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

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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, cetyl 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.

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 azidohymidine 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 as an emulsifying, gelling and water-binding agents in the several industries (Freitas et al., 2011; Patel, Michaud, Singhania, Soccol, & Pandey, 2009).
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, & Bouleguere, 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 KMBl578I, was isolated from the soil around Daegu area in Korea by using a medium consisting of 2% glucose, 0.3% Bacto-peptone, 0.05% MgSO4·7H2O, 0.03% KH2PO4, 0.07% K2HPO4 and 1.5% agar. Production of the biopolymer by the bacterium 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’-AGAGTTTGATCTMTGGCTCAG-3’ ) and 1492r (5’-TACGGYTACCTTGTTACGACTT-3’ ) (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 neighboring 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, cetyl 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 biopolymer 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 set 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 H2SO4 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 (ø 0.25 μm × 30 m). It was interfaced with an Agilent 5973N mass-selective detector configured in El 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 Mandal 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.
(2%, 1%, 0.5%). Shear rate range was $10^3$ to $10^5$, but data were statistically analyzed up to $6 \times 10^4$/s. The software Paar Physica was used to calculate the main flow parameters including temperature, pressure, shear rate, viscosity, and residence time (Vergnes & Berzin, 2010). Shear rate ($\gamma$) has been obtained from an applied shear stress. The shear stress ($\sigma$) is the force that flowing liquid exerts on a surface, per unit area of that surface, in the direction parallel to the flow. The shear viscosity ($\eta$) is defined as $\eta = \sigma / \gamma$. Xanthan gum was used as a control considering that it is one of the widespread commercialized water-soluble biopolymers.

Effects of temperature on the viscosity of the EPS were also investigated. The 2% sample was prepared same way as mentioned above and xanthan (Sigma–Aldrich Chemie Gmbh, Munich, Germany) were used for comparison. The experiment was performed using the cone-and-plate geometry in a dynamic rheometer (Mars, Thermo Haake, Karlsruhe, Germany). To prevent moisture loss during the experiment, silicone oil (Shin-Elsu Chemical Co. Ltd., Tokyo, Japan) was applied around the sample edge. Temperature sweep tests were conducted at 0.1 Hz to obtain the viscoelastic properties from 20 to 92 °C at a rate of 1 °C/min. The storage ($G'$) and loss ($G''$) were recorded at the range from 10 to 10,000. The experiment was performed in the linear viscoelastic region where in target stress 1.2 Pa was determined target strain 0.009 experimentally by stress sweep tests, as described previously (Yoon & Gunasekaran, 2007).

2.7. Measurement of syneresis

Syneresis was measured by the method of Viñarta, Malina, Figueroa, and Fariña (2006). Starch is one of the most abundant and widely distributed components in foodstuffs. Therefore, corn starch powder used as a control was first dispersed in cold water at a final concentration of 2%. The starch slurry was then cooked in a boiling water bath for 10–15 min with gentle stirring to make a starch paste. The purified biopolymer was dissolved in distilled water on a magnetic stirrer and heated at 60 °C for 48 h with continuous stirring until getting constant viscosity to make 2% biopolymer solution. Then, the starch paste and the biopolymer solution were mixed at the volume ratio of 9.9:1, 9.75:0.25, 9.5:0.5, 9.25:0.75 and 9:1 to give final EPS concentrations 0.02, 0.05, 0.1, 0.15 and 0.2%. Microbial activity was prevented by adding one mg/ml sodium benzoate. The tubes containing the hydrogel were incubated at 4 °C for 20 days. The syneresis properties of the mixtures as well as 2% corn starch paste and 2% biopolymer solution were evaluated after the incubation at 4 °C for 20 days. The extent of syneresis was estimated by measuring the volume of the liquid phase as the expelled water separated during the incubation at 4 °C. After the incubation, the expelled water was tipped out. And, the tubes containing the hydrogel were centrifuged at 4000 g for 10 min. The weight of the liquid phase separated by the centrifugation was measured as the absorbed water. After the absorbed water was removed, the remaining gel weight was measured. Portions of the weight of the expelled water released during the incubation, the absorbed water separated by the centrifugation and the remaining gel after the centrifugation were expressed as the percentage per the initial weight of the gel before the incubation. The same experiment was performed with xanthan gum (Sigma–Aldrich Chemie Gmbh, Munich, Germany) as a control.
2.8. Statistical analysis

All the experiments were carried out in triplicate. Data were analyzed by the variance (ANOVA) according to the general linear model procedure of the statistical analysis system (SAS Institute Inc., Cary, NC). Differences between means were separated by the least significant difference (LSD). P < 0.05 was considered statistically significant.

3. Results and discussion

3.1. Bacterial identification and biopolymer purification

A bacterial strain KMBL 5781, producing an extracellular biopolymer, was isolated based on the mucoid morphology on the NB agar plate. The bacteria were aerobic, gram positive, spore-forming, motile bacilli. The 16S rRNA sequences were similar to those of *Mit-suaeria chitosanitabida* type strain 3001 (Amakata et al., 2005). The sequence identity was 99.4% with eight mismatches in the 1420 bp nucleotide sequences. Phylogenetic analysis based on the 16S rRNA gene sequences resulted in that the isolate is genetically closest to *M. chitosanitabida* type strain 3001 (Fig. 1). *M. chitosanitabida* strain 3001 has been isolated as a chitosanase-producing bacterium in Japan and identified as a gen. nov., sp. nov. bacterial strain (Amakata et al., 2005). Although there have been a dozen of papers on the chitosanase produced by the strain, its EPS production has never been described thus far.

Production of biopolymer by the bacteria was confirmed in the liquid media as well as on the agar plate. The biopolymer produced by the *M. chitosanitabida* KMBL 5781 was purified from the culture filtrate with the sequential precipitation using ethanol, cethyl trimethyl ammonium bromide and then ethanol again. The purified biopolymer exhibited “cotton caramel” shape after freeze drying. Scanning electron microscopy (SEM) revealed that the biopolymer exhibits fibrous structure of which diameter is below five μm (Fig. 2a). Molecular weight distribution of the purified biopolymer was obtained by the MALDI-TOF MS spectrometry. Average molecular weight of the biopolymer was estimated to be 54.7 kDa (Fig. 2b).

In general, the molecular weights of the EPSs range over 1 x 10^6 with a few exceptions. Colloids of the EPS with a high molecular weight (>70 kDa) have been observed to contain side effects such as a high pressure in venous, where colloids with a low molecular weight (<40 kDa) gets rapidly rejected from organisms which start to develop secondary hemorrhagic shock (Rekha & Sharma, 2007). Molecular weights of natural dextrans produced by *Leuconostoc* spp. range from 10^4 to 10^5 Da (Freitas et al., 2011). However, dextrans are available in multiple molecular weights ranging from 10 to 150 kDa in the market. Dextran-40 (MW: 40 kDa) and dextran-70 (MW: 70 kDa) intravenous solutions have been approved by US FDA for plasma volume expansion (Atik, 1967; Rehm, 2010).

3.2. Chemical composition of the EPS

In order to understand chemical structure, the purified biopolymer was hydrolyzed, then methylated, which was applied to a GC–MS. Search of the GC–MS library resulted in the detection of several components including glucose, mannose, galactose, fructose, glycine and cis-1,2-dimethoxy cyclopropane (Fig. 3). The peaks of glucose, mannose, galactose and fructose were confirmed by using the standard sugars treated with same reactions. The analyses of the standard methylated monosaccharides showed the retention time of glucose with 15.27 and 16.20 min (key ions are m/z 75, 88, 101), mannose with 16.14 and 16.53 min (an intense ion at m/z 18, 28, 45, 59, 75, 88, 101, 149), galactose with 16.35 and 16.70 min (observed from m/z 45, 75, 101), and fructose with 8.16 min as a minor component. They were the same as those in the GC–MS spectra of the hydrolyzed biopolymer. When compared with the area of the peaks obtained from the standard sugars, it was found that the molar ratio of glucose:mannose:galactose was 70:24:3.9. These results suggest that the biopolymer produced by the strain KMBL 5781 is a novel polysaccharide containing glucose, mannose and galactose with an approximate ratio of 18:6:1. Compositions of several bacterial EPS have been elucidated including xanthan, gellan, alginate, glucans, hyaluronan, succinoglycan, levan, etc. Glucose appears most often, while mannose is seldom found as a component of the EPS. In particular, it is very rare that bacterial EPS contains both mannose and galactose (Freitas et al., 2011).

3.3. Viscosity of the EPS

Although highly viscous, the EPS solution showed no resistance to any deformation. The bacterial EPS solution in water was observed to be transparent and exhibit jelly-like properties. The dry EPS easily dispersed in cold water and provides water binding resulting in a high viscose solution (Fig. 4b). When the EPS and xanthan solutions used as a control were subjected to the shear force externally applied, viscosities of the two solutions increased according to the increase of the concentration. In lower concentrations such as 0.5% and 1%, the EPS solution was much more highly viscous than the xanthan gum. At the final range of shear rate (γ) which was around 5 x 10^4/s, 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. These results are representing that the EPS contains higher stress preventive properties than xanthan. However, no significant difference in their viscosities was observed between the two polysaccharides at 2% solution. It was thought that the EPS solution showed a typical non-Newtonian and pseudoplastic or shear-thinning behavior with an increasing shear rate system suggesting the EPS solution belong to a pseudoplastic gum, which deforms under the applied shear force (Fig. 4).

3.4. Effects of temperature on the viscosity of the EPS

Understanding the effect of temperature on the viscosity of the EPS solution is very important for food or non-food processing.
Therefore, changes in the viscosity of 2% of the EPS solution were assayed at a temperature range of 20–92 °C. The rheological properties of EPS and xanthan remain stable in room temperature until 60 °C. In comparison with xanthan they are both stable from 20 to 60 °C but viscous properties of EPS solution is higher. The viscosity of EPS solution and xanthan increased from 60 °C. From the plot, we could see the viscosity of EPS solution in 60 °C has changed dynamically while viscosity of xanthan changed sharply. Between 60 and 68 °C, the 2% EPS solution showed a same viscosity as 2% xanthan, which is about 450–490 Pa s with a slight increase according to the temperature raise. However, the EPS solution showed significantly higher viscosity than xanthan at 90 °C (Fig. 5).

3.5. Syneresis in the mixture with corn starch

Mixtures of the EPS and starch solutions with the final EPS concentrations of 0.02, 0.05, 0.1, 0.15 and 0.2% were incubated at 4 °C for 20 days. During the incubation, the degree of syneresis (percentage of the expelled water volume per the initial gel volume) in all the mixtures increased as the incubation time was prolonged (data not shown). After the incubation for 20 days, the expelled water was tipped off and the tubes containing the hydorgel were centrifuged. And, the weight percentages of the absorbed water and gel separated by the centrifugation were calculated as the percentage per the initial gel weight before the incubation. Similar experiment was performed by using xanthan gum instead of the EPS as a control (b).

![Fig. 6. Syneresis of the EPS in the mixture of the EPS (a) or xanthan gum (b) with corn starch. Mixtures (2%) of the EPS and corn starch (CS) solutions were prepared to give a final concentration shown below the figure, which were incubated at 4 °C for 20 days. After the incubation, the weight of expelled water was determined. In addition, the weights of absorbed water and gel were assayed after centrifugation of the tubes at 4000 × g for 10 min. Portions (%) of the weights of the expelled water (white bar), absorbed water (black) and remaining gel (gray) were calculated as the percentage per the initial gel weight before the incubation. Similar experiment was performed by using xanthan gum instead of the EPS as a control (b).](image)

Xanthan gum produced by *Xanthomonas campestris* is one of the most extensively and commercially exploited bacterial EPSs. About 96,000 tons of xanthan gum has been reported to be consumed worldwide every year. This is mainly due to its high viscosity yield at low concentrations as well as the stability over wide ranges of temperature, pH and salt concentrations (Freitas et al., 2011). However, the EPS of *M. chitosanitabida* KMBL 5781 showed a higher viscosity than xanthan at 0.5% and 1% (Fig. 4). The EPS solution showed a strong stability under the heat to 60 °C (Fig. 5) and water holding capacity under the refrigerating conditions (Fig. 6). Its 2% hydrogel was fully stable after incubation at 4 °C for 20 days with no release of water even after the centrifugation at 4000 × g for 10 min, although its water prevention was less than that of xanthan (Fig. 6). Therefore, the authors argue that the EPS produced by *M. chitosanitabida* KMBL 5781 may be a potential use in the hydrocolloid industry.

4. Conclusion

We have isolated a bacterial strain producing a high level of exopolysaccharide (EPS) and identified the bacteria as *M. chitosanitabida*. The EPS was purified by the sequential precipitation using ethanol, cetyl trimethyl ammonium bromide and then ethanol again. GC–MS analysis of the EPS hydrolysate resulted in that the EPS was composed of glucose:mannose:galactose (18:6:1) suggesting that it is a novel polysaccharide. The EPS exhibited a high level of viscosity in comparison with xanthan at the same concentrations of 0.5 and 1%. It was relatively stable at the
temperature range between 20 and 60 °C and 2% EPS showed higher viscosity at 92 °C in comparison with xanthan. A hydrogel containing 2% EPS showed a high level of water holding capacity, which released no water during the incubation at 4 °C for 20 days and even while the centrifugation at 4000×g for 10 min after the incubation. Therefore, it was proposed that the EPS produced by M. chitosanitabida KMBL 5781 is highly viscous and contains a high level of yield stress and water holding capacities.

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