydrolysis—then a 1 → 4-linked glucose residue is revealed by the generation of erythritol, whereas a 1 → 6-linked glucose residue gives rise to glycerol. These two polyhydric alcohols are readily separated by paper chromatography; their quantitative determination therefore provides an accurate estimation of the ratio of 1 → 4- to 1 → 6-linked glucose residues.

When this sequence of reactions was applied to AI, erythritol and glycerol were released in the ratio 1.93:1, in good agreement with a repeating unit of two 1 → 4- and one 1 → 6-linked glucose residues.

When fraction BII was subjected to periodate oxidation, 1.36 mol of periodate was consumed per glucose residue and 0.33 mol of formic acid was released, suggesting that this polymer is chemically similar to the major polysaccharide AI (or BIII). However, since this fraction contains only 81% total carbohydrate, it is likely that these data reflect a polymer with a greater proportion of 1 → 6 linkages than was found in AI or BIII.

The methods employed in this study do not yield information on the question of whether the polysaccharide has a regular repeating sequence of these two linkages or whether the arrangement is random. Evidence was provided, however, by the action of pullulanase on crude PP-floc (results obtained by Dexter French of Iowa State University). Pullulanase specifically cleaves α-(1 → 6) glucopyranosidic linkages and has no action on α-(1 → 4) bonds.

When this enzyme was applied to crude PP-floc, maltotriose was the main product formed. On the basis of all the evidence available, it is possible to postulate the following structure for the major polysaccharide in PP-floc in which there is an ordered arrangement of two consecutive α-(1 → 4)-linked glucopyranosyl units flanked by α-(1 → 6)-linked residues: → $_4G_1 \rightarrow {}_6G_1 \rightarrow {}_4G_1 \rightarrow {}_4G_1 \rightarrow {}_6G_1 \rightarrow_n {}_4G \rightarrow$ glucopyranose.

The chemical and physical properties of the PP-floc polysaccharide suggest that it is closely related to the previously studied polysaccharide pullulan produced by *P. pullulans* (1-5). The evidence suggests that there are a related group of polysaccharides present in PP-floc and that individual differences occur both in molecular weight and in the degree of regularity displayed in the typical repeating unit $[\rightarrow {}_4G_1 \rightarrow {}_4G_1 \rightarrow {}_6G_1 \rightarrow]$.

Marked variations in molecular weight or slight variations in the regularity of the repeating unit could have profound effects on certain physical properties of the polymer.

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