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Isolation and Characterization of a Novel Exopolysaccharide-Producing *Paenibacillus* sp. WN9 KCTC 8951P

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Abstract A bacterial strain WN9, which produced a new type of extracellular polysaccharide, was isolated from soil samples. By morphological, physiological, biochemical, and phylogenetic studies, strain WN9 was identified as a *Paenibacillus* sp. and it was named as *Paenibacillus* sp. WN9, which produced a high molecular extracellular polysaccharide from glucose. The molecular weight of the exopolysaccharide (EPS-WN9) was estimated to be about 31.5 mega-Da. The FT-IR spectrum of EPS-WN9 revealed typical characteristics of polysaccharides. EPS-WN9 consisted of D-glucose and D-mannose with a molar ratio of 1:1.4 being identified as a neutral sugar component. The acidic component of EPS-WN9 was tyrosine. Rheological analysis of EPS-WN9 revealed that the pseudoplastic property and its apparent viscosity remained stable at various temperatures and pHs.

Key words: *Paenibacillus* sp., novel polysaccharide

Many microorganisms including bacteria, yeasts, fungi, and algal cells excrete biopolymers outside the cell as an extracellular polysaccharide [7, 11]. These biopolymers have physicochemical and rheological properties which are different from those of synthetic polymers. They are biodegradable and generally not harmful to the environment. Therefore, microbial polysaccharides have recently attracted much attention as a subject for research. Some of these polysaccharides have found many applications in various areas of industry such as oil recovery, foods, detergents, textiles, adhesives, cosmetology, pharmacology, paints, concretes, and wastewater treatments [1, 3, 17, 23].

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It is not easy to test the useful properties of microbial polysaccharides and to select them from nature for its specific field of application. Therefore, it is necessary to find a suitable screening method for targeting the application field. Thus, hundreds of polysaccharide-producing microorganisms were isolated from soil, and some rheological properties, such as viscosity and stability in a broad range of pHs, were investigated. From this selection process, we discovered a new bacterial strain, *Paenibacillus* sp. WN9 KCTC 8951P, producing a unique polysaccharide which has a possible use as a thickner or flow controller.

This paper deals with the screening and identification of this novel polysaccharide-producing bacterium and also some characteristics of the polysaccharide it produced.

Materials and Methods

Isolation and Identification of the Strain

The microorganisms were isolated from soil samples obtained from various regions in Korea. The isolation medium (pH 7) consisted of 30 g glucose, 2.5 g yeast extract, 2.5 g malt extract, 1 g K_2HPO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, and 15 g agar in 1-l distilled water. Mucous colonies were isolated from the medium after three days of incubation at 30°C. For selection, mucoid materials which were prepared from each isolate were tested for properties such as viscosity and pH stability. The isolate was identified for its morphological, physiological, and biochemical properties, according to Bergey's Manual of Systematic Bacteriology [15], Manual of Identification of Medical Bacteria [6], and the procedure previously described [22].

Cultivation

The cultivation medium (pH 7.0) for extracellular polysaccharide (EPS) production contained 10 g glucose, 5 g yeast extract, 1 g K_2HPO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.5 g KCl, 0.1 g $CaCl_2 \cdot 2H_2O$ and 0.01 g $FeSO_4 \cdot 7H_2O$ in 1-l distilled water, and the cells were cultivated at 30°C. For the seed preparation, the cells were cultivated in 250-ml Erlenmeyer flasks containing 40 ml of the culture medium in a gyratory shaking incubator at 150 rpm for 48 h. For production of polysaccharide, 40 ml of seeds were inoculated into a 3-l jar fermenter (Bioengineering AG, Switzerland) containing 2 l of the cultivation medium. Agitation speed and aeration rate were 500 rpm and 0.5 vvm, respectively.

Purification of EPS-WN9

After cultivation, the viscous culture broth was diluted about four folds with distilled water, and cells were removed from the diluted broth by centrifugating at 22,000 ×g for 4 h. After discarding the cell pellet, EPS was isolated from the supernatant by precipitating with four volumes of cold ethanol. The precipitated crude EPS was dissolved in deionized water, and 10% cetylpyridinium chloride (CPC) solution was then added to isolate the acidic EPS until no further precipitate was formed. The resultant insoluble acidic EPS-CPC complex was collected by centrifugation and redissolved in 10% NaCl solution. After dialysis against deionized water, the EPS was precipitated by the addition of ethanol and was lyophilized. The purified EPS was designated as EPS-WN9.

Characterization of EPS-WN9

The molecular weight of EPS-WN9 was estimated by gel permeation chromatography (GPC) with a Polymer PL-Gel column (8 μm, 300 Å, 300×7.5 mm; Polymer Lab., U.K.), using a conventional HPLC system with a differential refractometer detector (Waters 410, U.S.A.). The mobile phase was water at 50°C at a flow rate of 1 ml/min. The columns were calibrated by a standard calibration method including dextran (Mw: 10 kDa-2 mDa; Sigma, U.S.A.) as a standard molecular weight. Twenty-five μl was injected into the HPLC via a sample loop. The infrared (IR) spectrum of EPS-WN9 was obtained with a FT-IR spectrophotometer (Bruker, IFS 66/cs, Germany) with KBr pellets. Complete hydrolysis of EPS-WN9 was carried out in 2 M trifluoroacetic acid (TFA) at 121°C for 1 h. After hydrolysis, TFA was removed by an evaporator at 40°C and then lyophilized. For analyzing neutral sugar components, HPLC with differential refractometer was employed with an Aminex HPX column (300×7.5 mm; Bio-rad, U.S.A.). The mobile phase was water at 50°C at a flow rate of 0.5 ml/min. The acidic component of EPS was analyzed with an amino acid analyzer (Biochrom 20; Pharmacia Biotech, U.K.).

Rheological Properties of EPS-WN9

The EPS-WN9 was prepared from the culture broth of *Paenibacillus* sp. WN9 KCTC 8951P. Apparent viscosities of EPS-WN9 were measured with a Brookfield digital viscometer model LVDV-III along with a small sample adapter (spindle No. 25; Brookfield Engineering Lab., U.S.A.). The rheological properties of EPS-WN9 were compared with those of xanthan gum (Sigma, U.S.A.). The consistence index or flow behavior index were calculated by using the Ostwald's Power-low equation from the measured shear rate and shear stress of EPS solutions [10].

Analytical Methods

Concentrations of cells and EPS were determined by measuring dry weight. The viscous culture broth was diluted four folds with distilled water and cells were harvested from the diluted culture broth by centrifugation at 22,000 ×g for 4 h. Harvested cells were washed and dried to a constant weight in a convection oven at 80°C for determining the cell dry weight. EPS that existed in the supernatant was precipitated by adding four volumes of cold ethanol, recovered, and then dried until it reached its constant weight. Glucose was determined by the enzyme kit (Sigma, U.S.A.) using glucose oxidase.

RESULTS AND DISCUSSION

Screening and Identification of the EPS-Producing Bacterium

Two-hundred bacterial strains producing the mucous material were isolated from soil samples on agar plates of the isolation medium. A mucoid colony on the agar plate presumed to be an EPS producer was cultured in a 250-ml Erlenmeyer flask containing 40 ml of the culture medium. For selection, materials produced from each isolate were tested for viscosity and pH stability (data not shown). Among the materials tested, mucoid from the strain WN9 showed the highest viscosity and relative stability in a wide range of pHs. From these results, strain WN9 was selected to be the most suitable candidate as a practical EPS producer. Additionally, it was isolated from Chinju in the Kyeong-Nam province.

The bacteriological characteristics of strain WN9 were investigated (Table 1). After incubating for two days on the YPD agar medium under dark, colonies were circular, convex, glistening, and opaque. Under irradiation with visible light, the color of the colonies changed to pink. Strain WN9 was gram-positive, rod shaped with peritrichous flagellar, and an aerobic bacterium. They produced ellipsoidal spores in a swollen sporangia. The cell size of the strain was 0.7-1 μm by 4-5 μm. Cells grow at temperatures between 15 and 45°C, but not at 50°C. Its optimum growth temperature was 35-40°C. The pH range for growth was 5 to 9 with an

Table 1. Bacteriological characteristics of strain WN9.

Characteristics	Results[1]
Gram staining	+
Endospore	+
Shape	rod
Cell size (m)	0.7-1×4-5
Mobility	+
Catalase	+
Oxidase	+
Oxidation/fermentation test	oxidative
Indole production	-
Methyl red reaction	-
Voges-Proskaur test	- (pH 6.6)
Citrate assimilation	-
Acid production from	
D-glucose	+/-
D-xylose	+/-
L-arabinose	+/-
D-mannitol	+/-
Growth temperature	
at 15-45°C	+
at 50°C	-
Growth concentration of NaCl	
at 1.0%	+
at 3.0%	-
Growth pH	
at 4.0	-
at 5.0-9.0	+
at 10.0	-
Potassium nitrate reduction	-
Urea hydrolysis	+/-
Starch hydrolysis	+/-
Casein hydrolysis	+/-
Gelatin liquifaction	+/-

[1]+, positive; +/-, positive, weak reaction; -, negative.

optimal growth at pH 7-8. The strain was able to hydrolyze starch, casein, and gelatin weakly and produced acid from glucose, xylose, arabinose, and mannitol. The strain showed a negative reaction in IMViC test. From these results, the strain WN9 was considered to belong to the *Bacillus* genus or a related one [15].

The cellular fatty acids of the strain WN9 were extracted and analyzed according to the instructions of the Microbial Identification System (MIDI; Microbial ID). Table 2 shows the cellular fatty acid composition of the strain. The strain WN9 is characterized by a major anteiso-$C_{15:0}$ (68.15%) which is found in aerobic, endospore-forming genera [18, 19] and by other minor fatty acids such as iso-$C_{14:0}$ (1.96%), iso-$C_{15:0}$ (3.84%), $C_{15:0}$ (4.34%), iso-$C_{16:0}$ (7.53%), $C_{16:0}$ (5.73), iso-$C_{17:0}$ (1.59%), and anteiso-$C_{17:0}$ (6.87%). Although anteiso-$C_{15:0}$ is a major fatty acid, the analysis of cellular fatty acid profiles could not establish a definitive taxonomic position for the strain WN9. The major isoprenoid quinone was MK-7, which is a major menaquinone generally found in aerobic, endospore-forming rods [18, 19].

In recent years, the nucleotide sequence comparison of the 16S rDNA sequence has been used as a powerful tool for identifying bacterial species and for determining exact phylogenetic and taxonomic positions of similar genera and species, since 16S rDNA is highly conserved in the evolutionary aspects [13, 22]. To establish the taxonomic position of the strain WN9, the primary structure of the 16S rDNA (1488 bp; GenBank accession number is AF1643345) was determined by sequencing the subcloned 16S rDNA of strain WN9 for phylogenetic analysis. As a result of its homology search with GenBank databases, the phylogenetic tree (data not shown) constructed from the sequence data showed that the strain WN9 appeared within the evolutionary radiation area encompassing the genus *Paenibacillus* species. Levels of 16S rDNA showed 92.4-95.8% similarities between the strain WN9 and the *Paenibacillus* species. The highest 16S rDNA sequence similarity of 95.8% was obesrved between the strain WN9 and *Paenibacillus validus*. The phylogenetic analysis clearly established that the strain WN9 was a member of the *Paenibacillus* species and it was therefore named as *Paenibacillus* sp. WN9. This strain was deposited into the Korean Collection for Type Cultures (KCTC) with the collection number KCTC 8951P.

Production of EPS

Figure 1 shows typical time courses of cell growth and EPS production from *Paenibacillus* sp. WN9 KCTC 8951P. The EPS production began in an early exponential phase of growth and continued until it reached the stationary phase of growth. It was apparent that EPS production by *Paenibacillus* sp. WN9 KCTC 8951P was definitely growth-dependent. Microbial polysaccharides are usually produced under a limited condition of nitrogen or phosphate [4, 5]. However, *Paenibacillus* sp. WN9 KCTC 8951P continued to produce EPS during cell growth under a nitrogen sufficient condition. Productions of some EPS such as A49-Pol from *Bacillus polymyxa* [9] and xanthan gum from *Xanthomonas campestris* [16] were also enhanced in higher nitrogen concentrations. After 44 h of cultivation, maximum concentrations of 2.7 g/l of EPS and 0.9 g/l of cell were obtained under 4.4 g of glucose consumption, respectively. In fact, there was a

Table 2. Cellular fatty acid composition of strain WN9.

Profiles of fatty acids	iso 14:0	iso 15:0	ante 15:0	*n* 15:0	iso 16:0	*n* 16:0	iso 17:0	ante 17:0
Content (%)	1.96	3.84	68.15	4.34	7.53	5.73	1.59	6.87

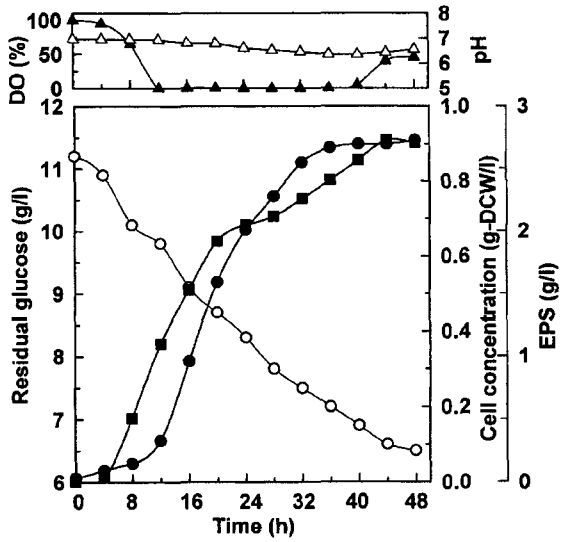

Fig. 1. A time course of fermentative production of EPS-WN9 by *Paenibacillus* sp. WN9 KCTC 8951P.
Symbols: ▲ dissolved oxygen; △ pH; ○ residual glucose; ● cell growth; ■ EPS production.

slight decrease in the pH of the culture medium (pH 0.6) during the course of cultivation. This pattern was different from those reported in other EPS productions such as A49-Pol, zooglan, and bacterial alginate [12, 20, 21].

Characteristics of Isolated EPS-WN9

The purified acidic EPS, named EPS-WN9, from its final preparation was investigated in terms of molecular distribution, IR spectrum, and its constituents. A GPC pattern of EPS-WN9 is shown in Fig. 2. The molecular weight of EPS-WN9 was estimated to be about 31.5 MDa,

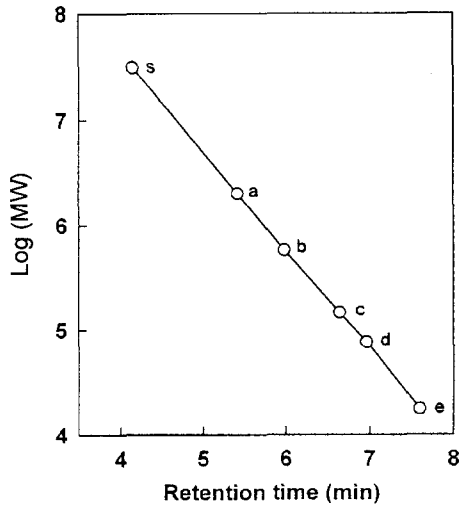

Fig. 2. Estimation of molecular weight of the EPS-WN9 using gel permeation chromatography with HPLC.
Standard marker: (a) 200 MDa dextran, (b) 500 kDa dextran, (c) 70 kDa dextran, (d) 40 kDa dextran, (e) 10 kDa dextran, (s) EPS-WN9.

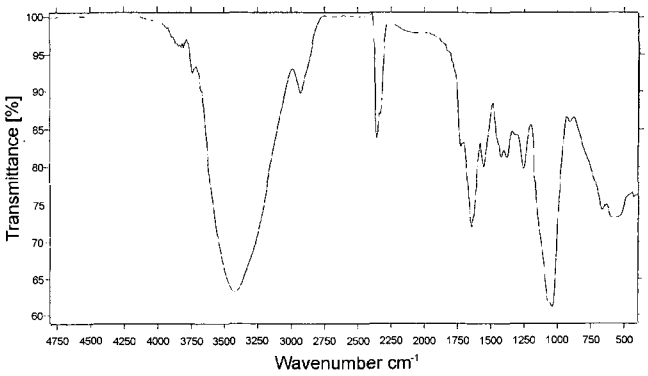

Fig. 3. Infrared absorption spectrum of EPS-WN9 in KBr.

and this was about twenty times higher than other microbial extracellular polysaccharides [2, 8, 14].

As shown in Fig. 3, the IR spectrum of EPS-WN9 exhibited a broad O-H stretching absorption band around 3,420 cm^{-1}, and a minor C-H stretching band at 2,930 cm^{-1} including a strong C-O stretching band at 1,037 cm^{-1}. The spectrum also showed bands of the carboxyl group near 1,645 cm^{-1} and 1,400 cm^{-1}.

Constituents of EPS-WN9 were investigated after TFA hydrolysis. HPLC analysis showed that neutral sugar constituents of EPS-WN9 were D-glucose and D-mannose The molar ratios of D-glucose over D-mannose were approximately 1:1.4. The acidic constituent was shown to be tyrosine by an amino acid analyzer. Therefore, these results suggest that EPS-WN9 is an acidic heteropolysaccharide that contains tyrosine. Further investigations are under way to elucidate the chemical structure of EPS-WN9.

Rheological Properties of EPS-WN9

The usefulness of polysaccharides is determined by their ability to alter rheological properties of water. Xanthan is the most widely used microbial polysaccharide and has a variety of applications in different areas of industry such as oil recovery, foods, detergents, cosmetology, pharmacology, paints, and other chemistries [3, 11]. Therefore, the rheological properties of EPS-WN9 were compared with those of xanthan (produced by *Xanthomonas campestris*; Sigma, U.S.A.).

Figure 4a shows the viscosities of both polysaccharides by examining changes of the shear rate. It showed the pseudoplastic behavior in which its viscosity decreased when the shear rate increased. EPS-WN9 solution showed a little higher degree of pseudoplasticity than xanthan. This suggested that the EPS-WN9 solution showed a somewhat different viscosity behavior when used with the xanthan solution. Figure 4b shows the change in viscosity in different concentrations of polysaccharides at a shear rate of 22 sec^{-1}. The viscosity of 1.0% EPS-WN9 (w/v) solution was 9-fold higher than its 0.2% solution, whereas the corresponding viscosities of xanthan solution had a 6-fold difference. The viscosity of 1.0% EPS-WN9 solution

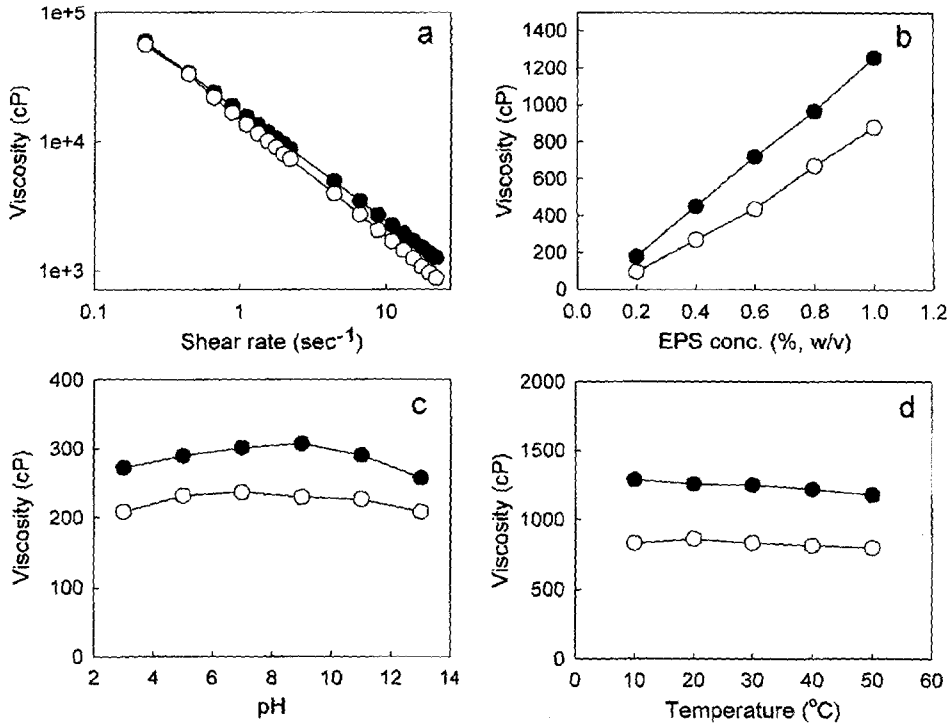

Fig. 4. Effect of shear rate (a; 1% solution), polysaccharide concentration (b; shear rate at 22 sec^{-1}), pH (c; 0.5% solution, shear rate at 28 sec^{-1}), and temperature (d; 1% solution, shear rate at 22 sec^{-1}) on the viscosity of EPS-WN9.
Symbols: ○ EPS-WN9; ● xanthan gum.

was 880 cp, which represented 70% of its viscosity rate of the same concentration with 1.0% xanthan solution.

The effects of temperature and pH on the viscosity of the solution are shown in Figs. 4c and 4d, respectively. The viscosities of the EPS-WN9 solutions were stable in the ranges of temperature and pH tested. Stabilities of the viscosity of EPS-WN9 solution at different temperatures and pHs were similar to those of xanthan.

Flow index and consistency index of EPS-WN9 are shown in Figs. 5a and 5b, respectively. EPS-WN9 is characterized by having a lower flow index and consistency index compared to xanthan. A lower flow index indicates a higher pseudoplasticity level which is defined by a rapid viscosity decrease as the shear rate increase. This property is beneficial in controlling the flow of aqueous systems.

EPS-WN9 was compatible to high concentrations of salts such as $CaCl_2$, $Ca(NO_3)_2$, $MgCl_2$, K_2HPO_4, H_3PO_4, and $CoCl_2$, and it was also compatible to cement extract and surfactants (data not shown). These properties of EPS-WN9 may be very useful for its practical application in various fields of industries like thickners, flow controllers, and cleaners.

From the above-described chemical identities and rheological properties, EPS-WN9 may be considered to be a

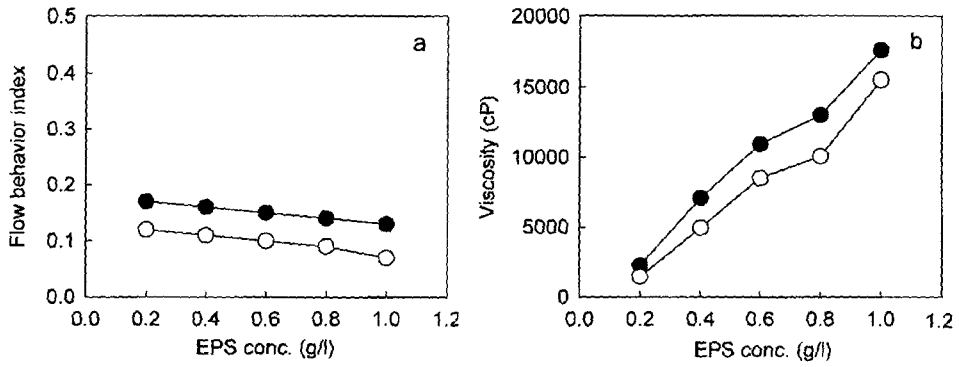

Fig. 5. Flow index (a) and consistency index (b) of EPS-WN9 and xanthan gum.
Symbols: ○ EPS-WN9; ● xanthan gum.

new anionic heteropolysaccharide. It has several important properties such as a high stability for temperature and pH with an excellent compatibility with salt. Further investigations are in progress to improve the yield of EPS-WN9 by optimizing cultivation conditions which include such physicochemical parameters as temperature, pH, DO level, and nutrients.

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