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Characteristics of a novel bacterial polysaccharide consisted of glucose and mannose as major components

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ABSTRACT

A bacterial strain, designated KMBL 5781, producing a high level of extracellular biopolymer was isolated from soil and identified as *Mitsuaria chitosanitabida* based on the 16S rRNA gene sequences. The biopolymer was purified by the sequential precipitation using ethanol, cethyl trimethyl ammonium bromide and then ethanol again. Its molecular weight was estimated to be about 54.7 kDa by the MALDI-TOF analysis. GC–MS analysis revealed that it is a polysaccharide consisting of glucose, mannose and galactose with approximate molar ratio of 18:6:1. In lower concentrations such as 0.5% and 1% viscosities of the EPS solution was higher than that of the xanthan gum used as a control. At the final range of shear rate the viscosity of 0.5% EPS was 34 cP while 0.5% xanthan showed 19.8 cP. The 1% EPS solution at the same rate has shown 59 cP and the viscosity of 1% Xanthan was 35 cP. When heated from 20 to 92 °C, the EPS solution (2%) remain stable from room temperature until 60 °C and showed significantly higher viscosity at 92 °C than 2% xanthan. The EPS hydrogel (2%) was strongly stable, which released no water during the incubation at 4 °C for 20 days and even after the tubes containing the gel was centrifuged at 4000× *g* for 10 min. Addition of the EPS into the starch solution resulted in the increase the weight and volume of the gel.

1. Introduction

Exopolysaccharides (EPSs) from microbial origin exhibit large applications in the pharmaceuticals, foods, cosmetics, minerals mining and oil refinery industries because of their peculiar physical and rheological properties. They can modify the flow characteristics of fluids, stabilize suspensions, flocculate particles, encapsulate materials and produce emulsions. Consequently, they can be used as drug delivering, thickening, stabilizing, emulsifying, gelling and water-binding agents in the several industry fields (Freitas, Alves, & Reis, 2011; Sutherland, 2002). There is evidence for the health promoting properties that are attributable to EPSs produced from certain bacteria (Welman, 2009). The application of the polysaccharides was also intensively documented because of their potential industrial interests (Freitas et al., 2011; Patel, Michaud, Singhania, Soccol, & Pandey, 2010).

Bacterial cellulose, an extracellular polymer synthesized by the bacteria belonging to *Gluconacetobacter* spp., has several applications as a component of dental and arterial implants, wound-healing tissues, sensitive diaphragms in audio speakers or headphones, archival documents repair as well as a coating agent of roots and plants to prevent desiccation (Bielecki, Krystynowicz, Turkiewicz, & Klinowska, 2002). Curdlan, a microbial exopolymer synthesized by *Agrobacterium* and *Alcaligenes* species, is used for biodegradable materials for medical and other important uses. Sulfated curdlan used as an immobilizing agent in combination with azidothymidine have been tested as an antiretroviral drug for the treatment of primarily HIV (Lee, 2002). The acidic extracellular succinoglycan produced from several strains of the bacteria belonging to the genera *Rhizobium*, *Agrobacterium*, *Alcaligenes* and *Pseudomonas* is used not only in foods and cosmetics but also to enhance oil recovery (Stredansky, 2002). Another polymer such as alginate synthesized by the bacteria belonging to *Pseudomonas* and *Azotobacter* can be used as stabilizing, thickening, gelling and immobilizing agents and can promote growth of *Bifidobacteria* species (Rehm, 2002). Dextran produced from *Leuconostoc mesenteroides* is used as an agent for the plasma therapies, a prebiotic compound in the food products, animal feeds and cosmetics as well

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as a separation compound based on gel filtration in the research industry (Leathers, 2002).

Among the microbial polysaccharides, the most widely used microbial exopolysaccharide is xanthan produced by *Xanthomonas* bacterium. Xanthan gum is a valuable texturizing agent due to its shear-thinning behavior and water-binding capacity, which is used in a wide range of industrial applications (Born, Langendorff, & Boulenguer, 2002).

The present study was undertaken to determine the taxonomic position of a certain novel strain that was isolated from the soil as an extracellular biopolymer-producing bacterial strain and to characterize the physicochemical properties of the biopolymer.

2. Materials and methods

2.1. Strain and culture conditions

A biopolymer-producing bacterium used in this study, designated KMBL 5781, was isolated from the soil around Daegu area in Korea by using a medium consisting of 2% glucose, 0.3% Bacto-peptone, 0.05% $MgSO_4 \cdot 7H_2O$, 0.03% KH_2PO_4, 0.07% K_2HPO_4 and 1.5% agar. Production of the biopolymer by the bacteria was carried out at 30 °C for three days with shaking (170 rpm) in the same media without agar described above. For the preparation of chromosomal DNA, the bacteria were cultured at 30 °C overnight in NB liquid media.

2.2. Bacterial identification

The bacteria were identified by using the phylogenetic analysis based on the 16S rRNA gene sequences. Chromosomal DNA, for the PCR template, was isolated from bacterial cells grown in NB media overnight by the general method described by Sambrook and Russel (2001, Chapter 8). Oligonucleotide primers for PCR were synthesized by a commercial company (Bioneer Co., Chongwon, Korea). The bacterial universal primer set 27f (5′ – AGAGTTT GATCMTGGCTCAG) and 1492r (5′ – TACGGYTACCTTGTTACGACTT) (Lane, 1991) was used for PCR to amplify the 16S rRNA gene. PCR was performed in 50 μl using the TaKaRa Taq DNA polymerase (Takara Shuzo Co., Otsu, Japan) with a GENE cycler (BioRad Co., Richmond, USA) by the general method (Sambrook & Russel, 2001, Chapter 8). Nucleotide sequences of the 16S rRNA gene region were compared with those available in the GenBank database by using the BLAST method to determine their approximate phylogenetic affiliation and their sequence similarities at the National Center for Biotechnology Information, USA (Altschul et al., 1997; NCBI, 2011). The sequences of the related taxa were acquired from the same web site. Nucleotide sequences were initially aligned using the CLUSTAL X program (Thompson, Gibson, Plewniak, Jeanmougin, & Higgins, 1997) and then manually adjusted. Distance matrices were calculated and phylogenetic tree for the data set was created according to the Kimura two-parameter model (Kimura, 1980) and neighbor-joining method (Saitou & Nei, 1987) by using the Mega 4 (version 4.02) software package obtained from the web site (MEGA, 2011; Tamura, Dudley, Ney, & Kumar, 2007).

2.3. Purification of biopolymer

After the bacteria were cultivated at 30 °C at 170 rpm for 3 days, the culture broth was homogenized by a blender (Hanil Electric Co. Blender Titanium, Seoul, Korea). Culture supernatant was obtained by high speed centrifugation at 11,000× *g* for 15 min at 4 °C, which was filtered through a membrane filter (0.45 μm) to remove bacterial cells. The biopolymer was purified by the sequential precipitation using ethanol, cethyl trimethyl ammonium bromide and then ethanol again. After each step of ethanol precipitation, the precipitants were washed with 70% of ethanol and dissolved in water. The final precipitants were dissolved in 10% NaCl solution to remove a trace of impurity and to improve the EPS precipitation with ethanol. The biopolymer finally precipitated with ethanol was dissolved in water and dialyzed against distilled water for two days. And then, the dialyzed bipolymer solution was lyophilized and used as a purified biopolymer by the method of Yun and Park (2000). Its morphology was examined by using a scanning electron microscope (Hitachi S 4300, Tokyo, Japan), after the biopolymer was coated with platinum.

2.4. MALDI-TOF mass spectrometer

The purified biopolymer was analyzed by using a MALDI-TOF MS (Bruker Daltonics, Bremen, Germany) equipped with a 337 nm nitrogen laser. For the analysis, the 2% biopolymer solution was mixed directly with matrix solution (2 mg 3,4-dihydroxybenzoic acid in 0.2 ml of 20% ethanol) with 1:2 ratio (v/v). The mixture was spotted on the sample target of the machine and allowed to dry at room temperature. After drying, the analysis was performed in the linear negative ion mode scanning from 5000 to 150,000 *m*/*z* and using ion suppression up to 2000 *m*/*z*. For all the experiments, the ion sources one and two were held at 20 kV and 16.35 kV, respectively. The guiding lens voltage was at 9.75 kV. The reflector detection gain was set up at 5.3 with pulsed ion extraction at 200 ns. The nitrogen laser power was set to the level necessary to generate a reasonable signal, which was about 60–80% laser energy. Three-point external calibration was performed, using the bovine serum albumin, cytochrome and trypsinogen.

2.5. GC–MS analysis

Components of the biopolymer, after being hydrolyzed and methylated, were analyzed using a GC–MS by the method of Stenutz, Jansson, and Widmalm (2004). The purified biopolymer (20 mg in 2 M H_2SO_4 solution) was hydrolyzed at 121 °C for 2 h, which were then dried on a speedvac. Methylation was carried out at room temperature for 30 min with 60 μl of methyl iodide in the presence of 120 mg of sodium hydroxide. The 50 μl of dimethyl sulfoxide was used as the solvent for the reagents and reactants (Ciucanu & Kerek, 1984). The methylated sample was precipitated with distilled water and thoroughly mixed with the same volume chloroform. The mixture was briefly centrifuged to separate the two layers and 1 μl of the chloroform layer was injected to a GC–MS (Agilent 7890A-5975C, Santa Clara, CA) which was attached with a silica column HP-5 (ϕ 0.25 μm × 30 m). It was interfaced with an Agilent 5973N mass-selective detector configured in EI mode. The injection port temperature was 250 °C and helium flow rate was 0.7 ml/min. The peaks were identified by W9/N08 library and confirmed with the standard monosaccharides. Molar ratio values were corrected to use quantitative analysis by comparison of various permethylated monosaccharides as the control.

2.6. Viscosity measurement

The viscosity of the biopolymer was measured by the method of Mandala and Bayas (2004) with some modifications. The purified biopolymer (0.4 g) was dissolved thoroughly in 20 ml distilled water on a magnetic stirrer. Air bubbles were removed by a gentle centrifugation prior to the measurements. Seven milliliters of the fluids or diluted solutions were introduced to a rheometer (Universal Dynamic Spectrometer Physica UDS 200, Stuttgart, Germany) at a fixed temperature of (25 ± 0.2 °C) with the plate–plate geometry at a distance of 1 mm. The diameter of the upper plate was 25 mm. All measurements have been used to measure the viscosity of shear-thinning solutions xanthan and EPS with the same concentrations