J. Microbiol. Biotechnol. (1998), **8**(2), 171 - 177

JOURNAL OF
MICROBIOLOGY AND
BIOTECHNOLOGY

Isolation and Characterization of a Novel Polysaccharide Producing *Bacillus polymyxa* A49 KCTC 4648P

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Received: December 26, 1997

Abstract The strain A49, which produces a new type of extracellular polysaccharide was isolated from soil samples. From morphological, physiological and biochemical tests, the strain A49 was identified as a *Bacillus polymyxa* and named *Bacillus polymyxa* A49. *Bacillus polymyxa* A49 was found to produce a highly viscous extracellular polysaccharide when grown aerobically in a medium containing glucose as the sole source of carbon. The polysaccharide (A49 POL) showed a homogeneous pattern on gel permeation chromatography (GPC) and its molecular weight was estimated to be about 1.6 mega dalton (mDa). The FT-IR spectrum of A49-POL revealed typical characteristics of polysaccharides. As a result of investigations with HPLC and carbozole assay, A49-POL was found to consist of L-fucose, D-galactose, D-glucose, D-mannose, and D-glucuronic acid, with the molar ratio of these sugars being approximately 1:2:7:50:12. Rheological analysis of A49 POL revealed that it is pseudoplastic and has a higher apparent viscosity at dilute concentrations than does xanthan gum. The consistancy factor of A49 POL was found to be higher, and the flow index of A49 POL lower, than xanthan gum. Its apparent viscosity was comparatively unstable at various temperatures. the A49 POL showed the highest apparent viscosity at pH 3. When salts were added to A49 POL solution, the solution was compatible with up to 10% KCl, 35% NaCl, 55% $CaCl_2$, 55% $MgCl_2$, 55% K_2HPO_4, and 110% $Ca(NO_3)_2$, respectively.

Key words: *Bacillus polymyxa*, novel polysaccharide

Many microorganisms have an ability to synthesize extracellular polysaccharides and excrete them out of the cell either as soluble or insoluble polymers. Many kinds of extracellular polysaccharides produced by microorganisms have been discovered and developed for commercial application [2, 11, 19].

Many polysaccharides produced by bacteria have characteristic rheological and physiological properties which are different from those of natural gums and synthetic polymers. They are also susceptible to biodegradation in nature and less harmful to the environment than are synthetic polymers. For these reasons, microbial biopolymers have recently attracted much attention as a subject for research. Microbial biopolymers, including polysaccharides, have been traditionally used in a wide range of applications. Such biopolymers can be employed as stabilizers, emulsifiers, thickener in foods, additives for the recovery of petroleum by water flooding, selective adsorbents, and rheological control agents [6, 20].

It is difficult to define useful properties of polysaccharides and select them from nature for specified application. So, it is necessary to find a suitable method for targeting the application field. Thus, hundreds of highly viscous polysaccharide-producing microorganisms were isolated from soil samples and some rheological properties of extracellularpolysaccharide were investigated for selection. From the selection step, we found that strain A49 produced a unique polysaccharide which is anticipated for use as a thickener or flow controller. Several studies using the polysaccharide as bioflocculant, emulsifier, and thickener have been done in our laboratory.

In this paper, the screening and identification of the polysaccharide-producing strain, and some characteristics of the polysaccharide from the isolate are reported.

MATERIALS AND METHODS

Screening of Polysaccharide-producing Bacteria and Identification of the Isolate

The two hundred and fifteen microorganisms included in this study were originally isolated from soil samples

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from various regions in Korea. The screening medium consisted of 40 g glucose, 1.0 g NH_4NO_3, 0.8 g K_2HPO_4, 0.8 g KH_2PO_4, 0.05 g $MgSO_4 \cdot 7H_2O$, 0.05 g $MnSO_4 \cdot 4\sim5H_2O$, 0.01 g soytone, 0.01 g tryptone, 0.01 g yeast extract, and 1.0 g $CaCO_3$ in 1 *l* distilled water. The pH was adjusted to 7.0 with 1.0 N NaOH. Mucoid strains were isolated after three days of incubation on agar screening medium at 30°C. For selection, mucous materials prepared from each isolate were tested for properties such as viscosity, heat stability, pH stability, and salt compatability. The isolate A49 was identified from its morphological and physiological properties, according to Bergey's Manual of Systematic Bacteriology [17], Macfaddin's Biochemical Tests for Identification of Medical Bacteria [12], and Cowan and Steel's Mannual for the Identification of Medical Bacteria [5]. Also, the fatty acid compositions of isolate A49 were analyzed by gas chromatography (Hewlett-Packard 5890A, Avondale, U.S.A.) with the microbial identification system (MIS) [21]. The cell mass required for MIS was prepared with late exponential cells after cultivation on tryptic soy agar (TSA) at 37°C for 24 h.

Measurement of Cell Growth and Apparent Viscosity

The optical density was measured at a wavelength of 660 nm using a spectrophotometer (UV-160A, Shimadzu, Japan) for estimation of cell growth. The apparent viscosity of the culture broth was measured by a rheometer (DV-II, Brookfield, U.S.A.) fitted with spindle No. 3 at 30 rpm and 25°C.

Determination of Polysaccharide Amount

Total carbohydrate accumulated in culture broth was measured by the phenol-sulfuric acid assay [4] after cells were removed by centrifugation. The amount of polysaccharide was expressed using an equivalent amount of glucose as a standard.

Culture Condition

The isolate was cultured in a 5-l jar fermenter (KF-5, KFC, Korea) for polysaccharide production using the basal medium. Impeller speed and aeration volume were controlled to 200 rpm and 1.0 vvm, respectively. Seed culture was performed in a 250-ml Erlenmeyer flask in a shaking incubater at 140 rpm and 30°C for 24 h. The working volume and inoculum size were 3.0 l and 3.0% (v/v), respectively. During cultivation, the culture broth was sampled every 4 h and cell growth, apparent viscosity, amount of polysaccharide, and pH were monitored.

Purification of Polysaccharide

After cultivation, the harvested culture broth was diluted ten fold with distilled water. 5.0 M NaCl and 0.5 M tetrasodium ethylenetetraacetic acid (4Na-EDTA) solution were then added to 2.0% (v/v). Most of the bacterial cells were removed by centrifugation (9,000×g, 40°C, 40 min). The cell-free culture broth was concentrated to the initial volume with a Sartocon Mini Modules membrane filter (Satorius, 300 kDa, Goettinggen, Germany). The concentrated supernatant was precipitated by the addition of 3 volumes of 95% chilled ethanol. The precipitated crude polysaccharide was dried with a vacuum evaporator and redissolved in distilled water. 5.0% cetylpyridinium cloride (CPC) solution was then added until no precipitate was formed. The insoluble acidic polysaccharide-CPC complex was collected by centrifugation and redissolved in a 10% NaCl solution. After dialysis against distilled water, the polysaccharide was precipitated by the addition of two volumes of ethanol and dissolved in distilled water. The polysaccharide was dialyzed against distilled water and lyophilized.

Physical and Chemical Properties of Polysaccharide

High performance liquid chromatography (HPLC) (ACS, UK) with a PL-GFC 1000Å column (7.5×300 mm) (Polymer Lab., USA) and an ERC-7522 Refrection Index Detector (Waters, Milford, U.S.A.) were employed for investigation of the molecular weight (MW) distribution of A49 POL. The mobile phase was water and the flow rate was 1.5 mm/min. For estimation of molecular weight, several types of dextran (Sigma, St. Louis, U.S.A., MW: 2 mDa, 500 kDa, 70 kDa, and 10 kDa) were used for standards. The infrared spectrum of A49 POL was measured using an IR spectrophotometer (RFX-65, Laser percison analytical, Irvine, U.S.A.) with KBr pellets. Complete hydrolysis of the A49 POL was carried out with 2.0 N Trifluoracetic acid (TFA) at 121°C for 2 h. TFA was then removed with an evaperator at 40°C. After hydrolysis, the solution was neutralized with a 1.0 N NaOH solution and lyophilized. The hydrolyzed A49 POL (1.0 mg) was dissolved in 10 ml of distilled water and the neutral sugar constituent was analyzed by thin-layer chromatography and HPLC. Thin-layer chromatography was performed by silica thin-layer chromatography. HPLC (Millipore, Waters 501, Bedford, U.S.A.) and a differential refractometer (Millipore, Waters 401, Bedford, U.S.A.) were empolyed with an Aminex HPX column (7.5×300 mm) (Bio-rad, Hercules, U.S.A.) for analysis of neutral sugar components. Pretreatment of the hydrolysate was performed with Florisil Sep-Pak cartridge (Waters, Milford, U.S.A.) for removal of anionic sugar derivatives and impurities. The mobile phase was water and the flow rate was 0.5 ml/min. Glucuronic acid was detected by the carbazole method [3].

Rheological Properties of A49 POL

The polysaccharide A49 POL was prepared from the culture broth of *Bacillus polymyxa* A49 and xanthan gum was purchased from Jungbunzlauer Xanthan

Gesellschaft m. b. h. (E415, Food grade, Wien, Austria) for the comparison. Apparent viscosities of A49 POL and xanthan gum solution were measured with Digital Rheometer (DV-II, Brookfield, U.S.A.) fitted with spindle SC4-21. At each concentration (from 0.05% to 0.5%, (w/v)) of A49 POL and xanthan gum solution, apparent viscosities were measured at different shear rates (10^{-1} sec^{-1}).

Consistency index and Power-low index or flow index were calculated by Ostwald's Power-low equation [11] from the measured shear rate and shear stress of A49 POL and xanthan gum solution.

$\tau=k(\gamma^n)$ τ: shear stress (D/cm^2)

k: consistency index, k (cP)

γ: shear rate (sec^{-1})

n: flow index, n

RESULTS AND DISCUSSION

Screening and Identification

For the screening of new polysaccharide-producing bacteria, 215 bacterial strains which excreted mucous material on the agar plate of the screening medium were isolated from soil. A mucoid colony on the agar plate culture presumed to be a biopolymer producer was cultured in 50 ml of the liquid culture. For selection, materials perpared from each isolate were tested for properties such as viscosity, heat stability, pH stability, and salt compatibility. Among the strains tested, the mucoid material from strain A49 was relatively stable in a wide range of pHs and temperatures, and was salt compatible (data not shown). Based on these results, strain A49 was considered a most suitable candidate for practical polysaccharide application.

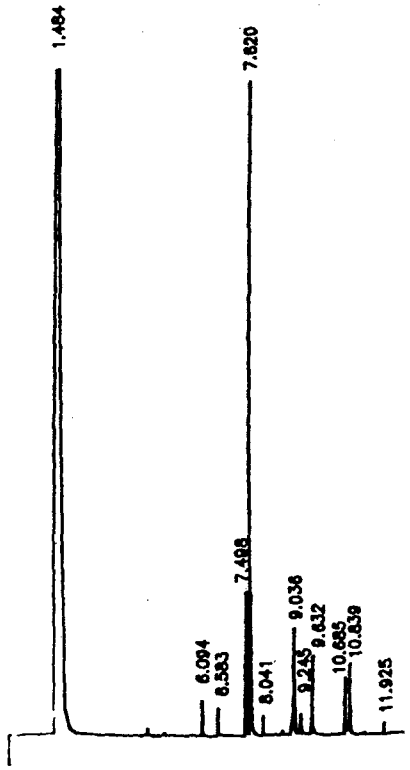
The morphological and physiological characteristics of strain A49 were investigated (Table 1). After incubation for 2 d at 30°C on glucose-nutrient agar medium, colonies were circular, convex, and milky-white. Strain A49 was rod shaped, gram-positive, and formed sub-terminal spores. The size of strain A49 was 0.5~0.7×2.0~2.5 μm. Growth occurred at 30°C and 40°C, but not at 50°C. The strain A49 was able to hydrolyze starch and gelatin. The strain showed a positive reaction in the Voges-Proskauer and catalase test, and produced acid from glucose, arabinose, xylose, and mannitol. Therefore, it was considered to belong to the *Bacillus* species. To elucidate the relationship between strain A49 and the genus *Bacillus*, its composition of fatty acids was examined. Strain A49's composition of cellular fatty acids and the dendrogram is shown in Figs. 1 and 2. The main fatty acids were found to be branched-chain fatty acids such as 13-methyl tetradecanoic acid (iso-15:0) and 12-methyltetradecanoic acid (anteiso-15:0); this finding is in agreement with that of Suzuki and Komagata [21]. The taxonomic position of strain A49 was concluded to be *B. polymyxa* and named *B. polymyxa* A49. This strain is stocked in the Korea Collection for Type Cultures (KCTC) with the collection number KCTC 4648P.

Table 1. Biochemical and physiological characteristics of strain A49.

Characteristic	Result
Gram staining	+
Endospore	+
Shape	rod
Cell size	0.5-0.7×2.0-2.5 μm
Motility	+
Catalase	+
O/F test	F
Voges-Proskauer test	+
Growth at 2.0% NaCl	+
Growth at 5.0% NaCl	+
Growth at 7.0% NaCl	-
Growth at 5°C	-
Growth at 10°C	+
Growth at 30°C	+
Growth at 40°C	+
Growth at 50°C	-
Carbohydrates, acid from:	
D-glucose	+
D-xylose	+
L-arabinose	+
D-mannitol	+
Growth at pH 6.8	+
Growth at pH 5.7	+
Starch hydrolysis	+
Casein hydrolysis	+
Gelatin hydrolysis	+
Indole reduction	-

Time Course of the Polysaccharide Production

When *B. polymyxa* A49 was grown at 30°C for two days on a solid culture medium containing glucose as a carbon source, translucent gelatinous colonies developed on the culture plate. It was also observed that the liquid culture medium became highly viscous with bacterial growth under aerobic conditions due to the formation of extracellular polysaccharide. Therefore, we investigated the time course of polysaccharide production and the changes of viscosity and pH of the culture medium with the growth of this strain. Figure 3 shows the time course of cell growth and polysaccharide production of *B. polymyxa* A49. The polysaccharide production started early in the exponential phase of growth and continued during the stationary phase. The concentration of polysaccharides increased in proportion to the increasing cell growth and reached its the maximum value of 1.7 g/l after 44 h of cultivation. The apparent viscosity of the culture broth at this time was 1,300 centiposie (cP). Decreases in the apparent viscosity of the culture broth and polysaccharide concentration were found after 48 h of cultivation. It was presumed that the



Retention time	Name	Content (%)
1.484	solvent	
6.094	iso 14:0	2.78
6.583	n 14:0	2.27
7.498	iso 15:0	11.14
7.620	anteiso 15:0	52.93
8.041	n 15:0	1.72
9.036	iso 16:0	9.27
9.245	unsturated 16:1	1.94
9.632	n 16:0	6.59
10.685	iso 17:0	4.99
10.839	anteiso 17:0	6.37

n: straight-chain acid
$CH_3 - (CH_2)_{14} - COOH$

iso: iso-branched acid
$CH_3 - CH(CH_3) - (CH_2)_{12} - COOH$

ante: anteiso-branched acid
$CH_3 - CH_2 - CH(CH_3) - (CH_2)_{12} - COOH$

Fig. 1. Gas chromatogram of cellular fatty acids of strain A49.

polysaccharide was partially degraded by depolymerase in the later stages of cultivation. The pH of the culture medium at the time of maximum accumulation of the polysaccharide was about 4.6. This pattern was similiar to that reported by other researchers [13, 14, 18, 22].

Molecular Weight and Infra-red Spectrum of Polysaccharide A49 POL

The acidic polysaccharide was isolated from the water-soluble mucilage *via* CPC-complex formation and subsequent column chromatography, and was then

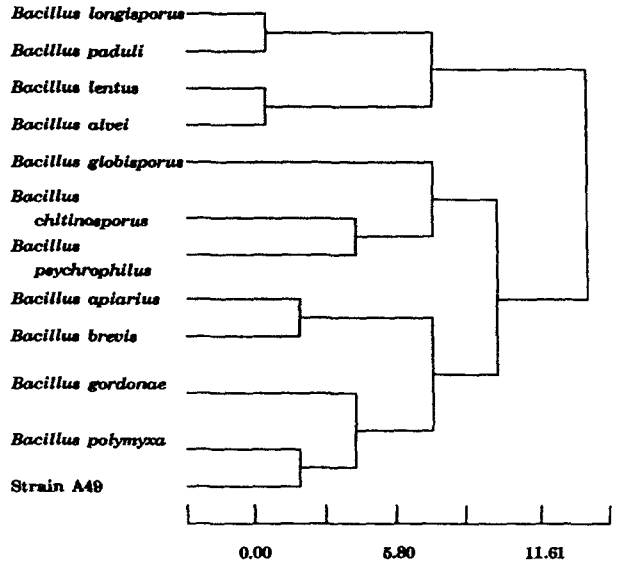


Fig. 2. Abridged dendrogram showing the relationship of *Bacillus* strains based on their cellular fatty acid profiles.

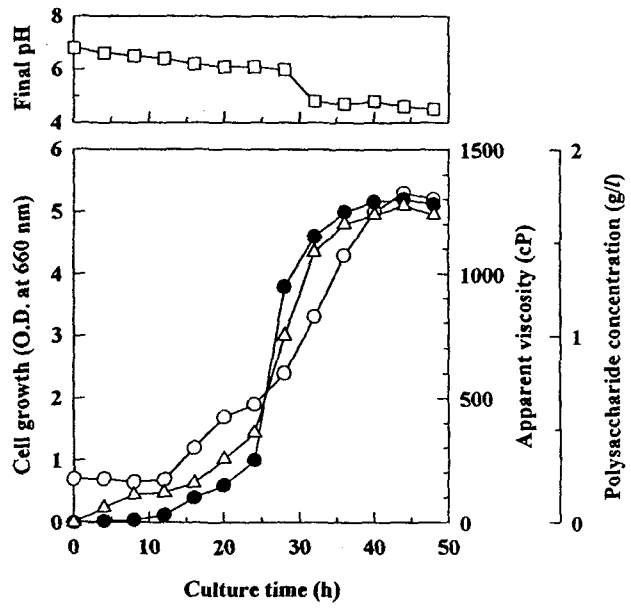


Fig. 3. Time course of cell growth, apparent viscosity, final pH, and the polysaccharide production in the jar fermenter.
The cultures were performed with a working volume of 3.0 l, stirred at 200 rpm, the initial pH was adjusted to 6.8. Aeration was maintained at a rate of 2.0 vvm. Symbols: (○) cell growth, (●) apparant viscosity, (△) polysaccharide concentration, (□) final pH.

submitted to both chemical and biological analyses. The purified acidic polysaccharide (the final preparation was named A49 POL) was investigated for its molecular weight distribution with HPLC. An HPLC pattern of the A49 POL is shown in Fig. 4. The molecular weight of A49 POL was estimated to be about 1.6 mDa. Generally, the molecular weight of a polysaccharide

influences the rheological properties of the polysaccharide solution, and also determines the molecular dimension in solution. The A49 POL produced in this study had a high molecular weight compared with other microbial polysaccharides [8-10]. The molecular weight of the A49 POL was almost the same as that of the xanthan gum (1.4~1.7 mDa) produced by *Xanthomonas campestris*.

From the infrared spectrum of A49 POL, the polysaccharide's chemical group characteristics were analyzed (Fig. 5). The absorption peak at 3,440 cm^{-1} was characteristic of OH stretching from the bound hydroxyl group and of adsorbed water molecules. The peaks in the range from 2,900 to 2,800 cm^{-1} were an indication of aliphatic C-H stretching. The absorption peaks around 1,620 cm^{-1} and 1,413 cm^{-1} were characteristics of the C=O and CH_2OH groups. The strong absorption peaks

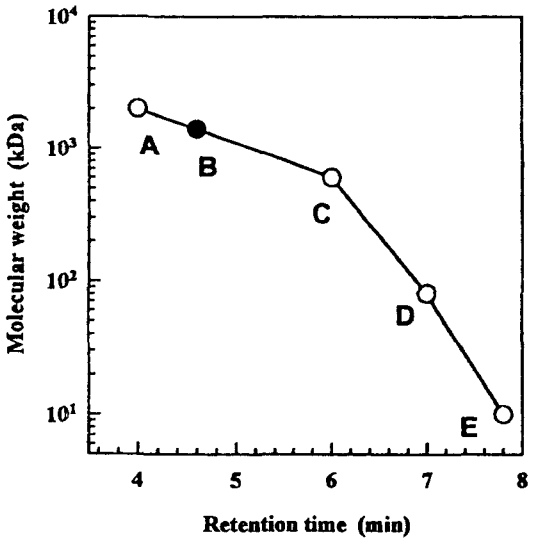


Fig. 4. Estimation of molecular weight of the A49 POL using gel permeation chromatography with HPLC.
Standard marker: (A) dextran (2 mDa), (B) A49 POL, (C) dextran (500 kDa), (D) dextran (70 kDa), (E) dextran (40 kDa).

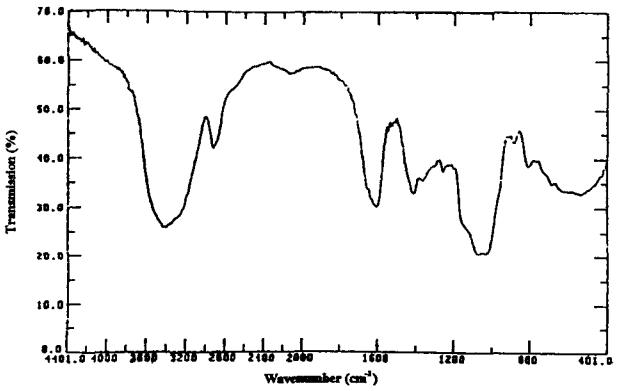


Fig. 5. Infrared absorption spectrum of A49 POL in KBr.

observed in the range from 1,200 cm^{-1} to 1,000 cm^{-1} are typically characteristics of all sugar derivatives. The infrared spectrum of A49 POL thus shows the presence of hydroxyl, aldehyde, and carboxyl groups. These functional groups are known to contribute to the industrial application of polysaccharides such as in bioflocculants [19] and bioabsorbents [16].

Sugar Constituent of A49 POL

Neutral sugar constituents of the A49 POL were analyzed by thin-layer chromatography and HPLC. Thin-layer chromatography was performed with silica thin-layer chromatography. Neutral sugars were detected by spraying with anisaldehyde-sulfuric acid. When a TFA-hydrolyzate of purified A49 POL was analyzed by thin-layer chromatography with a solvent system of acetonitrile-water (85:15, w/w), four compounds were detected on the plate with R_f values identical to those of authentic D-glucose, D-galactose, D-mannose, and L-fucose. Analysis of the TFA-hydrolyzate by HPLC indicated the presence of D-glucose, D-galactose, D-mannose, and L-fucose (data not shown). The relative ratios of the sugars based on peak area percentages were variable. The predominant neutral sugar for the TFA-hydrolyzate of A49 POL was D-mannose, but D-galactose, D-glucose, and L-fucose were also present in various amounts. Glucuronic acid content for TFA-hydrozate of A49 POL was identified by the carbazole method [3]. The molar ratios of L-fucose, D-galactose, D-glucose, D-mannose, and D-glucuronic acid were approximately 1:2:7:50:12. Therefore, these results suggest that the A49 POL is an acidic polysaccharide consisting of L-fucose, D-galactose, D-glucose, D-mannose, and D-glucuronic acid. Because the sugar components and composition of the A49 POL differ from those of other polysaccharides produced by the *Bacillus* species or other bacteria, A49 POL is believed to be a newly discovered polysaccharide. Notably, fucose has not been reported until now as a sugar component of other microbial exopolysaccharides produced from *Bacillus* sp. and other bacteria [1, 10, 13, 15].

Rheological Properties of A49 POL

Most commercial applications of microbial polysaccharides depend on their rheological properties which can be influenced by both their sugar composition and spatial structure of their basic units [11]. Among the rheological properties, viscosity is the important factor which can measure rheological characteristics of polymer solutions. The characteristic flow behaviour of the A49 POL solution has been studied in comparison with xanthan gum.

At a shear rate of 10 and 1 sec^{-1}, the apparent viscosity of A49 POL was found to be about 2~3 times higher

than xanthan gum at each concentration (0.05, 0.1, 0.15, 0.2, 0.3, and 0.5%) (data not shown). Especially, the important property of A49 POL is that it has a higher viscosity than xanthan gum solution at low concentration.

Consistency index (k) values of A49 POL solution were higher than those of xanthan gum solution. Furthermore, the flow index (n) values of A49 POL were lower than those of xanthan gum (Fig. 6). Thus, A49 POL is characterized by having a high consistency index and a low flow index. In general, a lower flow index indicates higher pseudoplastisity which is defined by rapid viscosity decrease as shear rate is increased. Therefore, the A49 POL solution exhibits higher thickening power and pseudoplastic (shear-shinng) properties than xanthan gum solution. These properties are advantageous to thickeners and flow controllers.

With regards to temperature effects (data not shown), the apparent viscosity of A49 POL was found to be higher than xanthan gum at every point in the temperature range (20~80°C). The changes in apparent viscosity of A49 POL were similiar to those of xanthan gum as temperature increased. However, the apparent viscosity of A49 POL decreased by about 1/2 at temperatures over 50°C and A49 POL was comparatively unstable at various temperatures.

The pHs of A49 POL and xanthan gum solution were controlled with 2 N NaOH and 2 N HCl, and their apparent viscosities were measured at 25°C. The change of apparent viscosity of A49 POL was similar to that of xanthan gum solution. Overall, A49 POL solution was less stable than xanthan gum in the pH range 2.0 and 11.0, but A49 POL solution was found to have higher apparent viscosity than xanthan gum solution. Especially, A49 POL solution was found to have the highest apparent viscosity at pH 3.0 (Fig. 7). In general, most microbial polysaccharides are unstable in acidic conditions, but A49 POL indicated the unique rheological characteristic of high viscosity under strong acidic conditions.

The viscosity of exopolysaccharide changed by binding of exopolysaccharide and salt. In generally, the higher salt concentration, the less the decrease in viscosity of exopolysaccharide. The high salt compatibility means that the native viscosity (rheological properties) of the exopolysaccharide does not change at high concentrations of salt. This property plays an important role on the finding of applicable field in the industry. In terms of salt compatibility (Fig. 8), A49 POL was compatible with up to 10% KCl, 35% NaCl, 55% $CaCl_2$, 55% $MgCl_2$, 55% K_2HPO_4, and 110% $Ca(NO_3)_2$, respectively. The high salt compatibility of A49 POL can directly dissolve a moderate concentration of a wide variety of salts and this property is very useful for application in thickeners, flow controllers, and cleaners.

From the results of its chemical identities and rheological properties, A49 POL can be considered to be a new anionic polysaccharide which shows a high apparent viscosity. It has several important properties such as a high viscosity at low concentration, low viscosity at high shear rate, high viscosity at low shear rate, good viscosity stability in strong acids and akalides, and excellent compatibility with salts. It is believed that A49 POL will be environmentally safe for many industrial applications, including textile printing, dyeing, cleaner, thickener, and flow controller.

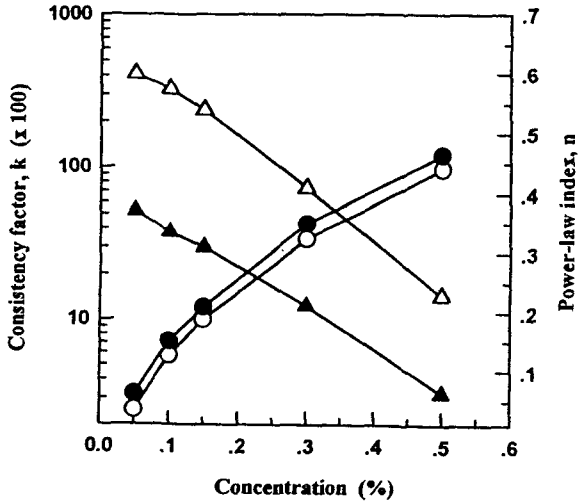


Fig. 6. Consistency factor and Power-law index of A49 POL and xanthan gum solution.
Symbols: (●) A49 POL, k, (○) xanthan gum, k, (▲) A49 POL, n, (△) xanthan gum, n.

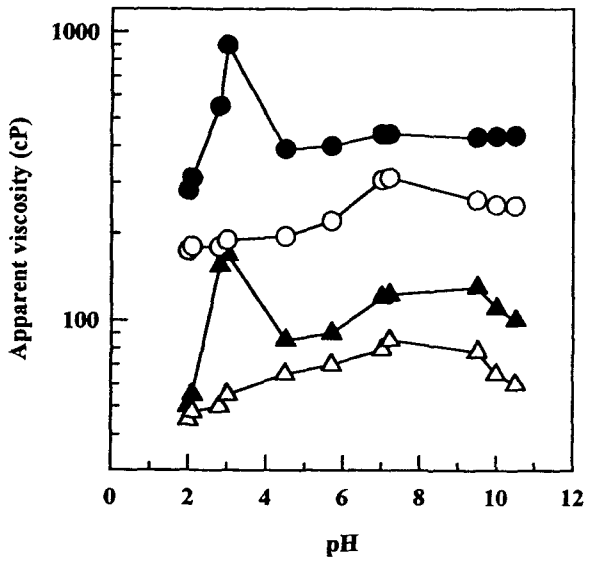


Fig. 7. Effect of pH on the apparent viscosity of A49 POL and xanthan gum solution.
Symbols: (●) A49POL (1 mg/l), (▲) A49POL (10 mg/l), (○) xanthan gum (1 mg/l), (△) xanthan gum (10 mg/l).

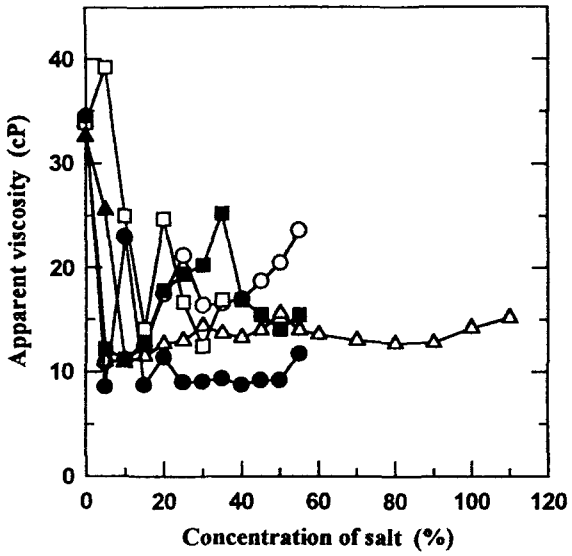


Fig. 8. Salt compatibility of A49 POL.
Symbols: (▲) KCl, (□) NaCl, (■) $CaCl_2$, (○) $MgCl_2$, (●) K_2HPO_4, (△) $Ca(NO_3)_2$.

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