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New Extracellular Biopolymer Produced by *Methylobacterium organophilum* from Methanol

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*Methylobacterium organophilum*에 의한 메탄올로부터 생성되는 새로운 생물고분자

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A new extracellular biopolymer was produced by *Methylobacterium organophilum* from methanol as a sole carbon and energy source. The purified biopolymer was found to have a high molecular weight of about 4-5 × 10^6 dalton and contained 66% (w/w) of carbohydrate but no polyhydro xybutyrate. Other organic constituents were consisted of protein, pyruvic acid, uronic acid, and acetic acid, whereas content of inorganic ash was 22%. Based on the chemical analysis of the biopolymer by TLC method, the polymer was consisted of glucose, galactose, and mannose with an approximate molar ratio of 2:3:2. The biopolymer solution showed a characteristics of pseudoplastic non-Newtonian fluid. The viscosity of the 1%-biopolymer solution was found to be 18,000 cp at a shear rate, 1 sec^{-1}, which was almost 10 times higher than that of a commercial xanthan gum.

C_1-compounds are attacting much attention as a convenient raw material in the fields of chemical industry and biotechnology. Especially, methanol is expected to be the most useful one for fermentation processes including the production of organic- and amino-acids as well as single cell protein (1). The advantages of methanol as a substrate are its low cost, high purity, complete water miscibility, and restricted use by certain microorganisms. Therefore, a kind of attempts has been performed to establish a new process for the fermentative production, in which both the unique and conventional metabolisms of methylotrophs are utilized (2, 3). Extracellular microbial biopolymers show a great diversities as well as novelty in their structures and properties (4, 5). Because of these reasons, microbial biopolymers have been utilized in a wide range of applications such as a stabilizer, emulsifier, or thickner in foods, as an additive for recovery of petroleum by water flooding, as a selective adsorbent, and a rheological control agent. Extracellular biopolymer production by methylotrophs has been reported both in methane utilizing bacteria (6, 7) and in methanol utilizing bacteria (8-11). Recently, the authors found that *M. organophilum* could accumulate extracellularily an unknown biopolymer under the specific culture conditions (12).

In the present study, the chemical identities and rheological properties of a new polysaccharide-biopolymer produced by *Methylobacterium organophilum* from methanol as a sole source of carbon and energy were investigated.

Materials and Methods

Key words: New biopolymer, *Methylobacterium,* methanol, high viscosity
* Corresponding author

Microorganism

Methylobacterium organophilum (NCIB 11278-KC-1) was used throughout this study (13).

Medium and culture conditions

M. organophilum was cultivated on the following basal medium (per liter): methanol, 0.5%(v/v); $(NH_4)_2SO_4$, 0.3g; KH_2PO_4, 0.63g; Na_2HPO_4, 1.06g; $MgSO_4 \cdot 7H_2O$, 0.45g; and trace elements (Ca^{+2}, Fe^{+2}, Mn^{+2}, Zn^{+2}, Cu^{+2}, Mo^{+2}, Co^{+2}) (12). The The medium was adjusted to pH 7.0 with 2 N-NaOH. Methanol was added aseptically after sterilization of other components.

Batch culture was performed in a 5 liter homemade jar fermentor equipped with standard control units and instrumentations. Fermentor was inoculated with 5% (v/v) inoculum and fermentation conditions were as follws: working volume, 3 *l*; agitation speed, 400-600 rpm; aeration rate, 1 vvm; temperature at 30°C; and pH 7.0 controlled with 3 N KOH/NaOH (1:1 mixture).

Isolation and purification of extracellular biopolymer

The bacterial cells were removed by a centrifugation (10,000 rpm). The cell-free biopolymer was precipitated by addition of 2 volumes of ethanol. The precipitated, fibrous biopolymer was washed with 75% (v/v) ethanol and then redissolved in distilled water. The biopolymer solution was dialyzed throughly against the distilled water for desalting. The purified biopolymer was obtained by freeze-drying the dialyzed polymer solution for the analysis.

Determination of chemical identity

The freeze-dried biopolymer was dried until constant weight in a 105°C-dry oven. The total carbohydrate contents of purified biopolymer were determined by Phenol-Sulfuric acid method (14) and expressed as glucose. The protein content of biopolymer was measured according to Lowry methods (15) and JASCO spectrofluorometer. The content of reducing sugar was determined by DNS method (16) after complete hydrolysis with trifluoroacetic acid. The carbazol method (17) for uronic acid, Friedemann method (18) for pyruvic acid, and hydroxamic acid method (19) for acetic acid were applied, respectively.

Elemental analysis was conducted with a Perkin-Elmer 240-C Elemental Analyzer and the infrared spectrum was obtained by using IR spectrometer (283B Perkin-Elmer) with KBr pellet.

Determination of molecular weight

The molecular weight of purified biopolymer was determined by a gel filtration chromatography using Sepharose 6B and dextrans of known molecular weight (10,000, 80,000, 500,000, 2,000,000 dalton) were used as standards.

Thin layer chromatography (TLC)

The biopolymer was completely hydrolyzed with trifluoroacetic acid at 121°C for 1 hour. The component sugars were identified by TLC using ascending method on a cellulose plate and the spots were visualized by spreading the alkaline silver nitrate solution. Individual monosaccharides were extracted from TLC plate and determined the molar composition. The following solvent systems were used; a) EtOAc: pyridine: acetate: H_2O=5:5:1:3; b) n-propanol: ethylacetate: H_2O=6:1:3; c) butanol: pyridine: 0.1 N HCl=5:3:2 (19-22).

Measurement of viscosity

A Haake Rotovisco Rheometer, Model RV2,

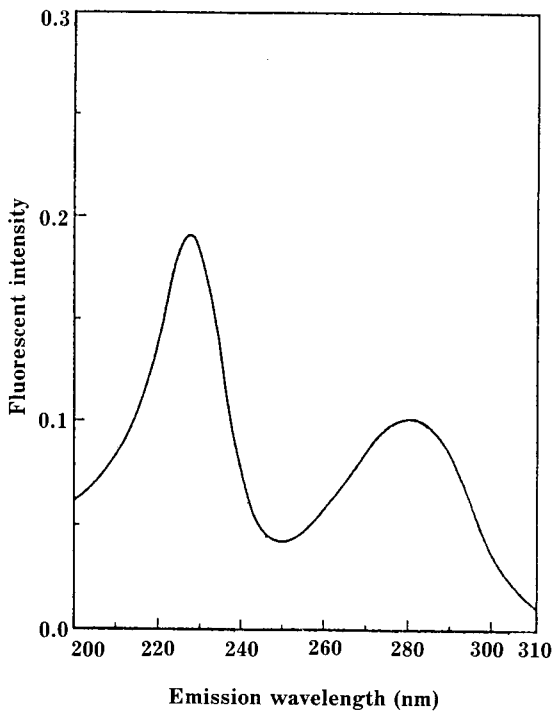

Fig. 1. UV fluorescence spectrum of the biopolymer.

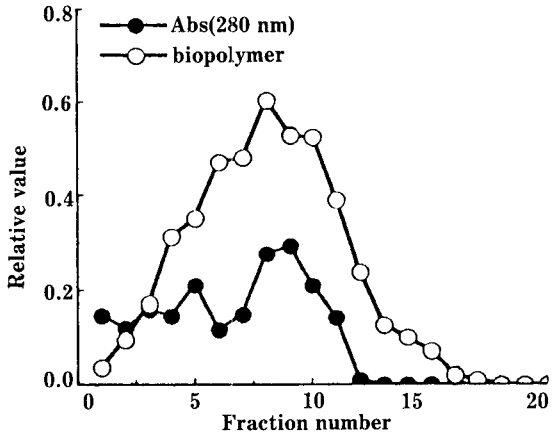

Fig. 2. Elution profiles of the biopolymer and protein.

equipped with MV1 sensor system was used for measuring the viscosity of the biopolymer solution at various concentrations. The temperature was controlled at 25°C by an external circulatory thermostat.

Results and Discussion

Determination of chemical identities of biopolymer

In order to determine the chemical identities of the purified biopolymer produced on methanol-containing medium by *M. organophilum*, a variety of tests were performed. By running a UV spectrofluorescence with the prufied polymer, it was observed that the biopolymer seemed to contain protein moiety and no nucleic acid since there was a absorption peak at 280 nm (Fig. 1). The presence of protein was further confirmed by Bradford reagent (23) and by the gel filtration chromatography using Sepharose 6B as shown in Fig. 2. It was confirmed that the protein was not contaminated but bound to biopolymer. The protein content was determined as 4.5% (w/w). Poly-β-hydroxybutyric acid was not detected. The phenol-Sulfuric acid method for total carbohydrates showed that the polymer contain-

Table 1. Chemical identity and elemental analysis of the purified biopolymer.

Constituent components	W/W(%)
Total carbohydrate	65.4
Reducing substance	57.6
Protein	4.73
Pyruvic acid	4.17
Uronic acid	9.2
Acetic acid	0.68
Inorganic ash	22.21
Polyhydroxybutyrate	Negative
Nucleic acid	Negative
Elemental analysis	
Ash	22.21
Carbon (C)	31.2
Hydrogen (H)	4.78
Oxygen (O)	41.01
Nitrogen (N)	0.56
Sulfur (S)	0.25

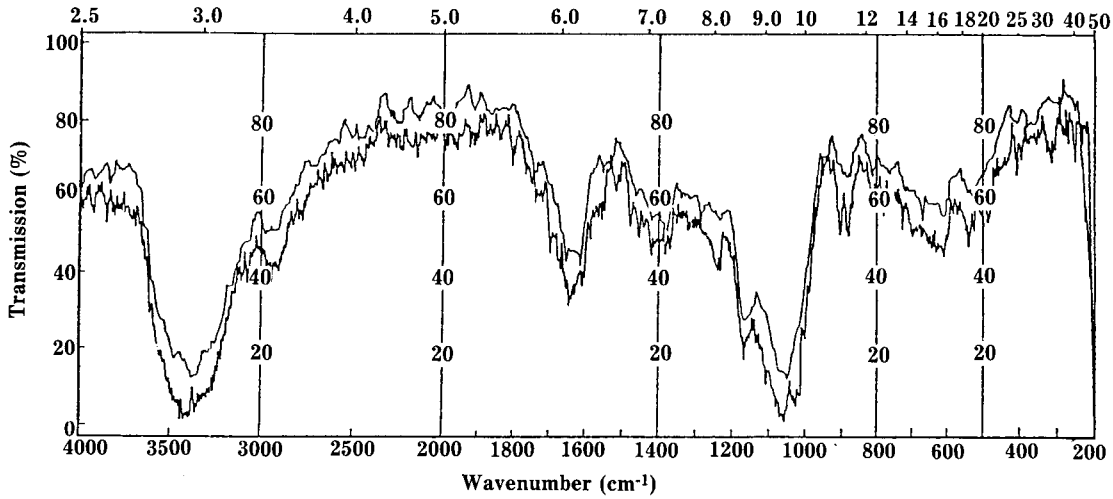

Fig. 3. Infra-Red spectrum of the biopolymer.

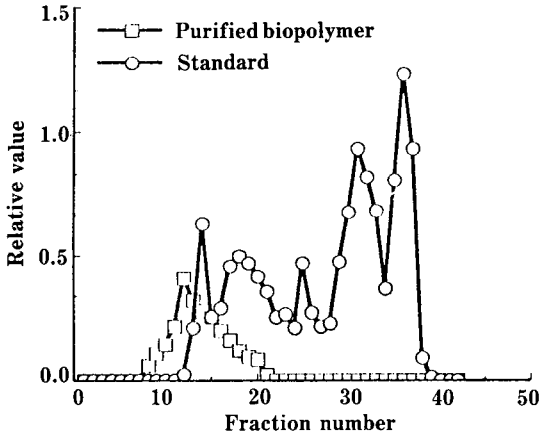

Fig. 4. Elution profiles of the purified biopolymer and standard molecular marker (dextran).

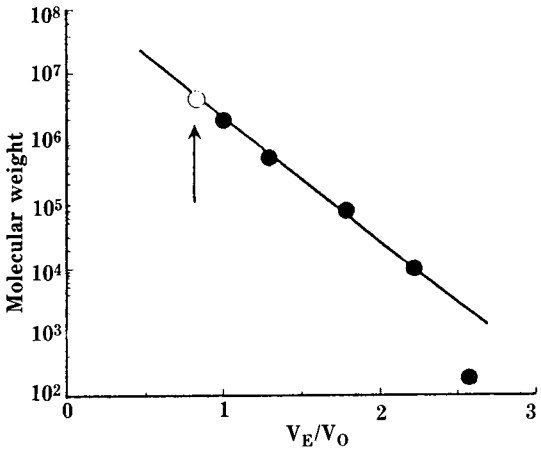

Fig. 5. Determination of molecular weight of the biopolymer.

ed 66% (w/w) of sugar and the DNS method indicated 57.6% (w/w) of reducing substances in the polymer. The Friedemann method for pyruvic acid, the hydroxamic acid method for acetic acid, and the carbazol method for uronic acid indicated that 14% of organic acids was contained in the polymer. Its molar ratio was found to be 4:1:8. The polymer contained also 22% of ash.

According to an elemental analysis, the constituent elements were carbon (31.2%), hydrogen (4.78%), and nitrogen (0.56%). Table 1 summarized the chemical identity and the elemental components of biopolymer. Typical IR spectrum of biopolymer produced by *M. organophilum* was represented in Fig. 3. The absorption peak at 3400 cm^{-1} was characteristic of OH streching from bound alcohol group and absorbed water, and the peaks in the range of 2900 to 2800 cm^{-1} were an indication of the C-H streching. The absorption peaks around 1720 cm^{-1} and 1060 cm^{-1} were characteristics of C=O and C-O (ketone) groups. The absorption peaks at 3400-3500 cm^{-1} and 1610-1655 cm^{-1} suggest the amine and amino group, respectively. The strong absorption peaks observed in range of 1,000 to

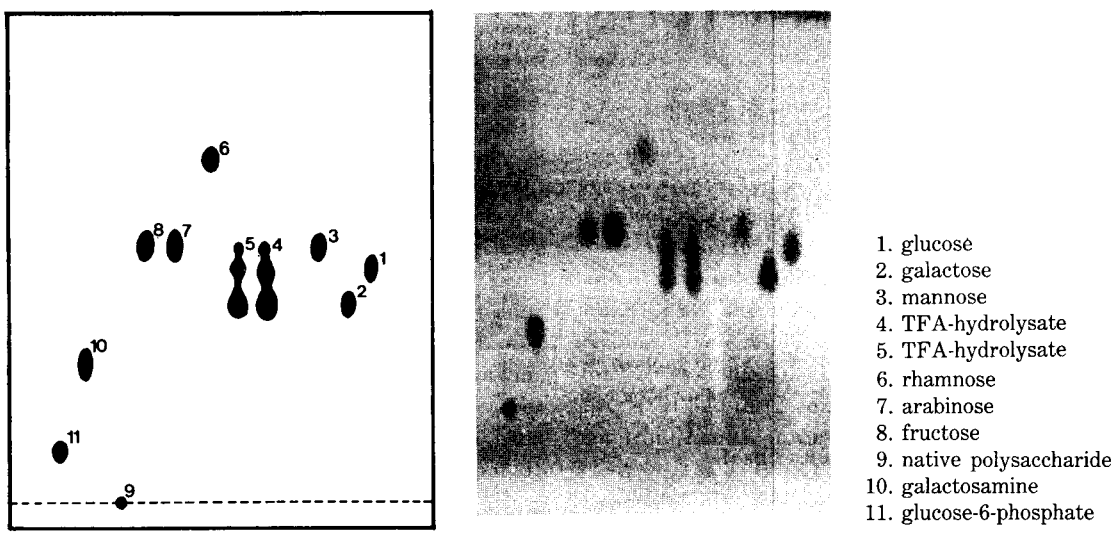

Fig. 6. Identification of the biopolymer by TLC.
Solvent system; EtOAc: pyridine: acetate: H_2O=5:5:1:3

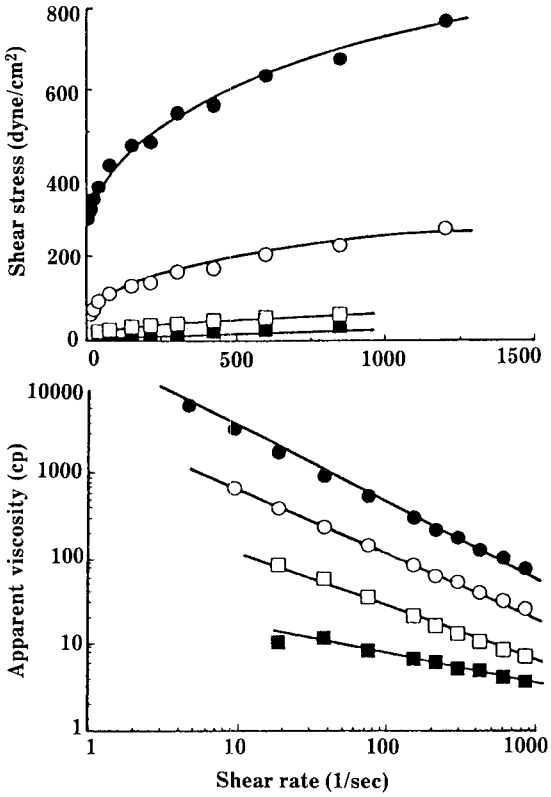

Fig. 7. Rheogram of the biopolymer solution.
Symbols; ■, 1g/*l*; □, 3g/*l*; ○, 5g/*l*; ●, 10g/*l*

1200 cm^{-1} was generally known as the typical characteristics of all sugar derivatives.

Determination of molecular weight

Molecular weight was estimated by gel permeation chromatography using Sepharose 6B. The elution profiles of biopolymer and dextrans as standard molecular markers are shown in Fig. 4. The average molecular weight of polymer was determined as about 4-5 × 10^6 dalton (Fig. 5). The molecular wight of this biopolymer was much higher than that of xanthan which was known as 2 × 10^6 dalton at maximum (24).

Sugar composition of biopolymer

After trifluoroacetic acid hydrolysis of the polymer, TLC were carred out in three different solvent systems described in Materials & Methods. The developed spots in TLC are shown in Fig. 6. The constituent sugars were determined as glucose, galactose, and mannose and it was found that the molar ratio of glucose, galactose, and mannose was 2:3:2.

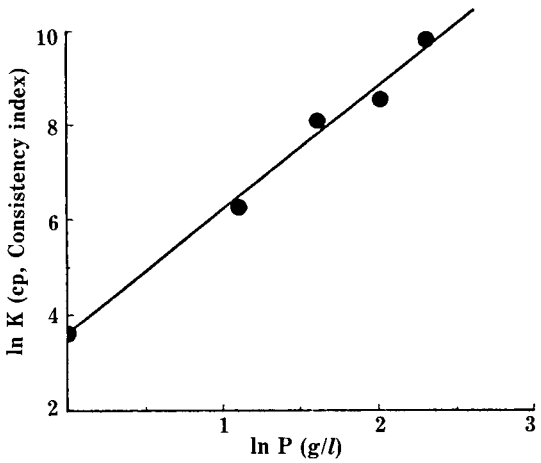

Fig. 8. Relationship between consistency index (K) and concentration of the biopolymer (P).

Rheological characteristics of biopolymer solution

The rheological properties of the biopolymer solution were examined in the range of 0.1 to 1.0% (w/w) at 25°C. The biopolymer solution showed the characteistics of pseudoplastic non-Newtonian fluid and higher degree of pseudoplasticity as the polymer concentration increased (Fig. 7). The consistency index (K) as a function of biopolymer concentration is shown in Fig. 8. The viscosity changes could be expressed as function of concentration in the following equation;

$K = AP^B$

$\ln K = \ln A + B \ln P$

where A and B are the constant and P is the concentration of biopolymer. From Fig. 8, A and B were determined as 35 and 2.6, respectively. The apparent viscosity of 1% solution at the shear rate of 1 sec^{-1} (K value) was about 18,000 cp, which was almost 10 times higher than that of xanthan gum.

Acknowledgement

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요 약

*Methylobacterium organophilum*이 탄소원과 에너지원으로서 메탄올로부터 생성하는 새로운 다당류계 생물고분자의 화학적 성질과 물성학적 성질을 관찰하였다. 분리정제한 다당류의 분자량은 약 4-5×10^6 dalton 정도로 고분자량을 지니고 있다. 다당류의 화

학적 조성은 건조중량의 66% 가 탄수화물로 구성되어 있으며 이들의 88% 가 환원당으로 구성되어 있다. 특이하게 본 고분자는 건조중량의 4.7% 가 단백질로 구성되어 있다. 다당류는 glucose, galactose, 그리고 mannose을 몰비로 2 : 3 : 2 함유하고 있으며 탄수화물 이외에도 pyruvic acid, uronic acid, acetic acid 등을 함유하고 있다. 정제된 다당류를 증류수에 용해시켜 농도별로 점도를 측정한 결과 비뉴톤성 성질 중 pseudoplastic 성질을 보였으며 농도가 증가함에 따라서 겉보기점도 증가가 월등히 높았다. 동일한 1% 용액의 경우 K(consistency index) 값을 비교하면 xanthan gum에 비해 약 10배 정도 높은 18,000 cp 값을 나타내었다.

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