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Extraction of novel sulfated polysaccharides from *Aphanothece sacrum* (Sur.) Okada, and its spectroscopic characterization*

Maiko Okajima-Kaneko[1], Masateru Ono[2], Kiyotaka Kabata[2], and Tatsuo Kaneko[1,‡]

[1]School of Materials Science, Japan Advanced Institute of Science and Technology, Asahidai, Nomi, Ishikawa 923-1292, Japan; [2]School of Agriculture, Kyushu Tokai University, 5435 Minamiaso, Aso, Kumamoto 869-1404, Japan

Abstract: We extracted polysaccharides (PS) from *Aphanothece sacrum* using a hot alkaline solution which degraded other biopolymers such as proteins and nucleotides. The spectroscopy and elemental analyses indicated the PS contain carboxyls and sulfate groups. The degree of sulfation was estimated as about 10 mol %. ^{1}H NMR studies demonstrated that the PS of *A. sacrum* had a dimethylated fucose unit. The combination of sulfate group and fucose in the prokaryotic PS was first evidenced by the direct spectroscopic studies. The PS showed efficient gelation behavior, binding to metal ions abundant in soil, and the swelling volume of the gel was approximately 250 times the dry volume. These results imply that PS of *A. sacrum*, which has been mass cultivated in Japan for a long time, may have potential as an environmentally benign water absorbent.

Keywords: polysaccharides; extraction; cyanobacterium; gels; spectroscopy.

INTRODUCTION

Bioresources of microbial origin have considerable potential to promote "green" alternatives to petrochemical feedstock exploitation. Water-absorbent polysaccharides (PS) have been obtained from seaweeds with a large amount of jelly matrix [1], but their production largely depends on the weather and the food chain. From cyanobacteria with jelly matrix [2], we have selected *Aphanothece sacrum* (Sur.) Okada, which has a huge jelly matrix, with a cluster size from several centimeters to several decimeters. *A. sacrum*, which was biologically classified in the 19th century by Suringar [3], is a freshwater unicellular cyanobacterium indigenous to Japan. Though *A. sacrum* has been known for more than 100 years, only 24 papers on it have been reported, shown in results of a literature search using the keyword "*Aphanothece sacrum*" (by Scifinder). Such a lack of research activity is due to the low availability of experimental material and lack of an efficient cultivation method. However, *A. sacrum* has higher water content than other popular jelly organisms such as *Nostoc commune*, according to the literature [4]. From this information, we can analogize that extracellular agar of *A. sacrum* has a high water absorption capacity. The fact that the agars of seaweeds [4] and *N. commune* [5] are comprised of PS easily leads to the assumption that a main construct of *A. sacrum* agar is PS. However, the PS in *A. sacrum* have never been reported, as far as we know.

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‡Corresponding author; E-mail: kaneko@jaist.ac.jp

Here we have extracted PS from *A. sacrum* and have clearly shown for the first time using the direct spectroscopic method that prokaryotic PS contain both fucose units and sulfate groups. The PS anions bind to various metal cations to form highly water-swollen gels.

METHODS

Microorganism samples

A. sacrum cultivated in a river farm was kindly provided by Kisendo Corp. (Amagi, Japan). Raw samples used for the extraction were wavy jelly sheets (thickness: 1–2 mm, area: 100–1600 mm^2) colored brown–green. Prior to the extraction operation, the raw samples were thoroughly washed using a large amount of tap water, and rinsed in pure water. Microscopic observation confirmed that no other microorganisms contaminated the inside of the jelly matrix for *A. sacrum* (Fig. 1).

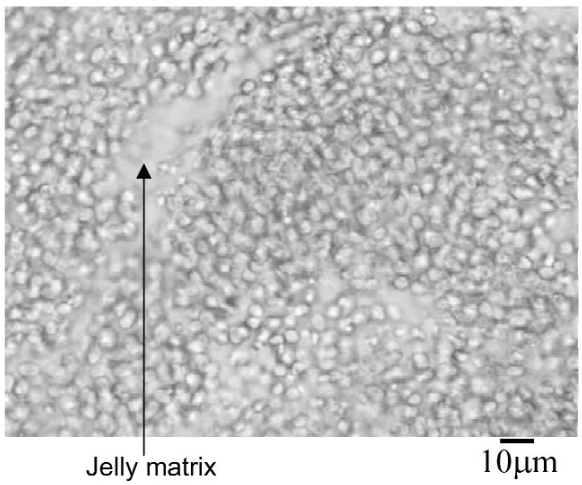

Fig. 1 Microscopic photo of *A. sacrum*, where many circular matters are cell bodies.

Extraction and purification of PS

PS were extracted from *A. sacrum* by the following procedure. The *A. sacrum* samples (2.01 g) were freeze-thawed and washed in pure water, followed by lyophilization, and were milled into small pieces. The milled samples were washed three times using a large amount of ethanol with shaking (120 rpm) overnight, and then collected by filtration using gauze. The ethanol-washed samples were put into 1 M NaOH aq (50 ml) at 100 °C, and agitated at constant temperature for 4 h to yield the transparent solution. The solution was dialyzed with pure water for more than 72 h using the regenerated cellulose membrane (MWCO:14 000) until the pH value decreased to 8.0–9.0, and then filtrated. Then the filtrate was concentrated by rotary evaporator to create a highly viscous solution. The viscous solution (10 ml) was slowly poured into 100 % ethanol (1000 ml) to precipitate white fibrous material. The fibers were dissolved in hot water again, concentrated, and reprecipitated, and these operations were repeated three times in total. The aqueous solutions of the fibers showed a pH value of about 6.0–7.0. Further purification was achieved by gel filtration chromatography (Sephadex LH-20, GE Healthcare) to produce a transparent solution containing PS solute (0.157 g, yield: 7.4 %). The aqueous solution showed no specific absorption in the wavelength range of 220–600 nm in UV–vis spectroscopy, which confirmed there was no contamination by proteins, nucleic acid, chromophores, and/or other chemicals with aromatic rings.

Spectroscopic analysis

Fourier transform infrared spectroscopy (FT-IR) spectra of PS were recorded at 25 °C on a Perkin Elmer Spectrum One spectrometer between 4000–600 cm^{-1} using a diamond-attenuated total reflection (ATR) accessory. UV–vis absorption spectra of PS aqueous solution were measured at 25 °C on a Perkin Elmer Lambda 25 UV/vs spectrometer. ^{1}H NMR of PS was measured at 25 °C in dimethylsulfoxide (DMSO-d_6) solution on a Varian FT-NMR spectrometer (UNITY 500plus, 500 MHz), using the residual proton resonance of water as an internal standard (4.79 ppm). Electron spectroscopy for chemical analysis (ESCA) was performed with a Physical Electronics PHI 5600 ESCA system employing Mg Kα radiation (1253.6 eV) and a pass energy of 31.5 eV. Polarized-microscopic observation was made by an Olympus microscope BX51 equipped with a digital camera DP70. Elemental analysis was made by Yanako CHN coder MT-6 (at the Center for Organic Elemental Microanalysis at Kyoto University). Rotation viscosity of PS aqueous solution was made by Anton Paar Physica MCR 301. The sample (gap: 1 mm) was statically sheared by rotation of a cone plate at 30 °C. The optical rotation of sugars was made by JASCO DTP-1000KUY digital polarimeter. ^{1}H and ^{13}C NMR spectra of monosaccharides were measured by JEOL α-500 spectrometer in chloroform-*d* or pyridine-d_5 at room temperature using tetramethylsilane as an internal standard (0.00 ppm).

Monosaccharide analysis

Monosaccharides constituting the jelly matrix were analyzed by the following procedure. The lyophilized samples of *A. sacrum* (3.81 g) were washed by methanol twice at room temperature for 14 days, and the residue was degraded in a mixed solvent (100 ml) of methanol/35 % HCl aq (80/20 v/v) in a reflux condition for 5 h. The supernatant was neutralized with 4 N NaOH aq and evaporated to yield dried powder. The monosaccharides were extracted by methanol addition into the powder, followed by centrifugation at 3000 rpm for 5 min. The supernatant was applied to silica gel column chromatography (Merck Art. 7734) eluted by a mixture of chloroform/methanol/water while the solvent composition was changed from 14:2:0.1 → 10:2:03.1 → 8:2:0.2 → 7:3:0.5 → 6:4:1 to methanol, to create eight fractions. First fractions (59 mg) were further divided into two fractions (SU-1: 3 mg and SU-2: 7 mg) by silica gel column chromatography (Merck Art. 9385) eluted by hexane/ethyl acetate 1:1 mixture. Of every fraction, only one ^{1}H NMR analysis of SU-2 was successfully made. SU-2 was obtained as a white powder, and showed an optical rotation value of $[\alpha]_D^{16} = -123.0°$ (c = 0.6, methanol).

RESULTS AND DISCUSSION

A. sacrum is a cyanobacterium with a large quantity of jelly matrix. Figure 1a shows the microscopic image of *A. sacrum*. One can see that many cell bodies with a diameter of 3–4 μm are dispersed over the jelly matrix, forming a group with a size range less than 80 μm. In addition, about half of the area of the matrix contains no cells. From the results of the microscopic observation, we could roughly estimate the volume percent of the extracellular jelly matrix as 70–85 vol %. The swollen weight of *A. sacrum* compared to its dry weight was 62–64.

PS extraction

The raw sample of *A. sacrum* is brown–green, but the freeze-thaw treatment made it easy to remove the purple pigments such as phycoerithrine and phycocyanine by simple water washing. The following ethanol washing removed the green lipid pigment, such as chlorophylls. The sample surface was not slimy, which made us imagine no spontaneous PS elution from the jelly matrix. Actually, the hot water treatment of the *A. sacrum* sample gave no PS. We used alkaline solution for PS extraction, since cyanobacterial PS generally have uronic acid units, according to the literature [5–8]. As expected, the

sample dissolved in the NaOH solution (1 M), and the solution was agitated for 4 h in order to degrade other biopolymers such as proteins and ribonucleotides. After some insoluble matter was filtrated, the solution was dialyzed with pure water to remove low-molecular-weight impurities. We confirmed impurity removal by recording the sufficient electric resistance (more than 10^5 Ω) of the solution outside of the dialysis membrane. When the solution inside the dialysis membrane was concentrated, the solution became highly viscous, which implies the existence of PS. Slow pouring of the solution into ethanol created fibrous precipitates. Reprecipitation was performed twice more to give colorless fibers, which were confirmed as PS by the spectroscopic study shown in the next section. The yield of extracted PS from *A. sacrum* was 7 %/dry weight. The aqueous solution of PS (0.5 g/dL) showed a zero-shear viscosity of 1900 Pa·s at 30 °C, which is such a high value that the molecular weight may exceed 1000 kDa. After the extracted sample was further treated by gel filtration (Sephadex LH-20), we confirmed by gel permeation chromatography (GPC) that the trace of low-molecular-weight impurity (<6 kDa) was not contained. When the concentrated PS solution was poured into ethanol or acetone, the cotton-like precipitates appeared. The polarized microscopic observation showed that the precipitates were composed of oriented fibers with a length of <3 cm and a diameter of <50 μm (Fig. 2). Figure 2b shows a microscopic photo of the fibers taken under the cross-nicol, and Fig. 2c shows a corresponding photo taken using a first-order retardation plate (λ = 530 nm) inserted in the light path. The fiber birefringence was negative, as evidenced by subtractive birefringence (blue color) in the fiber lying from upper left to lower right. The negative birefringence strongly suggests the PS main chains along the fiber axis.

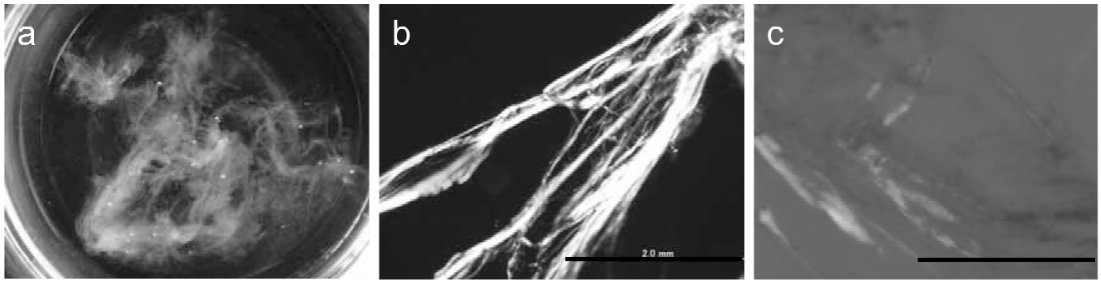

Fig. 2 Photos of PS fibers prepared by pouring its aqueous solution into ethanol: (a) appearance in ethanol; (b) crossed-polarizing microscopic photo. (c) crossed-polarizing microscopic photo taken under a first-order retardation plate. Scale bars: 2 mm.

Spectroscopic study

The UV spectra did not show any absorption peaks of protein, nucleotides, or pigments in the wavelength range of 600 to 230 nm (Fig. 3a), but rather the absorption widely detected in PS samples which is clearly visible at <230 nm, indicating PS isolation. The elemental analysis of desalinated PS by a cation exchange resin (DOWEX 50W-X8, 50–100 mesh, H form) showed results as follows; C; 36.04 %, H: 5.91 %, N: 0.30 %, S: 2.07 %. If average molecular weight for a sugar unit is assumed to be 162, the molar composition of S to the total PS can be estimated as 10 mol %.

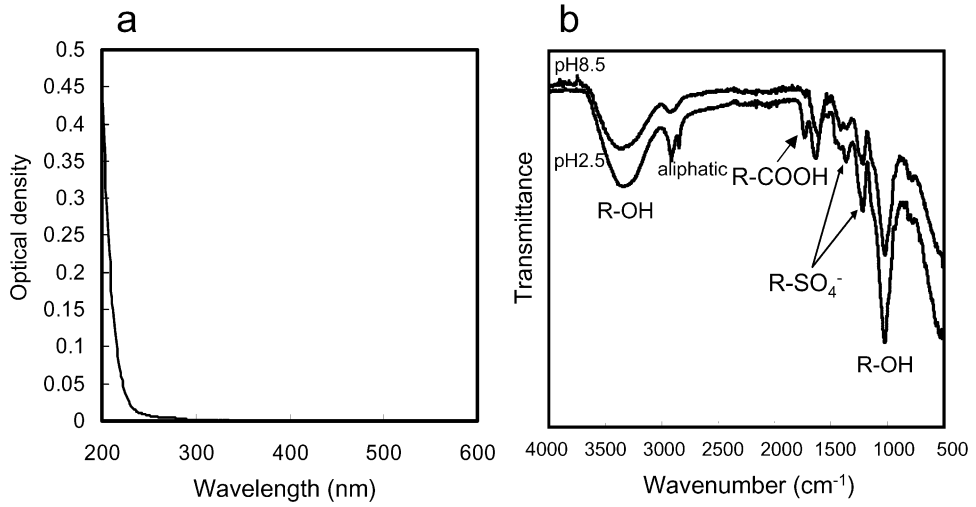

Fig. 3 Spectroscopy of PS extracted from *A. sacrum* (a) UV–vis spectrum showing no absorptions of protein, nucleotides, or chlorophylls. (b) FT-IR spectra showing carboxyl and sulfate groups as well as hydroxyl and aliphatic groups.

FT-IR spectra of PS showed several distinct peaks at wavenumbers of 3354 (hydroxyls), 2924 (aliphatic chains), 1613 (carbonyls), 1415 (aliphatic chains), 1363 (sulfates), 1223 (sulfates), and 1022 cm^{-1} (hydroxyls) (Fig. 3b). The spectra indicated that PS has carbonyl and sulfate groups, as well as the typical groups of sugars. Since the carbonyl type was unclear, the FT-IR spectrum of acid-treated PS was studied. The peak appeared at 1736 cm^{-1}, indicating that PS from *A. sacrum* have carboxyls of uronic acid. Uronic acid content was estimated as 22 % by the carbazole-sulfuric acid method (525 nm). Figure 4 shows the ESCA diagram of PS, showing a peak at a binding energy of 169 eV, with a shoulder around 170 eV, which is characteristic of the sulfate peak in ESCA.

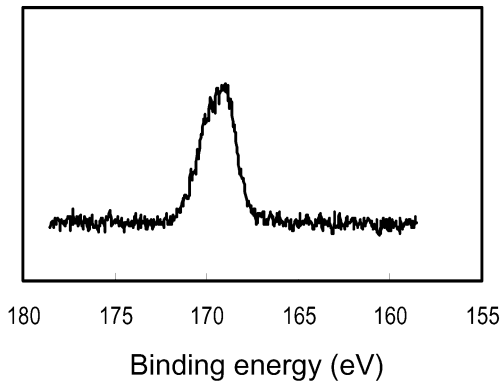

Fig. 4 XPS diagram of PS extracted from *A. sacrum*, showing the sulfur peak assigned to sulfates.

We tried to measure ^{1}H NMR spectra of PS in DMSO-d_6. Figure 5 shows the ^{1}H NMR spectra of the PS in DMSO-d_6, showing a distinct signal, whose maximum lies at a chemical shift of δ 1.24 ppm, and multiple broad signals at δ 2.8–3.6 ppm. All the signals were difficultly shown because the peak at δ 1.24 ppm was much smaller than multiple broad signals at δ 2.8–3.6 ppm. While a signal at δ 1.24 ppm may imply the presence of 6-deoxy sugar unit, the broad signals at δ 2.8–3.6 ppm may

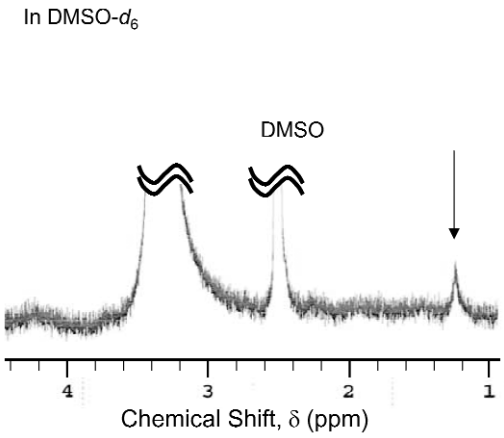

Fig. 5 ^{1}H NMR spectrum of PS extracted from *A. sacrum*. Arrow: the signal of 6-deoxy sugar units.

imply the presence of methoxyls, methylenes, and methines. Since all other cyanobacterial PS contain at least six units of different monosaccharides [5–9], one can analogize that the PS from *A. sacrum* may also contain several different monosaccharides and the multiple signals at δ 2.8–3.6 ppm may be overlapping of too many signals. Then it is impossible to make an in-depth investigation of the multiple signals in the ^{1}H NMR spectra of non-hydrolyzed PS.

Next, we tried to analyze the constituent sugars which were obtained by a direct degradation of the jelly matrix, since the PS hydrolysis to monosaccharides was restricted by insolubility in acidic water over the course of hydrolysis. Lyophilized sample of *A. sacrum* was degraded by methanol/HCl mixture, and the solutes were divided into nine fractions by silica gel column chromatography. One of them was successfully confirmed as a fucose derivative. Figure 6 shows ^{1}H NMR spectrum of SU-2, where nine signals appeared at δ 4.88 (1 H, d, J = 3.5 Hz), 3.96 (1 H, dd, J = 3.5, 9.5 Hz), 3.91 (1 H, q like, J = 6.5 Hz), 3.63 (3 H, s), 3.50 (3 H, s), 3.48 (1 H, dd, J = 3.5, 9.5 Hz), 3.41 (3 H, s), 3.38 (1 H, d like, J = 3.5 Hz), and 1.29 (3 H, d, J = 6.5 Hz). These signals were assigned to five oxygenated methines (δ 4.88, 3.96, 3.92, 3.48, 3.38), one secondary methyl (δ 1.29), and three methoxyls (δ 3.63, 3.50, 3.41). From these data, SU-2 was considered to be a trimethylated derivative of α-fucopyranoside. The ^{13}C NMR spectrum of SU-2 showed nine signals at δ 97.2, 82.1, 78.7, 70.4, 66.0, 62.3, 58.2, 55.4, and 16.5 ppm, supporting this inference. In order to clarify the non-methylated position, SU-2 was acetylated with acetic anhydride and pyridine, and its ^{1}H NMR spectrum was measured. As a result, an acylation shift appeared only in the signal [δ 5.55 (1 H, dd, J = 3.0, 10.5 Hz)] assignable to 3-positioned methine proton, indicating that three methoxyls were connected to 1-, 2-, and 4-position carbons. Thus, it was concluded that SU-2 was 1,2,4-trimethyl α-fucopyranoside, as shown in the inset structure of Fig. 6. Since the PS show the ^{1}H NMR signals at chemical shifts near the positions of SU-2 signals, we can presume that the PS have the fucose derivative as a constituent. Most of the sugar units in general PS connect to the nearest-neighbor unit via the 1-position carbon [7]. If this fucose derivative also connects via the 1-position carbon, the fucose unit for the *A. sacrum* PS might receive 2-, 4-dimethylation. Since it is difficult for only 2- and 4-hydroxyls to be selectively methylated by the methanolysis process, the PS may be dimethylated in the natural state, or as a result of transformation from other groups such as sulfates and/or sugars. Although SU-1 was speculated as the anomer of SU-2, the structure is still under investigation. Then, one can say that the PS extracted from *A. sacrum* may have not only sulfate among its groups but also the fucose derivative as one of its sugar units. Biomedical research on fucoidan contained in seaweed has been widely conducted, showing that the combination of fucose and sulfate group is quite important in terms of health, and may be useful in the pharmaceutical industry. The structure in cyanobacterial PS is first found with direct spectroscopic evidences, although

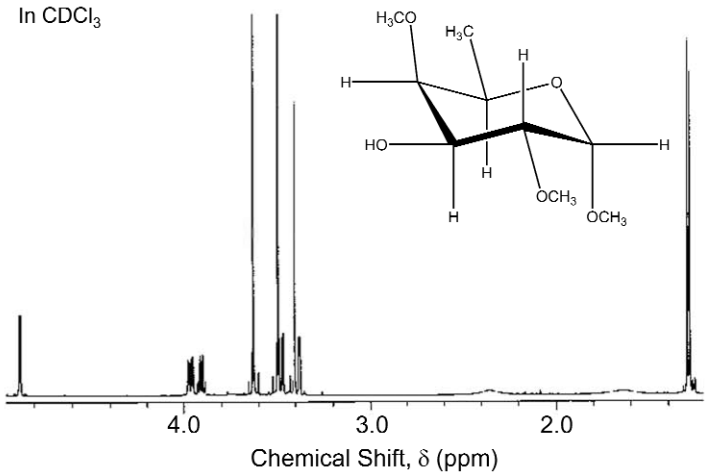

Fig. 6 ^{1}H NMR spectrum of a fraction (SU-2) obtained using silica gel chromatography of the *A. sacrum* sample degraded by methanol/HCl. The inset is a structure of 1,2,4-trimethylated fucose.

just one paper showed the coexistence of sulfate group and fucose unit in PS from cyanobacteria, *Synechocystis* PCC 6803 and 6714, using only chromatographic methods [9]. Studies of other monosaccharide constituents are under investigation.

Finally, we investigated PS gelation behavior in the aqueous solution of metal ions abundant in soil. After the addition of the aqueous solution for *A. sacrum* PS (0.5 % w/v) to the individual solutions of Sr^{2+}, Ba^{2+}, Al^{3+}, Fe^{3+}, Ce^{3+}, La^{3+}, Yb^{3+} ions (concentration: 1 mg/2 ml, counter ion: Cl^-), gel beads with a diameter of 5–8 mm were formed. The absorption efficiency of individual metal ions is under investigation and will be shown in the next paper. The swelling ratio of *A. sacrum* PS gel beads to the dry weight did not depend on the kind of metal ions (250 g water adsorbed/g dry weight), which was much higher than that of the jelly matrix in the natural state, presumably due to the removal of too many cations such as proteins and minerals in the process of PS purification. On the other hand, sodium alginate solution (used as a positive control [10], 0.5 % w/v) did not form gel beads in the solutions of Ce^{3+}, La^{3+}, Yb^{3+}, Al^{3+}. The excellent gelation behavior of *A. sacrum* PS may be attributed to the structure of the metal ion binding sites containing carboxyls and sulfates.

CONCLUSION

We successfully extracted PS from *A. sacrum* using hot alkaline solution, while degrading other biopolymers, for the first time. It was found that the PS have carboxyls and sulfate groups, as shown by FT-IR, X-ray photoelectron spectroscopy (XPS), and CHN elemental analysis. The sulfation degree was estimated as about 10 mol %. The PS solution showed an extremely high viscosity of 1000–2000 Pa·s, suggesting a high molecular weight >1000 kDa. ^{1}H NMR studies demonstrated that the PS of *A. sacrum* have disubstituted fucose unit. The combination of sulfate group and fucose in the prokaryotic PS was first evidenced by direct spectroscopic study and is very important in terms of health and pharmaceutical medicines. The PS showed an efficient gelation behavior, binding to metal ions abundant in soil, and maximal swelling degree of the gel compared to the dry weight was very high, 250. These results imply that PS of *A. sacrum*, which has been mass cultivated in Japan for a long time, have potential as an environmentally benign water absorbent.

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