New Bacterial Polysaccharide from *Arthrobacter*[1]

M. C. CADMUS, HELEN GASDORF, A. A. LAGODA, R. F. ANDERSON, AND R. W. JACKSON

Northern Regional Research Laboratory, Northern Utilization Research and Development Division, Agricultural Research Service, U.S. Department of Agriculture, Peoria, Illinois

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ABSTRACT

CADMUS, M. C. (U.S. Department of Agriculture, Peoria, Ill.), HELEN GASDORF, A. A. LAGODA, R. F. ANDERSON., AND R. W. JACKSON. New bacterial polysaccharide from *Arthrobacter*. Appl. Microbiol. **11:** 488–492. 1963.—A bacterial strain (NRRL B-1973) isolated from soil at Guatemala City and tentatively identified as an *Arthrobacter* species produced a polysaccharide with unusual properties. Conditions were studied for the production of this microbial gum in shaken flasks and 20-liter fermentors. Suitable nutrients for optimal polysaccharide production included 3% glucose, 0.3% enzyme-hydrolyzed casein, magnesium sulfate, manganese sulfate, and potassium phosphate buffer (pH 7.0). Polysaccharide yields ranged from 40 to 45%, based on initial dextrose in the medium in 3- or 4-day fermentations. The gum was readily recovered from culture fluid by alcohol precipitation in the presence of an electrolyte. The *Arthrobacter* gum exhibited characteristics unique for a polyelectrolyte. Viscosity of solutions was not decreased by heating in the presence of salt, and the gum withstood a temperature of 121 C for 30 min. At polysaccharide levels above 0.75%, gels were formed when solutions were autoclaved with KCl. There was no significant change in viscosity over a pH range of 5.0 to 10.0.

Previous publications from this laboratory described the fermentative production of polysaccharides by the yeasts, *Hansenula holstii* (Anderson et al., 1960) and *Cryptococcus laurentii* var. *flavescens* (Cadmus, Lagoda, and Anderson, 1962), and by the bacterium *Xanthomonas campestris* (Rogovin, Anderson, and Cadmus, 1961). Research was undertaken in the laboratory and pilot-plant to define a practical fermentation process with a species of the genus *Arthrobacter* (NRRL B-1973) that produces a polysaccharide composed of glucose, galactose, and a uronic acid; acetyl groups are also present as part of the structure. Some physical properties of the *Arthrobacter* polysaccharide were also investigated.

MATERIALS AND METHODS

The organism was isolated from a soil sample obtained from the Guatemala City airport and was tentatively identified as a species of *Arthrobacter*. Profuse, viscous growth was obtained in 24 hr on yeast-malt agar slants (Haynes, Wickerham, and Hesseltine, 1955). The bacterium was maintained on this medium, stored at 8 C, and transferred every 7 days to preserve an active, viable culture. Cultures prepared for laboratory experiments were grown in 300-ml Erlenmeyer flasks containing 75 ml of medium. Flasks were incubated at 25 C on a rotary shaker, which had an eccentricity of 2¼ in. at 200 rev/min. Test media were varied as to pH, glucose concentration, organic nitrogen sources, phosphate buffer, and minerals. Inocula for laboratory experiments were grown in media of the same composition as used for polysaccharide production in the test flasks. Yeast-malt broth with 3% glucose was also suitable for an inoculum medium. Experiments with 20-liter fermentors were limited to a study of agitation, aeration rates, sugar utilization, and temperature. Sterilization procedure and fermentor conditions used were those described by Cadmus et al. (1962).

The polymer from crude cultures was purified as follows. The viscous culture liquor was diluted 1:4 with water, and 0.5 volume of absolute methanol was added to expedite centrifugation of cells and other debris. After centrifugation for 30 min at 20,000 × *g*, the supernatant liquid was decanted and the small-cell volume discarded. The polysaccharide was precipitated by adding 1 g of potassium acetate per 100 ml of aqueous solution and methanol until a total of 2.5 volumes of alcohol was added. The bulk of the precipitate was removed from the surface with a strainer; the remainder was separated by partial decantation, centrifugation, and complete decantation. The entire precipitate was redissolved in a suitable volume of water, and the precipitation procedure was repeated. Finally, the precipitate was redissolved in water and lyophilized to obtain a dry, spongelike, white product. Drying in a vacuum oven was more rapid, although the polymer was slightly discolored. Physical properties of the polysaccharide, however, were not altered with oven drying. Slight differences in the physical properties of the gum were observed because of variations between batches and the methods of isolation used. Data presented in this communication are representative of some typical characteristics of the polysaccharide.

Viscosities of polymer solutions were measured with a viscometer (model LVF; Brookfield Engineering Laboratories, Stoughton, Mass.), which had a range of 0 to

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100,000 centipoises. (The mention of trade names is for the purpose of identification and does not constitute an endorsement by the U.S. Department of Agriculture.) Most measurements were made at 25 C and 30 rev/min in bottles 10 cm high and 5 cm in diameter, containing 100 ml of solution, or in 400-ml beakers with 300 ml of solution. No differences in readings were encountered by interchanging the containers.

Polymer yields in crude culture were determined by removing a 10-ml sample from the diluted, uncentrifuged culture fluid to a glass tube (25 by 150 mm). The polymer was precipitated by adding 0.1 ml of saturated KCl and 2.5 volumes of 95% ethanol. Tubes were centrifuged for 15 min at 1500 rev/min, and the alcoholic supernatant was carefully decanted. The precipitate was partially dehydrated with acetone and then dried to constant weight in an air oven at 110 C. Weights of the purified precipitates were about 20% less than those of the crude precipitates. Hence, a factor of 0.8 was used to approximate yields from weights of crude precipitates.

Glucose utilization during the course of a fermentation was determined by measuring the free reducing sugar of the whole culture initially and at daily intervals by the method of Somogyi (1945). Yields were calculated on the basis of initial sugar incorporated in the medium.

Results and Discussion

Several different substrates were examined for polysaccharide production by *Arthrobacter* NRRL B-1973. In a comparison of glucose, technical maltose, and pure maltose, the technical maltose afforded polymer yields two to three times those of pure maltose or glucose. Paper chromatography (Moffat and Lytle, 1959) of hydrolyzed and unhydrolyzed samples of pure and technical maltose revealed two amino acids present in the crude sugar but absent in the pure maltose. Further experimentation to ascertain whether these amino acids, when added to the glucose medium, had any effect on increasing polymer yields gave negative results. Sugar separations made with a butanol-pyridine-water solvent showed a trace of glucose in the technical maltose. However, small increments of glucose with larger amounts of pure maltose did not increase yields.

Ash from a sample of technical maltose was added to a glucose-containing medium; this mixture increased polymer yields. Qualitative tests for several minerals indicated that magnesium was largely responsible for the increased polymer yields. Experiments showed that 0.08% $MgSO_4 \cdot 7H_2O$ was the optimum for maximal viscosity production with glucose as the substrate. Manganese ion was also required in trace amounts, but polymer production was not affected by the presence of Na^+, Fe^{+++}, Co^{++}, and Mo^{+6}. Hence, by adjusting the mineral concentration of the medium, glucose was satisfactorily substituted for technical maltose. Also, tap water could be substituted for distilled water without any apparent difference in yields.

After mineral requirements were adjusted, a comparison was made between technical maltose and dextrose. Table 1 shows the percentage conversion of these two sugars to polysaccharide. The yield from corn sugar was about the same as that from technical maltose when the higher concentration of magnesium sulfate was used. Apparently, under optimal conditions good yields may be obtained with corn sugar as the carbon source.

The requirement for a low-cost nitrogen source, which would afford good yields of polysaccharide with a minimum of extraneous material, was met by enzyme-hydrolyzed casein (Amber EHC; Amber Laboratories, Inc., Milwaukee, Wis.); other nitrogen sources were less effective. Figure 1 shows the effect of crude nitrogen concentration

TABLE 1. *Effect of $MgSO_4$ on polysaccharide production by Arthrobacter NRRL B-1973*

$MgSO_4 \cdot 7H_2O$	Carbohydrate	Polysaccharide
g/100 ml	*3 g/100 ml*	*g/100 ml*
0	Technical maltose	0.66
0	Corn sugar	0
0.02	Technical maltose	1.48
0.02	Corn sugar	0.90
0.08	Technical maltose	1.45
0.08	Corn sugar	1.43

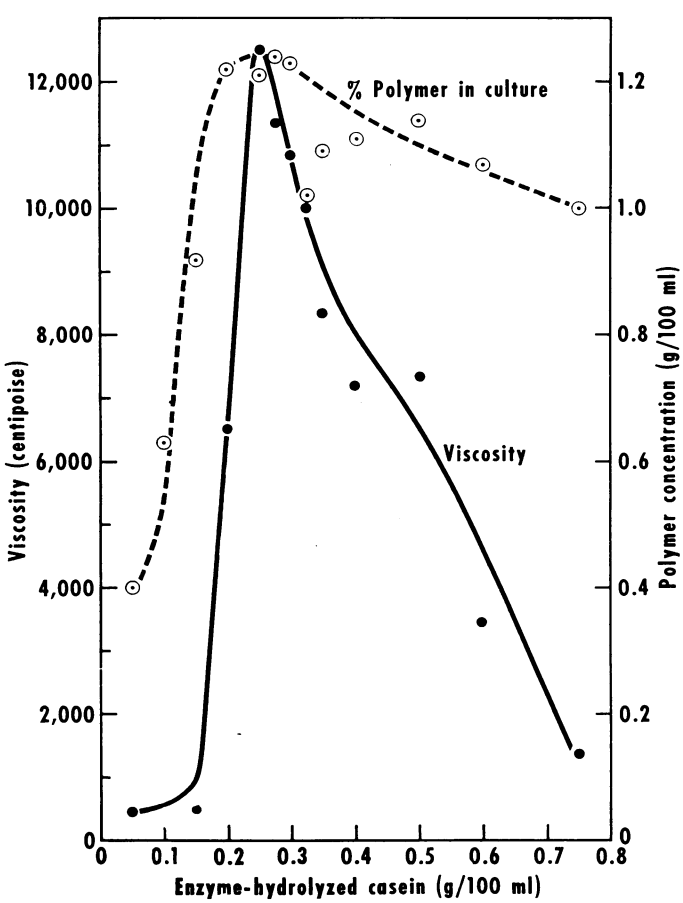

FIG. 1. *Effect of crude nitrogen concentration on polymer production by Arthrobacter NRRL B-1973.*

on polysaccharide production with glucose as the substrate. The optimum near 0.3% Amber EHC is equivalent to 0.04% total nitrogen in the medium.

The medium was buffered with dibasic potassium phosphate, and the presterilization pH was adjusted to 7.0. Buffer concentration optimum, as indicated by polymer yield and sugar utilization (Fig. 2), was determined to be 0.4%. Maximal viscosities were also obtained at this level of K_2HPO_4 and declined rapidly at higher levels. When 0.8% K_2HPO_4 was used, slightly higher yields of polymer were realized, but this substance was more crystalline in nature and probably had lower molecular weight in view of the reduced viscosities obtained. When more than 0.4% buffer was incorporated in the medium, the final pH was above 6.5 (Fig. 2).

Initial glucose concentration was varied at levels from 1 to 6% (Table 2). The concentration of polysaccharide in crude culture increased as the initial sugar level was increased but not sufficiently to warrant the use of more than 3% corn sugar. Above this level, the residual glucose concentration was too high for practical purposes, and the percentage conversion of sugar to polymer sharply decreased.

A range of initial pH of the medium from 5 to 9 was investigated for effects on polysaccharide production by the organism. The optimal initial pH range was 6.8 to 7.2. A sharp drop in yield was noted at ±0.5 pH units from the optimum. The pH change of the medium during sterilization was negligible when 0.4% phosphate buffer was present.

Aeration requirements of strain NRRL B-1973 in shaken flask experiments necessitated the use of a rotary shaker. Under no condition was a reciprocal shaker adequate, probably because of poor aeration after the culture fluid began to thicken, even though the initial oxygen-absorption rate (Corman et al., 1957) was higher than with the rotary shaker. Satisfactory aeration in the 20-liter fermentors was obtained by use of the following agitation schedule: first day, 200 rev/min; second day, 300 rev/min; third and fourth days, 500 rev/min. Reduced polysaccharide yields were encountered when fermentations were started at 500 rev/min.

Air-flow rates through the small-scale fermentors were varied between 0.1 and 0.75 volumes of air per liter per min. Results indicated that the rate of air flow was not critical under the conditions used. The fermentors were unbaffled, but equipped with double paddles to aid stirring of the viscous material, and were maintained under 1 atm of pressure. A lower air flow (0.25 volumes of air per liter per min) was used to keep evaporation at a minimum. The lower rate also slowed glucose consumption somewhat without reducing final polymer concentration.

The most suitable temperature for production of this gum was 25 C; final yields were slightly lower at 22 and 28 C; practically no product formed at 32 C.

No antifoam agents were necessary in the 20-liter fermentors; although some foam was encountered at 24 hr, it was easily controlled by increasing the rate of agitation.

In Fig. 3 are shown the results of a typical 20-liter fermentation which was essentially completed in 3 to 4

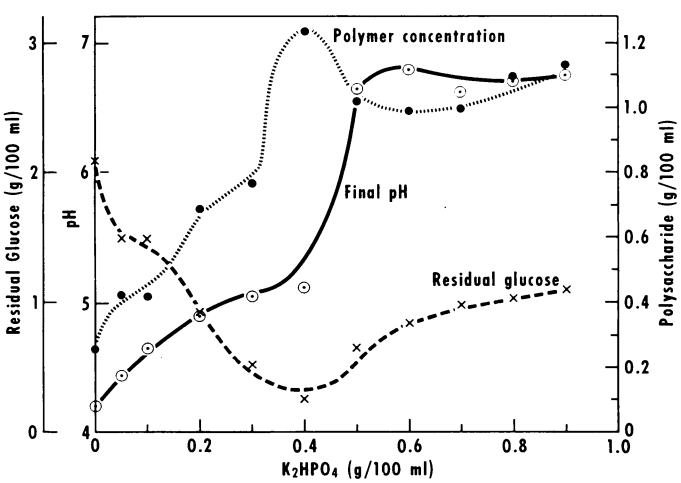

FIG. 2. *Effect of varying levels of phosphate buffer on polymer production by Arthrobacter NRRL B-1973.*

TABLE 2. *Glucose concentration versus polymer yield from Arthrobacter NRRL B-1973*

Initial corn sugar	Residual glucose	Polymer concn	Polymer yield*
g/100 ml	*g/100 ml*	*g/100 ml*	
1	0	0.55	55.0
2	0	0.94	47.0
3	0.34	1.23	41.0
4	1.40	1.23	30.8
5	2.16	1.37	27.4
6	3.00	1.44	24.0

* Expressed as per cent of initial glucose.

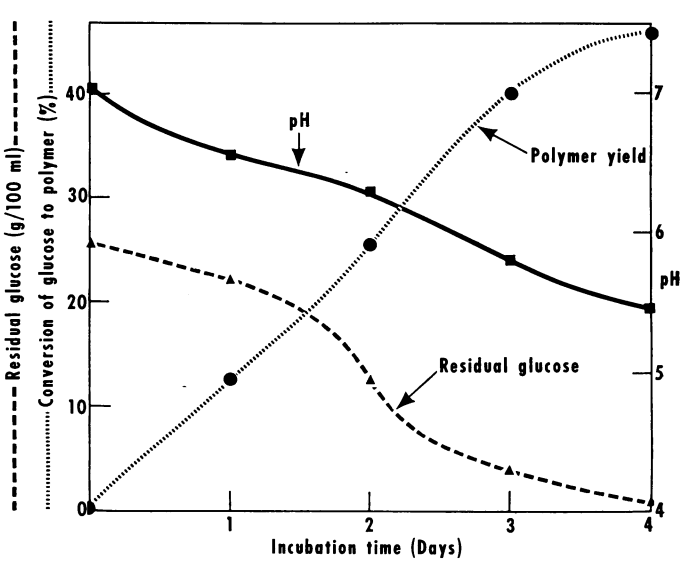

FIG. 3. *Course of a typical 20-liter fermentation with Arthrobacter NRRL B-1973.*

days. After this time, polymer concentrations remained relatively constant along with pH, probably due to limitation of oxygen diffusion to the cells caused by the high viscosity of the culture liquor. With an initial corn sugar concentration of 2.5 to 3.0%, utilization of glucose was essentially complete in 4 days. Final pH of the crude culture was between 5.2 and 5.5. Yields were 40 to 45% based on initial commercial corn sugar in the medium.

Concentration versus viscosity of both heated and unheated polysaccharide in either the presence or absence of KCl is shown in Fig. 4. Autoclaved solutions appeared to be more viscous than the unheated gum above 1% concentration, but lower in viscosity below 0.5% polymer. When 2% KCl was added, the viscosity at all concentrations of polymer was increased considerably, but the most significant increase was realized at the higher concentrations of the autoclaved material.

An unusual property of this gum is its ability to form a gel after autoclaving and cooling. The product is very cohesive and tends to retain the shape of the container in which it is heated, particularly at concentrations of 1.5% or more.

The effect of various salts on polysaccharide B-1973 was also investigated. Figure 5 shows the effect of $Al_2(SO_4)_3$, NaCl, and $CaCl_2$ at concentrations from 0 to 20%. At low concentrations of sodium chloride or calcium chloride, viscosity doubled, but at higher levels of either salt it was not significantly changed. However, aluminum sulfate

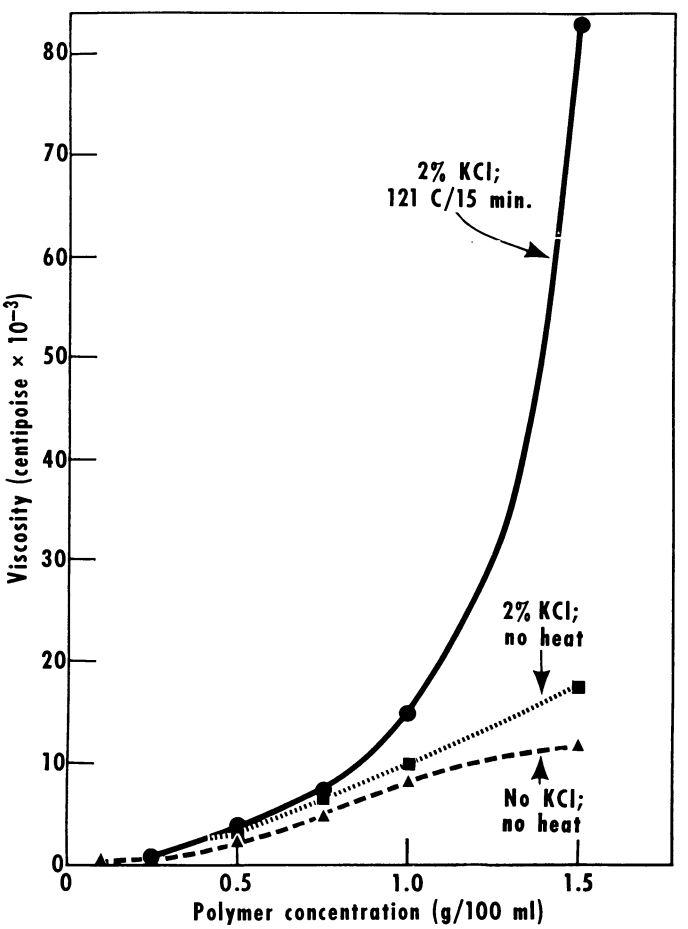

FIG. 4. *Effect of concentration on viscosity of polysaccharide B-1973.*

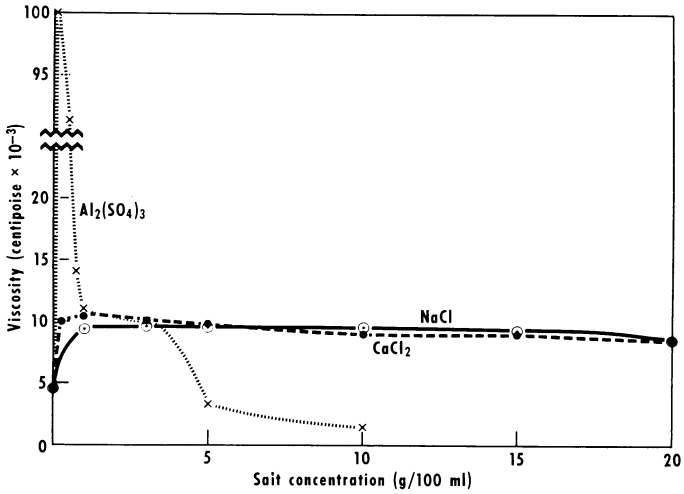

FIG. 5. *Effect of salts on a 1% solution of polysaccharide B-1973*

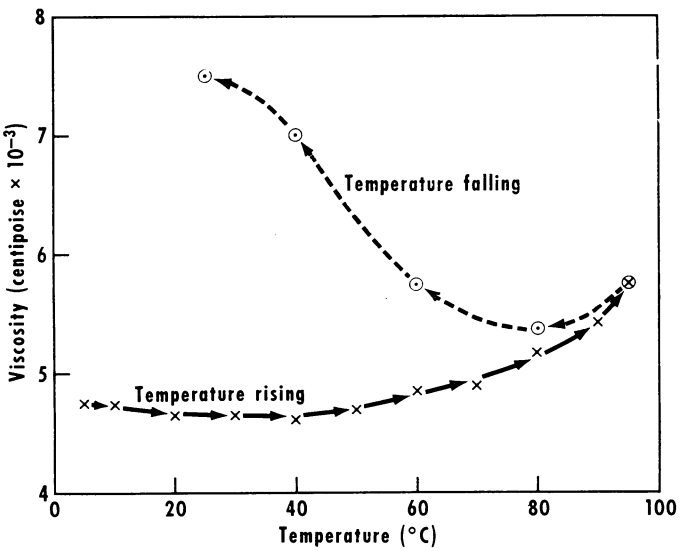

FIG. 6. *Effect of temperature on viscosity of polysaccharide B-1973.*

TABLE 3. *Effect of heat (121 C) on viscosity of polysaccharide from Arthrobacter NRRL B-1973 (1.25% in water)*

Heating time	Viscosity readings at 25 C (centipoises)
min	
0	5900
5	6640
10	10,380
15	67,900
20	>100,000
25	>100,000
30	>100,000
35	10,100
40	980

had a most unusual effect on the polymer solution. At an extremely low concentration (0.05%), the viscosity of the solution increased 20-fold and then decreased very sharply as the salt level was increased beyond 0.25%. Also, the extremely viscous material containing aluminum sulfate was more gel-like in consistency than any of the other salt solutions. Increasing amounts of sodium borate were added to a 1% solution of the gum. Only a very small concentration of $Na_2B_4O_7 \cdot 10H_2O$ doubled the viscosity of the solution. All the salts tested, except the aluminum sulfate, showed that a concentration of 0.25% was adequate to achieve nearly maximal viscosity with no significant decrease at higher concentrations.

This bacterial polysaccharide was also examined to determine viscosity at various temperatures from 5 to 95 C. As the temperature was increased at several intervals for a 1% solution of the gum, Brookfield readings were recorded; then the solutions were cooled, and viscosities were again determined as the temperature decreased (Fig. 6). The viscosity of most polysaccharide solutions decreases as the temperature rises, but in this experiment the viscosity of the solutions actually increased as the temperature rose. Subsequently, upon cooling, the viscosity increased still further. This phenomena may be attributed to the gelling effect of heat on this gum; once the gel forms, the solution has properties similar to many other viscous solutions with respect to temperature effect.

Table 3 shows the effect of autoclaving time (121 C) on the viscosity of a 1.25% solution of polysaccharide. Solutions were cooled to 25 C before viscosity measurements were made. Maximal viscosity was obtained between 20 and 30 min, after which time the polymer began to degrade. A precipitate was observed after solutions were autoclaved for 40 min.

An alcohol precipitation curve on a 0.25% polymer solution containing 1% KCl was prepared to ascertain the homogeneity of the product. Results showed a clear-cut, essentially complete precipitation at 2.1 volumes of 95% ethanol when precipitation was carried out at room temperature. All polymer was precipitated in 2 hr from solutions to which 2.0 volumes of alcohol had been added. At 1.8 and 1.9 volumes, the alcohol-polymer solution mixture formed a stable agarlike gel. This effect was also observed with as low as 1.5 volumes of alcohol, if the mixtures were cooled to 10 C.

A 0.25% solution of the gum was adjusted to various pH levels, ranging from 1 to 12, and viscosities of each solution were measured at 0, 3, and 11 days. Solutions were maintained at 25 C during this period; the polysaccharide appeared to be stable, as judged by viscosity measurements, over the range of pH 5 to 10. It was more unstable to basic than acidic conditions, even though some drop in pH did occur at pH 9 and 10; the degradation of polymer was more rapid at the higher pH levels.

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