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Isolation of Biopolymer-producing Bacterium and Its Growth Pattern

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Biopolymer 생산세균의 분리 및 증식패턴

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A soil bacterium synthesizing an extremely viscous biopolymer was isolated and identified as *Pseudomonas delafieldii*. The optimal pH and temperature for the growth were 6.5 and 30°C, respectively. Maximum specific growth rate was 0.24 h^{-1}. The specific polysaccharide productivity, growth yield and product yield were 6.25 mg/g-cell/h, 54.5% and 38.39%, respectively. The polysaccharide was presumed to be β-glucan containing glucose and gluconolactone (1.9: 1.0 in molar ratio) and 1.35% acetyl group. Element analysis showed that it contained carbon (31.85%) and hydrogen (5.15%). The weight average molecular weight by GPC was 5.64×10^7. The intrinsic viscosity was 42.84 dl/g.

Microbial exopolysaccharides have been a subject of research because they are a source of rheological agents with novel properties sometimes superior to those of traditionally used plant and seaweed gums (1-5). The polysaccharides have a potentiality in applicationn to food, pharmaceutical, oil, paint, paper and cosmetic industry as viscosifying, emulsifying, gelling and encapsulating stuff (6,7).

A wide range of bacteria are known to produce copious quantities of polysaccharide which can be reflected as large mucoid colony on agar plate or highly viscous culture broth (8). Many papers were reported in relation to developing new and novel polysaccharide for the forementioned purpose and some are commercialized (9,10) or proposed for commercialization (6).

In this laboratory, a mucoid Gram negative soil bacterium synthesizing an unusually viscous polysaccharide was isolated in screening procedure. In this paper, several characteristics of the isolate and physicochemical properties of biopolymer will be reported.

Materials and Methods

Isolation and screening of bacterium

For isolating and screening of polysaccharide producing bacteria, 854 samples representing many regional environments were collected. After serial dilution of collected sample with physiological saline solution, aliquot of each preparation was inoculated on plate count agar (oxiod) by using the pouring method. After incubating 2-3 days at 30°C, mucoid colonies (11) were selected, purified, maintained on nutrient agar slant and subcultured every month. Final test organism was screened by viscometry and visual inspection of alcohol precipitatable slime of cell-free culture supernatants after 72 hours of incubation at 30°C in flask culture containing a glucose-peptone basal medium (12). A soil bacterium from Honseong, Chungnam, coined as BT-4, produced a very viscous culture broth (1511 m Pa.s,

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Brookfield LVT Viscometer, Spindle No. 3, 30 rpm, 25°C) and was therefore finally selected for this study.

Starter culture

Starter culture was grown in YM broth (Difco) at 30°C for 18 hours and the inoculum size used was 5%

Medium and cultivation

The mineral medium contained the following components per liter of distilled water: NH_4Cl (10 mM), KH_2PO_4 12.8 mM; K_2HPO_4 2.8 mM; citric acid 1 mM; $MgCl_2.6H_2O$ 4 mM; Na_2SO_4 11.5 mM; $CaCl_2$ 100 μM; $MnCl_2$ 50.5 μM, all analar grade from British Drug House Chemicals (England); polypropyleneglycol 2025 (BDH) 0.2 m*l*; glucose (Sigma Chem. Co., U.S.A.) 25g. The carbon source was added into the mineral medim after separate autoclaving (121°C, 15 psi, 30 min).

Culture was performed in Bioflo C-30 fermentor (working volume: 1500 m*l*, agitation 400 rpm, aeration: 1 vvm, New Brunswick Scientific Co., U.S.A.). The pH of the medium was controlled with 3N KOH (pH 40 controller, NBS, U.S.A.).

Dry cell weight (11,13,14)

Membrane filtration technique was introduced to collect the culture biomass on the preweighed membrane filter (47 mm diameter, 0.45 μm pore size, Millipore division, U.S.A.). The biomass on filter was washed twice with distilled water and dried to constant weight under infrared heat lamp.

Growth rate measurement (15,16)

The optical density at 660 nm of culture broth was measured periodically with Spectronic 21(Baush and Lomb Inc., U.S.A.) and plotted logarithmically against fermentation time. The specific growth rates (h^{-1}) and generation times were calculated from the slope of straight-line portion of the growth curve.

Nitrogen assay

Ammonium ion content of culture broth was assayed after centrifugation by the colorimetric phenate method described by Franson *et al.* (17).

Identification of bacterium (18)

The bacterium was identified by the characteristics described in Bergey's manual of systematic bacteriology. The morphology was examined by transmission electron microscopy (100 kV, Hitach Model H-700, Japan).

Preparation of polysaccharide

BT-4 was grown at 30°C for 72 hours in glucose-peptone medium (19) in fermentor (working volume: 1500 m*l*, agitation: 200 rpm, aeration: 1 vvm, New Brunswick Sci. Co., U.S.A.). The culture broth was centrifuged at 95,400 × g to separate the cell after dilution and the polysaccharide was harvested by isopropanol precipitation (19-21).

Purification of polysaccharide (16, 22-24)

Polysaccharide recovered from the culture broth as above was redissolved in 500 volumes of distilled water and recovered by 2 volumes of isopropanol twice. The aqueous solution of the polysaccharide was dialyzed against distilled water in cold room (4°C) with cellophane dialysis sack (250-11, Sigma Chem. Co., U.S.A.) and then freeze dried.

Preparation of polysaccharide hydrolyzate (25,26)

1 g of purified polysaccharide sample was digested with 160 m*l* of 2 N sulfuric acid (Kanto Pure Chem. Co., Japan) in boiling water bath for 24 hours and neutralized with barium hydroxide (Kanto Pure Chem. Co., Japan). The hydrolyzate was filtered with an aid of charcoal and distilled water. The filtrate was loaded on Amberlite IR-120 resin [H-form, 16-45 mesh, previously washed with 2 N hydrochloric acid and distilled water (pH 5.0), Fluka Chem. Co. Switzerland] and eluted with distilled water (pH 5.0).

Analysis of sugar component

Sugar component in polysaccharide hydrolyzates was analyzed with high performance liqiuid chromatograph [carbohydrate anlaysis column (3.9 mm diameter × 30 cm length), refractive index detector, mobile phase; acetonitrile (85)/ water (15) (LC grade, E. Merck, U.S.A.), flow rate 1.5 m*l*/min., Waters, U.S.A.]

Infrared spectroscopy (27)

Polysaccharide sample (2.5 mg) was completely ground with potassium bromide (400 mg, infrared

grade, Wako Pure Chem. Co., Japan) in mortar and the mixture was pressed to form pellet. The IR spectrum of the sample was obtained by using Infrared Spectrometer (Shimadzu Model IR 435, Shimadzu, Seisakusho, Ltd. Co., Japan).

Determination of acetyl content (28)

Acetyl content in the polysaccharide sample was determined by colorimetry. The polysaccharide sample was deacetylated with alkaline hydroxylamine solution. After thorough mixing, ferric perchlorate was added to form a pigmented complex. The optical density of reaction mixture was measured at 520 nm after filtration. The acetyl content was calculated by using glucose pentaacetate as standard material.

Determination of molecular weight

High performance gel permeation chromatography was performed by using ALC/GPC 150-C Chromatograph (Millipore, Waters, U.S.A.) with μ -bondagel E-linear column and RI detector. The The solvent was purified water injection volume was 10 μl. The flow rate of solvent was 1.0 ml/min and the operating temperature was 27°C. The retention time of sample was compared with that of standard carbohydrates for estimating the molecular weight of polysaccharide. The standards were dextran (Sigma Chem. Co., U.S.A., Mw; 2,000,000, 488,000, 73,400, 35,600), raffinose (Sigma Chem. Co., U.S.A., MW; 504) and maltose (Sigma Chem. Co., U.S.A., MW; 360). The molecular weight was also estimated by determining reducing power (1).

Viscosity

Viscosity was measured with the automatic capillary tubing viscometer designed by Kilp *et al*(29).

Elemental analysis

Elements in the polysaccharide sample was analyzed by elemental analyzer (Perkin-Elmer, 240-C, U.S.A.).

Results and Discussion

Identification of bacterium

The selected polysaccharide-synthesizing bacterium BT-4 was identified. Strain BT-4 had circular, entire, pulvinate, filliform and opaque colonies on both nutrient and YM agar. It was Gram negative strictly aerobic, motile rod (0.68-0.86 $\times$ 2.3-2.88 μm) which had lophotrichic flagella (Table 1, Fig. 1). It did accumulate polybetbetahydroxybutyrate as carbon reserve material and was not pathogenic. Gelatin and starch were not hydrolyzed by this bacterium. Strain BT-4 utilized glucose. Therefore, strain BT-4 was identified as *Pseudomonas delafieldii*.

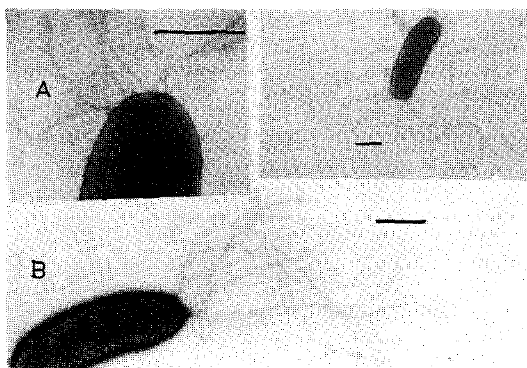

Fig. 1. Transmission electron photomicrograph of strain BT-4 (A and B: higher magnification, Bar: 0.5 μm).

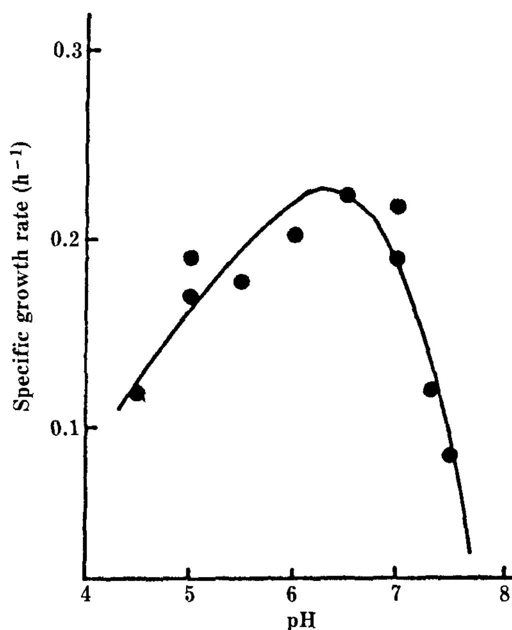

Fig. 2. pH dependency of growth of *Pseudomonas delafieldii* BT-4 in mineral salt medium at 30°C.

Growth study *Ps. delafieldii* BT-4 in salt medium

For studying physiology of microorganism and giv-

Table 1. Morphological and biochemical characteristics of strain BT-4.

Characteristics	Record	Characteristics	Record
Gram staining	−	DNase	−
Cell size	0.68-0.86×	LDC	−
	2.30-2.88 μm	ODC	+
Motility	+	Argininedihydrolase	+
Flagella	polar (several)	Acid and gas form	
Colony	opaque	Glucose	+
Growth	strictly aerobic	Fructose	+
Metabolism	oxidative	Galactose	+
Catalase	+	Xylose	+
Oxidase	+	Mannose	+
PHB	formed	Rhamnose	+
Levan	not formed	Mannitol	+
Growth factor	not required	Ribose	+
Growth at 41°C	−	Arbinose	+
at 12% NaCl	−	Adonitol	+
at pH 3.6	+	Inositol	+
on McConkey agr	+	Malonate	+
Fluorescent		Lactose	−
pigment	not formed	Sucrose	−
Hydrolysis of		Trehalose	−
Starch	−	Raffinose	−
Gelatin	−	Utilization of	
Urease	−	Alanine	+
Indole	−	Dulcitol	−
Methylred	−	Salicin	−
Voges-Proskauer	+	H_2S (TSI)	−
Nitrate reduction	−	Plant pathogenecity	−

LDC: lysine decarboxylase, ODC: ornithine decarboxylase

ing more feasibility to newly found microbial polysaccharide, it is necessary to develop chemically defined lean medium for the process organism of interest. In order to formulate this kind of fermentation media, growth parameter of test microorganism should be examined first.

Fig. 2 shows the change of specific growth rate of *Pseudomonas delafieldii* BT-4 as affected by pH. Growth profile with pH change of culture medium was symmetrical shape and had sharp pH optimum. The optimum pH for growth was 6.5. The maxmum specific growth rate at the optium pH was 0.25 h^{-1} and doubling time was 160 minutes. Williams and Wimpenny (30) reported that doubling time of a *Pseudomonas* sp. was 110-140 minutes and Mian *et al* (31) found the specific rate of *Ps. aeruginosa* to be 0.27 h^{-1}.

Fig. 3 shows the temperature dependency of specific growth rate. The temperature dependency of specific growth rate is normally expressed in Arrhenius equation as follows; $\mu = A \cdot EXP(-E_a/RT)$ where μ is specific growth rate, A is frequency factor, E_a is activation energy for growth, R is gas constant (1.987 cal/mole-°K) and T is absolute temperature. Hence, a plot of log μ against 1/T should be a straight line. As shown in this result, the growth of *Ps. delafieldii* BT-4 was found not to be sensitive to the change of growth temperature used.

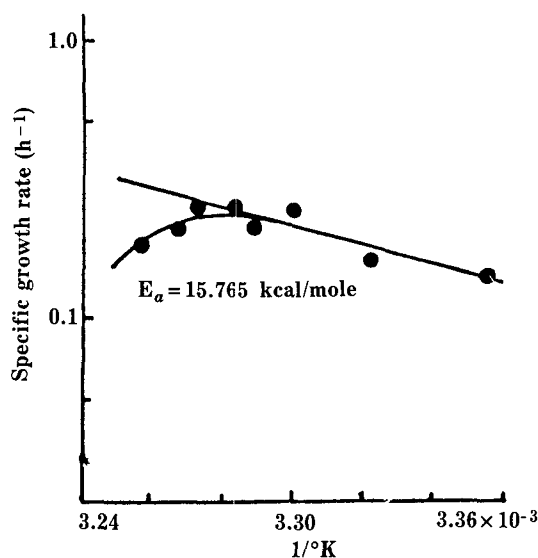

Fig. 3. Arrehenius plot of specific growth rate of *Pseudomonas delafieldii* BT-4 in mineral salt medium.

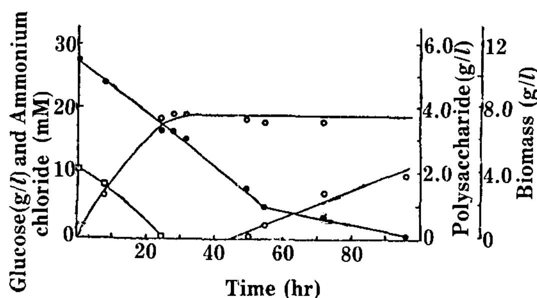

Fig. 4. Time course of polysaccharide production in mineral salt medium by *Pseudomonas delafieldii* BT-4 at 30°C.
(●:Residual glucose, □:NH_4Cl, ○:biomass, o: polysaccharide, initial glucose concentration: 25 g/l, NH_4Cl: 10 mM/l, agitation: 400 rpm, aeration: 1 vvm, working volume 1500 ml)

The optimum temperature was 30-32°C. The activation energy for growth was 15.765 Kcal/mole. Typical activaon energy for growth of microorganism was reported to be 15-20 kcal/mole (32)

Fig. 4 shows the growth pattern and polysaccharide production in bath culture of *Ps. delafieldii* BT-4 in mineral salt medium when using 10 mM of ammonium chloride. The growth in the medium signalled the stationary phase after 25 hours of elapsed fermentation time, when ammounium chloride was completely exhausted. The growth yield based on the glucose consumed was 54.51%. The maxmum biomass production was 7.5 g/l. The initial rate of glucose consumption was 0.4133 g/l/h. The rate in the later phase was 0.0922 g/l/h. The specific glucose uptake rate was 12.29 mg/g-cell/h. the biosynthesis of polysaccharide was also clarified to be commenced when ammonium chloride was completely depleted and thus the growth ceased. Hence it was thought that the polysaccharide might be secondary metabolite (non-growth associated) (14). The specific rate of polaysccharide production was 4.72 mg/g-cell/h and product yield was 38.39%.

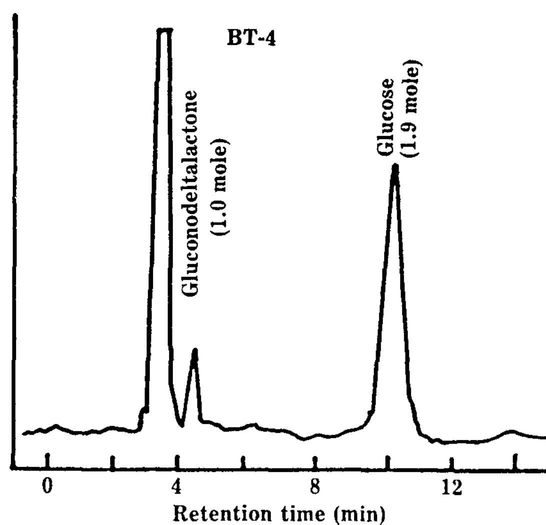

Fig. 5. High performance liquid chromatogram of polysaccharide hydrolyzate (Mobile phase: acetonitrile/water: 85/15).

Analysis of polysaccharide

Purified polysaccharide samples produced in a glucose medium by *Ps. delafiedii* BT-4 was subjected to analysis.

Elemental components were analyzed with Perkin-Elmer 240-C elemental analyzer. The polysaccharide was found to contain carbon (31.85%) and hydrogen (5.15%). For analysis of sugar components, liquid chromatographic analysis was conducted after complete acid hydrolysis and the result was compared with authentic sugar standards. The results are shown in Fig. 5. The polysaccharide was found to be composed of glucose and gluconodeltalatone at the molar ratio of 1.9: 1.0.

Constituent sugar components of microbial polysaccharides are reported to be very much different depending upon the microorganisms (1,16,23,33,34) which produce the polysaccharides

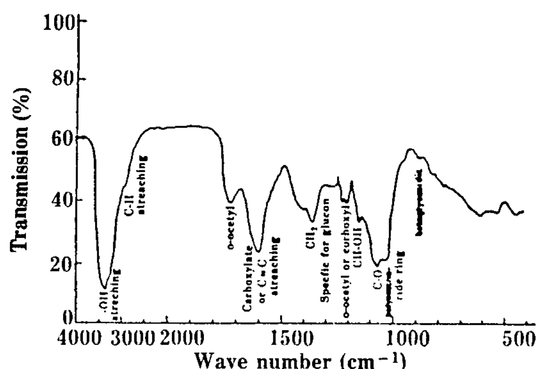

Fig. 6. IR spectrum of polysaccharide produced by *Pseudomonas delafieldii* BT-4.

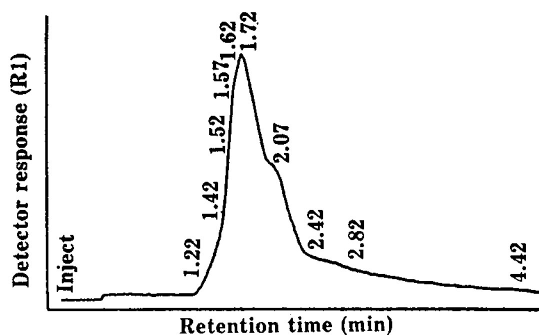

Fig. 7. Gel permeation chromatography of polysaccharide produced by *Pseudomonas delafieldii* BT-4.

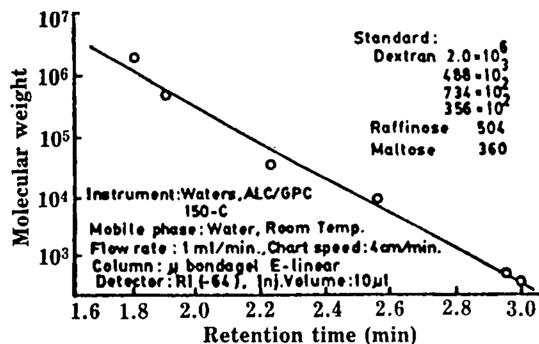

Fig. 8. Molecular weight calibration curve in gel permeation chromatography.

and the carbon sources which they utilized for the production (8,24,35). However, it cannot also be excluded that same polysaccharides are able to be produced by different genus or species of microorganisms (36). BT-4 polysaccharide was very similar in sugar composition to those produced by *Ps. solanacearum* (37), *Ps. aeruginosa* (37), *Acetobacter* sp.(16).

Infrared analysis of polysaccharide was carried out by Shimadzu IR-435 spectrometer. Typical spectrum of native polymer produced by *Ps. delafieldii* BT-4 is shown in Fig. 6. The large peaks at 3400 cm^{-1} are typical of OH streching from bound alcohol group and absorbed water (38,39). The band between 2980-2880 cm^{-1} is indicating the C-H bond (27,38). The peak at 1725 cm^{-1} is characteristic of absorption by o-acetyl group (16,31,40) and band at 1600 cm^{-1} is attributable to C=C ring streching (41) or carboxylate ion (1,42). Heyn (27) reported the absorption below 1400 cm^{-1} was specific for gucans which is possibly due to CH_2 bending. Absorption at 1250 cm^{-1} is characterisitic of o-acetly group (31) or carboxyl group (43). The acetyl group in the polysaccharide was confirmed by chemical analysis (28) and the content was 1.35%(w/w). The strong absorption at 1000-1200 cm^{-1} is reported to be the typical characteristics of all sugar derivatives. The absorption peak at 1160 cm^{-1} in this study must be due to tertiary CH-OH group (38) and the band at 1085 cm^{-1} must be due to C-O streching (27) or typical of polysaccharide ring (38). A peak at 880-890 cm^{-1} occurring in finger print region of sugars indicates that polysaccharide includes predominatly beta glycosidic linkages (1,16,33,34).

Molecular weight was estimated by high performance gel permeation chromatography. The result was as in Fig. 7 and 8. The polysaccharide was of extremely high molecular weight. The number and weight average molecular weight were 157,026 and 56, 413, 600 respectively. The dispersity was 359. The molecular weight of xanthan (44) and pullulan (36) was reported to be 2-15 $\times 10^6$ and 1.7 $\times 10^5$. The molecular weight was also estimated by determining the the number average degree of polymerization. The DP of the polysaccharide was 207. Therefore, the molecular weight could be calculated to be 37260. This kind of difference between physical method and chemical method was reported for the polysaccharide produced by *Bacillus subtilis* by Morita *et al.*(1)

Intrinsic viscosity and relative viscosity

To study the polysaccharide on the molecular level, dilute solution viscometry is commonly introduced (45). The purified polysaccharide was dissolved in deionized water and subjected to capillary viscometry. After measuring and compar-

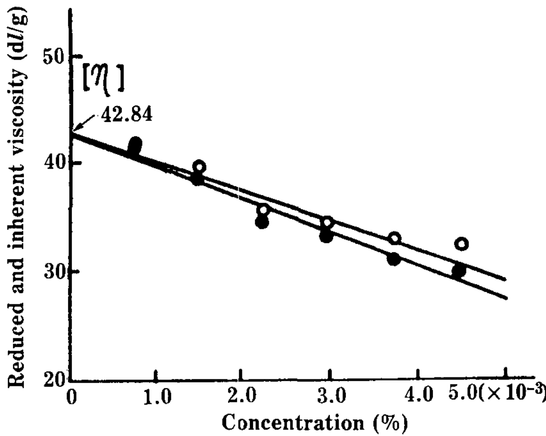

Fig. 9. Reduced and inherent viscosity of polysaccharide produced by *Pseudomonas delafieldii* BT-4.
(○: reduced viscosity, ◐: inherent viscosity)

ing the flow time of polysaccharide solution against distilled water, reduced viscosity (η_{sp}/C) and inherent viscosity [(ln η_{rel})/C] were calculated. The viscosities were plotted against concentration and the relationship could be expressed in Huggin's equation (46) and Kraemer equation (45) as follows;

$\eta_{sp}/C = [\eta] = K'[\eta]^2C$ …… Huggins' Eq.

$(\ln \eta_{rel})/C = [\eta] + K''[\eta]^2C$ …… Kraemer's Eq.

where K is interaction coefficient.

The intrinsic viscosity could be derived by extrapolating the straight line to the zero concentration. The quantity can be regarded as the fractional increase in the viscosity of unit volume of solvent due to the addition of 1g of non-interacting polymer molecules. The intrinsic viscositiy of polysaccharide produced by *Ps. delafieldii* was 42.84 dl/g (Fig. 9) and the relative viscosity at 0.003% concentration of BT-4 polysaccharide was 1.10.

요 약

물성조정제로서 고점도의 다당류를 생산하는 토양세균을 분리 선발하여 동정한 결과 *Pseudomonas delafieldii*로 판단되었다. 이 세균의 증식을 위한 pH와 온도는 6.5 및 30℃이었다. 최대 비성장속도는 0.25 h^{-1}이며 염류배지에서 다당류의 비생산속도, 증식수율 및 다당류 생산수율은 6.25 mg/g-cell/h, 54.5%, 38.39% 이었다. 본 다당류는 포도당과 gluconolactone 을 몰비로 1.9 : 1.0 로 함유한 *β*-glucan 으로 추정되었으며 원소 분석결과 탄소 31.85% 수소 5.15%를 함유하고 아세틸기를 1.35% 가지고 있었으며 고유점도는 42.84 dl/g, 분자량은 5.64×10^7이었다.

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