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Production of an Extracellular Polysaccharide by *Haloferax mediterranei*

JOSEFA ANTÓN, INMACULADA MESEGUER, AND F. RODRÍGUEZ-VALERA*

División de Microbiología, Universidad de Alicante, Alicante, Spain

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The extremely halophilic archaebacterium *Haloferax mediterranei* produces an exocellular polymeric substance that gives the colonies a typical mucous character and is responsible for the appearance of a superficial layer in unshaken liquid medium. This exocellular polymeric substance can be obtained from the supernatant of shaken liquid cultures by cold ethanol precipitation, and yields as high as 3 mg/ml have been detected. The substance was produced under all the conditions tested and with all substrates assayed, although higher yields were obtained with sugars, particularly glucose, as carbon and energy source. The total exocellular polymeric substance produced was proportional to the total biomass. The polymer is a heteropolysaccharide containing mannose as the major component. Glucose, galactose, and another unidentified sugar were also present, as well as amino sugars, uronic acids, and a considerable amount of sulfate, which accounts for the acidic nature of the polymer. The infrared spectrum and specific assays showed the absence of acyl groups. The rheological properties of polymer solutions were studied, showing a pseudoplastic behavior and a high apparent viscosity at relatively low concentrations. Viscosity was remarkably resistant to extremes of pH, temperature, or salinity. These characteristics make this polymer interesting for enhanced oil recovery and other applications for which a very resistant thickening agent is required.

Halobacteria are members of the archaebacteria that require extremely high concentrations of NaCl to grow and survive. Although classically halobacteria were considered organisms of very slow growth able to utilize only a restricted range of organic compounds, mostly amino acids, as energy sources, some groups described more recently utilize a wide range of substrates, including sugars, and grow more rapidly. One of these organisms is *Haloferax mediterranei*, originally included in the genus *Halobacterium* but separated into a new genus after a recent taxonomic restructuring (26). This organism has very characteristic large mucous colonies and in unshaken liquid medium produces thick superficial layers in which the cells appear to be enclosed in an amorphous matrix (22). All of these features pointed towards an exocellular polymeric substance (EPS; 10) being produced in considerable amounts. The first evidence suggested that this was a polysaccharide, and therefore its study seemed promising for a number of reasons. Bacterial polysaccharides are widely used in industry and are considered to be of immediate or prospective utility (4). Halobacteria, due to their extreme salt tolerance, can be grown without sterile precautions, which can obviously reduce production costs. Also, *H. mediterranei* has been shown to produce considerable amounts of poly-β-hydroxybutyrate granules (8), another industrially interesting product, as biodegradable plastic. Finally, the use of bacterial polysaccharides for enhanced oil recovery (21) often requires the resistance of the substance or, even better, the organisms to elevated salinities found in oil reservoirs. In this work we have purified and analyzed this compound, studied the effect of some culture conditions on production, and measured rheological properties of this substance. These properties are of paramount importance in determining possible applications.

* Corresponding author.

MATERIALS AND METHODS

Production of extracellular polysaccharides. (i) Culture conditions. *H. mediterranei* ATCC 33500 was used in this study. A Braun Biostat M fermentor with an effective volume of 1.3 liters was used. During growth, the pH (7.2), stirring (550 rpm), temperature (38°C), and aeration (≈100% O_2 saturation) were maintained constant.

(ii) Growth media. The compositions of the various media used in the production study are given in Table 1. All media contained a mixture of marine salts (SW) (22) at a total concentration of 25%, and 0.0005% (wt/vol) $FeCl_3$. A medium with 25% SW and 0.5% yeast extract as carbon and energy source was also used; pH of all media was adjusted to 7.2 with 1 N KOH. The media were sterilized by autoclaving for 20 min at 110°C.

(iii) Polysaccharide assay. Samples, 40 ml, of bacterial suspension were taken throughout the growth of the culture. After centrifugation at 8,000 rpm for 30 min, the supernatant was passed through 0.45-μm filters and the filtrate was dialyzed with distilled water to eliminate any sugars present. The dialyzed supernatants were precipitated in the cold with ethanol, and the dry weight of EPS was determined after drying in an oven at 110°C to constant weight. The amount of total sugars in the supernatant was also measured by using Kochert's phenol-H_2SO_4 method (14).

EPS purification and characterization. (i) isolation and purification. Cells were grown in 20.5-liter batches of a medium containing 25% SW, 0.5% yeast extract, and 0.1% peptone or minimal medium 1. The medium was autoclaved in 25-liter cylindrical glass flasks and inoculated with 200 ml of a preculture of the strain in the same medium. The cultures were magnetically stirred, aerated by a flow of humidified air through a glass sparger, and incubated at 38°C. The pH of the glucose-containing medium was checked regularly and readjusted to 7.2; otherwise, the acidification stopped growth much earlier. On reaching the stationary phase, the culture was subjected to tangential filtration, using the Pellicon system (Millipore Corp.) with

TABLE 1. Minimal media used for EPS production by *H. mediterranei*

Medium	Component (%)				mg of EPS per ml[a]	Optical density[a]
	Glucose	Saccharose	NH_4Cl	KH_2PO_4		
1	1		0.1	0.03	2	6.7
2	1		0.05	0.03	1.2	3.8
3		1	0.01	0.03	1.2	3.9
4	1		0.1	0.015	3	9.2
5	1		0.1	0.0075	2.6	9.0
6	1		0.1	0.00375	2.1	7.5

[a] Maximum values.

0.45-μm ultrafilters, to eliminate the cells. Then the EPS of the filtrate was concentrated (0.5-liter final volume) by using the same system with 100,000-dalton ultrafilters. After dialysis of the concentrate with distilled water, the EPS was precipitated by the addition of 4 volumes of cold ethanol (2). The precipitated EPS was redissolved in distilled water and precipitated again. This was repeated twice more. Finally, the EPS obtained was lyophilized, and this material was used for all analytical work.

(ii) Analytical procedures. The infrared (IR) spectrum was obtained by the KBr technique on a Perkin-Elmer 457 IR spectrophotometer. The VIS-UV spectrum was performed on a Spectronic 2000 (Bausch & Lomb, Inc.) spectrophotometer. The EPS was subjected to return-flow hydrolysis with 2 N HCl at 100°C for 4 h (10 mg of EPS per ml of HCl). The solution was reduced to dryness by evaporation in a water bath at 100°C under a nitrogen current followed by drying under vacuum. The hydrolysate was dissolved in water and passed through a Dowex 50W-X8 column, using water as eluant (H^+ form, 200–400 mesh; Sigma Chemical Co.) to isolate the neutral sugars. They were identified by thin-layer chromatography in cellulose (E. Merck AG), using the following solvent systems: acetone-butanol-water (7:2:1) and butanol-pyridine-water (3:2:1.5). The sugar composition was confirmed by descending paper chromatography on Whatman no. 1 paper, using the same solvent systems. The sugars were visualized by spraying the chromatograms with either aniline-phthalic acid (4 g of phthalic acid, 0.2272 ml of aniline, 250 ml of water-saturated *n*-butanol) or $AgNO_3$ (1.4 g of $AgNO_3$ and 4 ml of water diluted with acetone to 400 ml), in the latter case followed by alcoholic NaOH (2 g of NaOH in 20 ml of water diluted with 95% ethanol to 400 ml).

The following quantitative analyses were carried out: total carbohydrates (14), uronic acids (9), amino sugars after hydrolysis with 1 N HCl for 15 h (12), proteins (16), sulfates after hydrolysis overnight with 1 N HCl (5, 6), and acyl residues (17). The presence of acidic radicals (pyruvate, succinate, and sulfate) was also tested by thin-layer chromatography (13).

(iii) DEAE-Sepharose chromatography. Samples, 2 ml, of an EPS solution, 20 mg/ml in 0.001 M Tris, pH 7, were passed through a 50-cm^3 DEAE-Sepharose column (7). The sample was eluted by a linear gradient of 0 to 2 M NaCl with a 0.6-ml/min flow. Portions of 10 ml were collected.

(iv) Electron microscopy. For this study, *H. mediterranei* was grown in 0.5% yeast extract in 25% SW supplemented with 1% glucose in an assay tube and maintained without agitation. The superficial layer formed after 7 days of incubation was gently collected with a spatula to avoid rupture and placed in a tube, where it was washed three times with 25% SW by gentle swirling and decantation. The preparation of samples for thin section and negative staining was as described previously (18).

(v) Rheology. The EPS obtained from the glucose-containing medium was subjected to a detailed rheological study, using a Ferranti-Shirley viscometer with a cone-and-plate system (Ferranti Ltd., Moston, Manchester, England). A 2-ml sample was used, and measurements were done at 25°C unless stated otherwise.

RESULTS

Polymer production. The presence of an exopolymer was first detected by observation of the cells in unshaken cultures in which they appeared surrounded by an amorphous matrix. They formed thick superficial layers on top of the cultures. Under the microscope, the cells of grown cultures show a tendency to aggregate, forming reasonably sized clumps. The superficial layer formed has been studied by electron microscopy, using thin sections and negative staining, showing the presence of a thick layer of EPS surrounding the cells (Fig. 1). Considerable amounts of a whitish precipitate were produced when the dialyzed cell-free supernatant was precipitated with cold ethanol. The addition of a small quantity of NaCl (a few drops of a 3 M solution) is sometimes required to enhance precipitation. To study polymer production during growth under different conditions, samples were withdrawn from the fermentor. The cell-free dialyzed supernatants were precipitated with ethanol, and the dry weight and total neutral sugars were determined. The production of EPS was barely influenced by the growth conditions studied. The production curve in minimal medium 6 is shown in Fig. 2. As with other EPS, production started early during growth, increasing with increasing cell density, reaching a maximum at the beginning of the stationary phase, and then decreasing. Production in yeast extract standard medium for halobacteria was significantly lower (about 20% less than in glucose medium). With sucrose, production was intermediate. The amount of nitrogen or phosphorus source in minimal medium did not influence the amount of EPS produced except when the concentration became limiting, in which case it decreased with cell density. Although the amount of polymer produced with yeast extract or glucose was not strikingly different, the aggregation of the cells on top in unshaken cultures depended on the presence of glucose in the medium (Fig. 3).

Characteristics of the polymer. The cold ethanol precipitate of dyalized supernatants grown in yeast extract or minimal medium 1 had the composition given in Table 2. No acyl residues were found; the assay for acyl groups produced a precipitate characteristic of the presence of uronic acids. Thin-layer chromatography did not show the presence of acyl residues (pyruvate or succinate) either, but instead confirmed the existence of sulfate esters. The polymer was retained by 100,000-dalton ultrafilters, indicating a molecular weight in excess of this value. The UV absorption spectrum of a 10-mg/ml solution did not absorb strongly at wavelengths specific for proteins or nucleic acids. A marked peak was found at 230 nm. Sugar analysis by paper and thin-layer chromatography showed that mannose was the major component of the hexoses present. Three neutral sugars, glucose, galactose, and mannose, and an unidentified compound were detected. The IR spectrum of the EPS is shown in Fig. 4. This spectrum is similar to those of other bacterial exopolysaccharides found in the literature (1, 15, 19, 23). A strong absorption band at 1,240 cm^{-1} is found, attributable to S=O in sulfate esters. The absorption of the EPS at 830

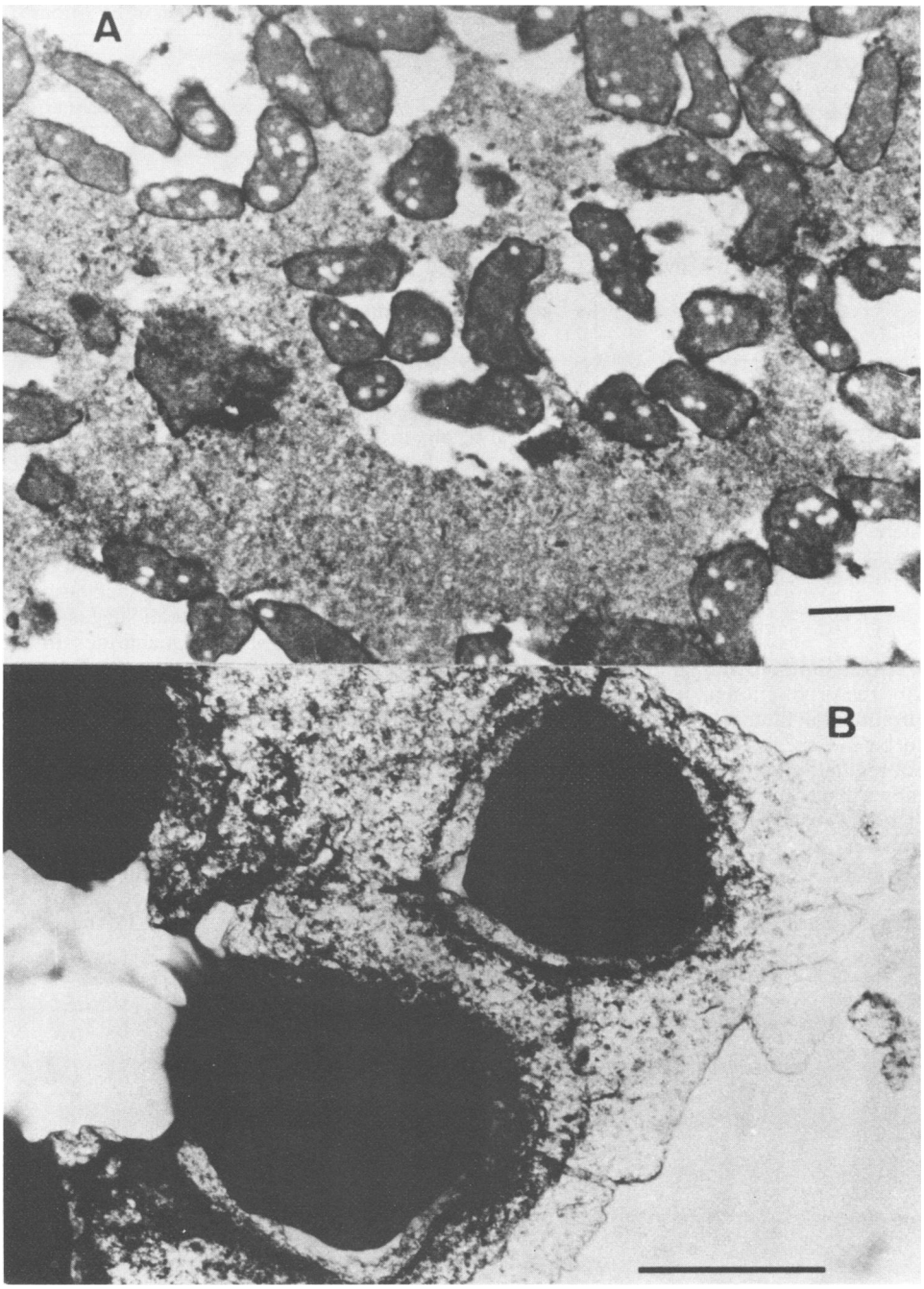

FIG. 1. Electron micrographs of thin sections (A) and negative stain preparations of whole cells (B) of the superficial layer formed by *H. mediterranei* in unshaken cultures. Bars, 1 μm.

cm^{-1} indicates the presence of sulfate ester in a secondary equatorial conformation. There was a strong absorption band at 1,610 cm^{-1}, characteristic of carboxylate ions. The IR spectrum showed a lack of absorption at 1,750 cm^{-1} (this band is characteristic of the carboxyl group of sterified organic acids).

Chromatography through DEAE-Sepharose showed a complex elution profile with two major peaks, one at the beginning and the other at the end.

The study of rheological properties is shown in Fig. 5. The rheograms obtained were typical of a pseudoplastic behavior. They indicated a high apparent viscosity, increasing markedly with the concentration, and also that the viscosity remained quite constant over wide ranges of pH and temperature. The same applies to different salinities, as expected.

DISCUSSION

The EPS of *H. mediterranei* is the first exopolymer produced by an archaebacterium to be described. However, in spite of the wide phylogenetic gap separating archaebacteria from eubacteria, the polymer of *H. mediterranei* resembles in many aspects the most common exopolymers of

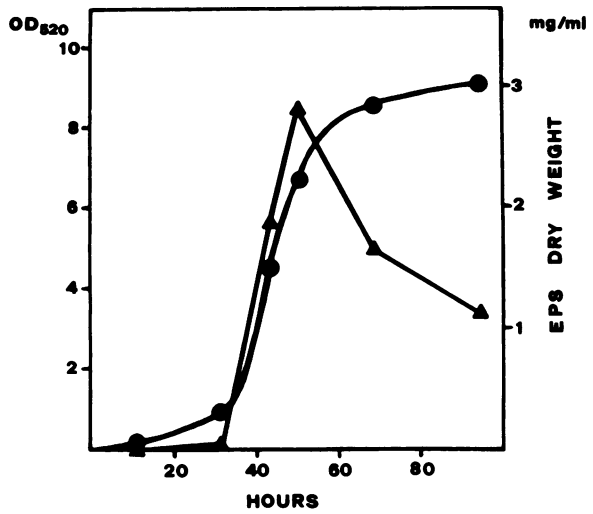

FIG. 2. Growth curve and EPS production in 1% glucose minimal medium (see Materials and Methods). EPS was determined by the dry weight of the ethanol-precipitated material from the supernatant. Symbols: ●, optical density at 520 nm (OD_{520}); ▲, EPS dry weight.

eubacterial origin. Like xanthan and a number of other eubacterial EPS (20), the production appears to be independent of most environmental factors. The amount of EPS produced seems to be mostly linked to the total biomass. The apparent lack of regulation of production of the polymer could be an advantage from the viewpoint of application. Although the total amounts estimated are low compared with the production obtained for some eubacterial EPS, the viscosity of the cultures is considerable (unpublished results). Furthermore, no attempts to optimize the production have been made; it is possible that, by greatly increasing the amount of carbon source, as is usually done when large amounts of EPS are sought, a much higher concentration of polymer could be found.

TABLE 2. Composition of EPS from *H. mediterranei*

Components	Carbon source (% of total wt of polymer)	
	Yeast extract	Glucose
Hexoses	55.75	17.70
Hexosamines	2.50	2.15
Uronic acids	2.28	0.70
Proteins (reference 16)	7.50	15.00
Sulfate	6.80	6.00

The presence of the sugar mannose as a major component is common in other exopolymers, xanthan, for example; the inclusion of uronic acids is more unusual and indicates that this substance is an acidic heteropolysaccaride (10). Even more rare is the large percentage of sulfate in the polymer (3, 20). These characteristics recall the cell envelopes of some halobacteria which contain a glycoprotein, the glucidic part of which is rich in uronic acids and sulfate (27). Sulfate is also found in the heteropolysaccharide of the *Halococcus* cell wall (24, 25). It has been suggested that the presence of sulfated structural polysaccharides in these bacteria indicates a possible phylogenetic relationship between the cell wall of this extremely halophilic procaryotic coccus and those of the eucaryotic marine algae (25). This could also be applied to the EPS of *H. mediterranei* because sulfated polysaccharides are common constituents of the muscilagous layer encapsulating algal cells of the *Phaeophyceae* and *Rhodophyceae* (10). A considerable part of the composition, close to 40%, of the polymer was not accounted for with the analytical procedures followed. However, this is not unusual and happens with other bacterial exopolysaccharides. A similar situation was found in the cell wall of the same

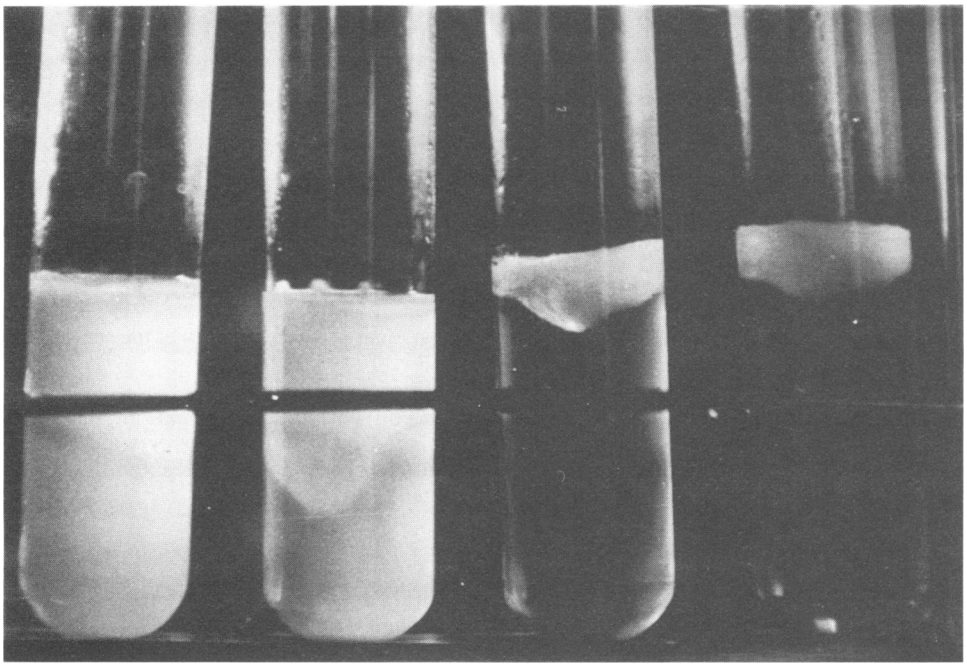

FIG. 3. Formation of superficial layer of cellular aggregate in unshaken cultures. From left to right, tubes contained increasing amounts of glucose (0, 0.1, 0.5, and 1%), all with 0.1% (wt/vol) yeast extract.

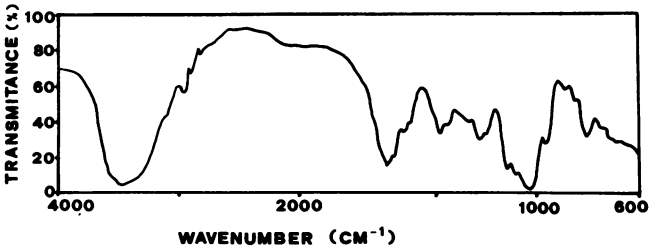

FIG. 4. IR spectrum of purified EPS.

organism (22). This could be due to either a failure of the analytical methods used to determine the correct amounts of components or the existence of other types of compounds not analyzed. The archaebacterial nature of the strain could justify the existence of unusual compounds, undetectable by the methods used.

In cultures which were agitated while incubating, under electron microscopy the cell wall of *H. mediterranei* showed a structure different from that of other rod-shaped halobacteria, with a thick irregular layer instead of the homogeneous thin layer as found in *Halobacterium salinarium* (22). As shown previously (22), when the culture is not agitated during the incubation period, a thick superficial layer surrounding the cells is formed over the medium. Electron microscopy reveals an amorphous matrix enveloping the cells. This structure is similar to that described for other procaryotic eubacterial microorganisms (3). It is possible that the polymer is linked partially or initially to the cell wall and probably becomes detached from the cell due to the agitation. The complex elution profile shows that the EPS studied is not a homogeneous sample, but rather a mixture of various components with different lengths or composition or both. The retention in the DEAE-Sepharose column indicated that there are anionic groups, which is consistent with the presence of sulfate and uronic acids.

Concerning rheological properties, it was apparent that the polymer had a pseudoplastic behavior with a high viscosity particularly resistant to salinity, which makes this EPS of possible use in various applications. From this point of view, this exopolymer offers a number of advantages for industrial production. As with other products of organisms which live in extreme environments, the cultures are very difficult to contaminate, which allows these organisms to be grown with few sterile precautions if the inoculum is large enough. *H. mediterranei* is also a producer of poly-β-hydroxybutyrate, which is accumulated as granules, and this production can be compatible with that of the EPS. Poly-β-hydroxybutyrate is a biodegradable plastic with multiple applications (11). Finally, the extreme salt tolerance of this polysaccharide and the producer organism makes it an interesting candidate for use in enhanced oil recovery, when the salinity of the oil deposits is high, as often happens. If production of the polymer in situ in the oil well is wanted, it is essential that the microorganism be capable of growing anaerobically. *H. mediterranei* requires oxygen, but can grow at very low O_2 concentrations; therefore, the possibility is at least worthy of consideration.

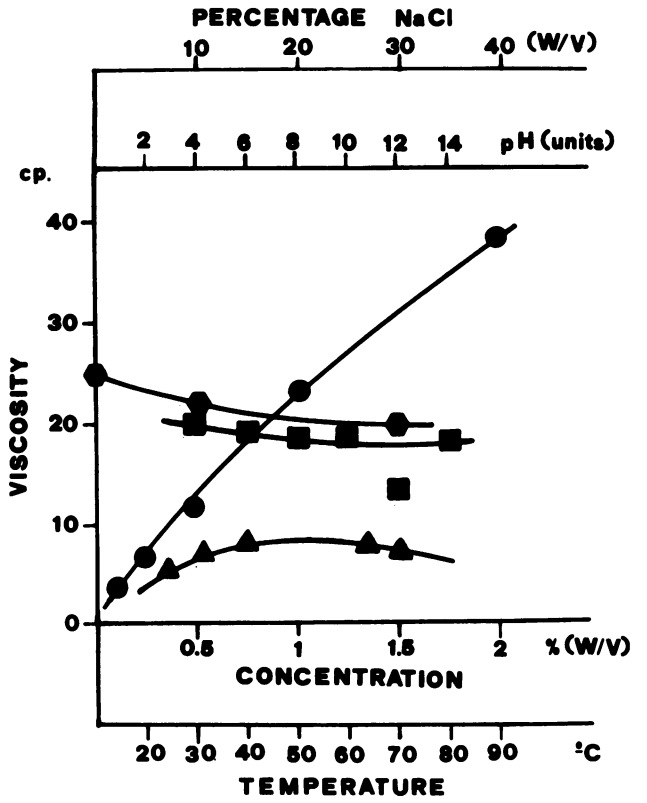

FIG. 5. Apparent viscosity of EPS solutions for a shear rate of 4,362.5 s^{-1}, varying the EPS concentration (●), temperature (■), pH (▲), or salinity (⬣).

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