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Characterization of the Extracellular Polysaccharide Produced by a Marine Cyanobacterium, *Cyanothece* sp. ATCC 51142, and Its Exploitation Toward Metal Removal from Solutions

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Abstract. Cyanobacterium, *Cyanothece* sp. ATCC 51142 produces an exopolysaccharide at a high level. Physical analysis of the exopolysaccharide (EPS), such as nuclear magnetic resonance, infrared spectrum, were done to determine its possible structure. Thermal gravimetric analysis, differential scanning calorimeter, and differential thermal analysis of the polymer were done to find out the thermal behavior. Calcium content within the sample was found out. Some of the physicochemical properties, such as relative viscosity, specific viscosity, and intrinsic viscosity of the EPS were studied under different conditions. The phenomenon of gel formation by the EPS was investigated for its potential application in metal removal from solutions.

Microbial polysaccharides have found a wide range of applications in industries. They are incorporated in food as thickeners and used as suspending or gelling agents to improve food quality and texture [4]. In the pharmaceutical industry it can be used as hydrophilic matrix for controlled release of drugs [3], for development of bacterial vaccines [4, 6], as an anti-HIV agent [15], and to enhance nonspecific immunity [13]. However, in the near future the biggest market is expected to be the oil industry, where polysaccharide will serve as mobility controllers in enhanced oil recovery process from natural reservoirs [1]. The advantages of microbial polysaccharides over plants or marine macroalgal polysaccharides are its novel functionality, constant reproducible chemical and physical properties, and a stable cost and supply [7].

Cyanobacteria, which can be a potential source of polysaccharide, is yet unexploited [10]. A marine nitrogen-fixing cyanobacterium, *Cyanothece* sp. ATCC 51142, has an ability to produce extracellular polysaccharide (EPS) at a high level. The present investigation is aimed at characterizing the physical and chemical properties of the EPS to ascertain its industrial exploitation.

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Materials and Methods

Microorganisms. *Cyanothece* ATCC 51142 was obtained as a gift culture from Prof. D. O. Hall, King's College, London. It was maintained at 27°C in ASN III medium with light/dark cycle of 16/8 h under fluorescent light of 3,000 lux light intensity.

Exopolysaccharide (EPS) production. The EPS was separated by centrifugation at 10,000 *g* for 10 min at 4°C using 30-day-old *Cyanothece* culture grown in ASN III medium. The supernatant was pooled, and an equal volume of methanol was added. The mixture was kept at 4°C overnight and the precipitate was then spooled using a spatula. The EPS was then washed thrice with 50% (v/v) methanol and dried to constant weight at 37°C.

Analytical methods

Nuclear magnetic resonance (NMR) and infrared (IR) analysis. Pellet for infrared analysis was obtained by grinding a mixture of polysaccharide with dry KBr and then pressing in a mold. FTIR spectra was recorded on FTIR Impact 400D. Spectra were run in the 4,000–500 cm^{-1} region. H^1 NMR spectra of the samples were performed on drx 500 using D_2O as the solvent.

Calcium determination. For analysis of calcium content in the EPS, 50 mg of the EPS was taken and digested with the acid solution of perchloric and nitric acid. It was then diluted to 10 ml with deionized water and calcium analyzed with Systronics Flame Photometer (model no. 121). 100 ppm solution of Ca^{2+} equivalent was used as standard.

Thermal gravimetric analysis (TGA). The TGA analysis of polysaccharide was done on a Mettler TA 4000 instrument. The TGA trace was

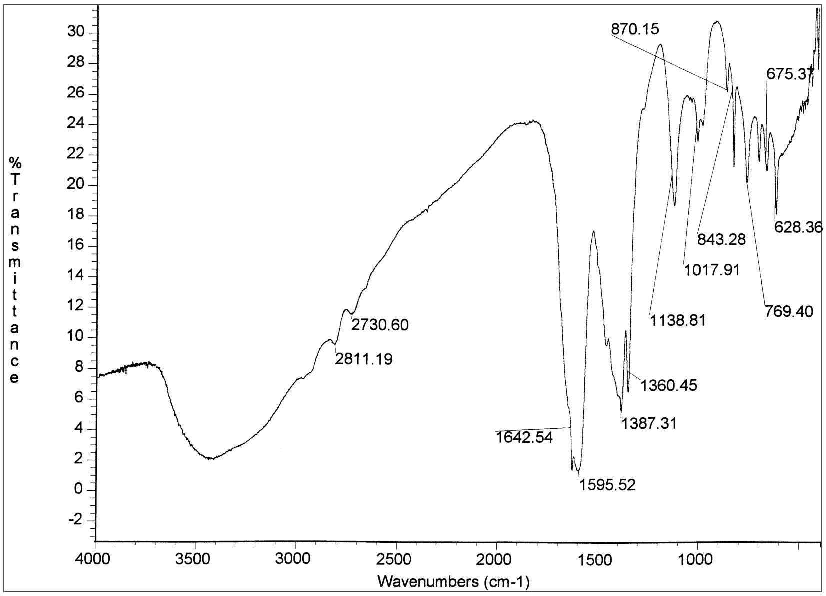

Fig. 1. The infrared spectra of the exopolysaccharide obtained from 1-month *Cyanothece* sp.

obtained in the range 40–400°C under nitrogen atmosphere at rise of 10°C/min.

Differential scanning calorimeter (DSC) and differential thermal analysis (DTA). The DSC and DTA analysis of EPS were done on Shimadzu Thermal Analyser (model no. DT-30). Both the analyses were carried out in nitrogen atmosphere with flow rate of 30 ml/min and heating rate of 10°C/min. Chart speed was kept at 5 mm/min.

Viscometric analysis. Viscosity measurements were performed at 25°C in an Ubbelohde capillary viscometer. 0.8% (w/v) polysaccharide solutions were prepared in water, 1% (w/v) $MgSO_4$, 1% (w/v) $CaCl_2$, and 1% (w/v) NaCl aqueous solutions, respectively, and viscosity was measured. The dilutions were made directly in the viscometer with respective solutions.

Gel formation. The supernatant obtained by centrifugation (as described earlier) of 30-day-old *Cyanothece* culture was used for gel formation. pH of the EPS was adjusted to between 10 and 11 by either 2 *N* NaOH or with ammonium buffer and gel was formed almost instantaneously upon mixing.

Study of metal removal. The study was concentrated on the ability of the EPS to remove copper, nickel, and cobalt solutions with metal concentration of 900 µg/ml, 700 µg/ml, and 200 µg/ml, respectively.

In a typical experiment 20 ml (2 mg of polysaccharide) EPS was mixed with increasing quantity of metal solution. The gel formed was filtered and the amount of metal left in the filtrate was determined by Perklin Elmer atomic absorbtion spectrophotometer (model no. 3110). Percentage removal was calculated by subtracting the amount of metal left from the amount of metal initially taken.

Results and Discussion

The IR spectrum of polysaccharide obtained from 1-month culture is shown in Fig. 1, and the spectrum of the polysaccharide from 2-week culture is shown in Fig. 2. There is some obvious difference between the two, indicating that the polysaccharide becomes more complex with the passage of time.

In Fig. 2, the peak around 3,410 cm^{-1} is assigned to υ OH stretching frequency and as expected it is broad. The weak absorbtion at ~2,910 and ~2,812 cm^{-1} are attributed to C–H stretching frequency. Absorbtion at 1,622 cm^{-1} is assigned to the stretching vibration of carboxylate group. The absorbtion at 1,387 cm^{-1} is possibly due to symmetric CH_3 bending. The bands due to asymmetric CH_3 bending and due to CH_2 bending could not be identified in the spectrum. However, in Fig. 1, a band at 1,450 cm^{-1} due to asymmetric CH_3 bending and bending of CH_2 could be observed. The strong absorbtion at 1,138 and 1,120 cm^{-1} are coupled vibrations of $S = O$ (sulfite moiety) and OH bending, $>$ C–O–C $<$ stretching might also contribute at this frequency [8, 14].

The spectra remained same for the polysaccharide obtained from the culture grown in presence of NaCl concentration ranging from of 0 to 5% (w/v), under nitrogen-deficient condition, and in pH from 5 to 11.

H^1 NMR spectrum of the polysaccharide obtained from culture grown in nitrogen containing medium is shown in Fig. 3, and the tentative structure of the EPS is illustrated in Fig. 4, which shows the numbering system used in this account. The structure shown in Fig. 4 does not demonstrate the structure or arrangement of the sugar moieties, but illustrates the type of protons that are expected in the EPS. The peak at δ 4.5 ppm is due to H1-type protons [5, 7]. The peak at δ 2.75 ppm is due to H2-type proton, and the peak at δ 2.15 ppm may be assigned to protons of hydroxyl group (H5). Absorbtion

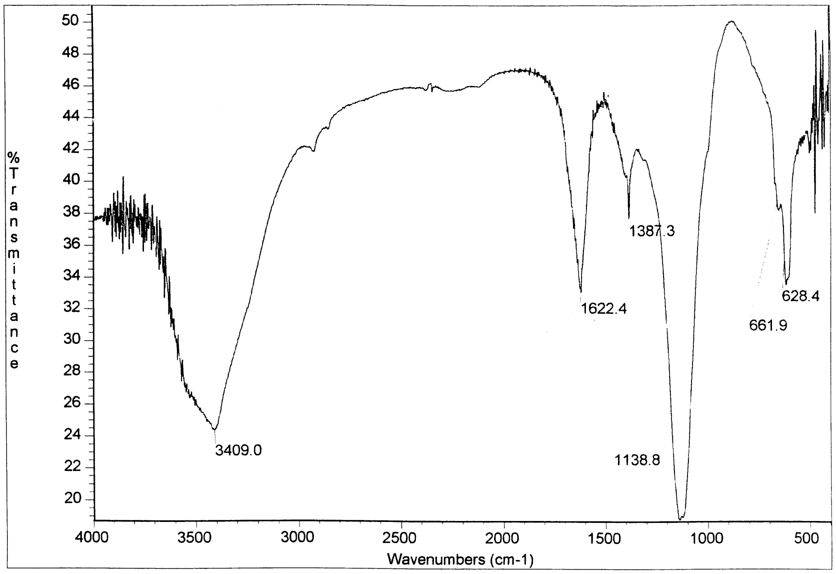

Fig. 2. The infrared spectra of the exopolysaccharide obtained from a 2-week *Cyanothece* sp.

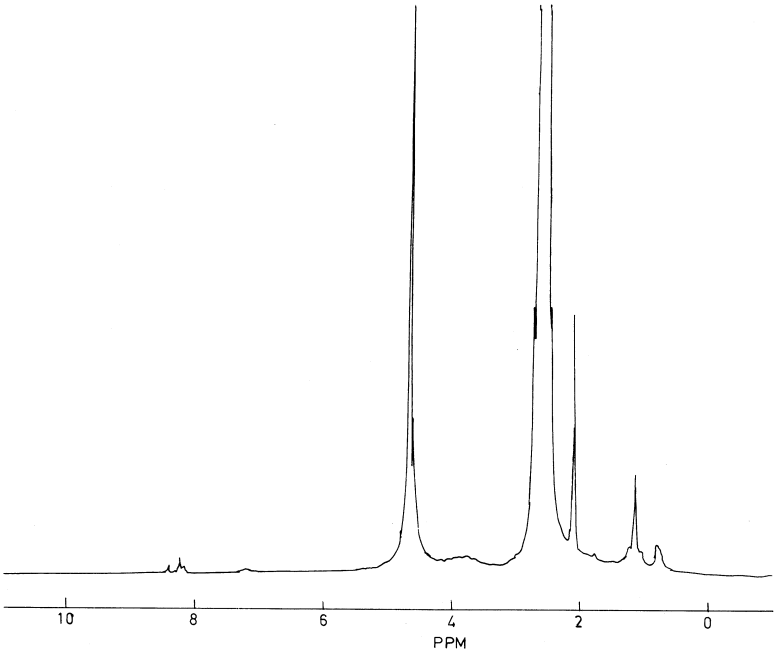

Fig. 3. H^1 NMR spectrum of the exopolysaccharide obtained from *Cyanothece* sp. grown in nitrogen-containing medium.

at 2.5 ppm may be due to H3-type protons. The δ 1.25 ppm peak can be due to methyl group protons, which are not directly attached to oxygen or any other electron withdrawing group (H4). The peak at around δ 4.2 is assigned to H6 type of protons [5, 7].

The H^1 NMR spectrum of the polysaccharide obtained from culture grown in nitrogen-deficient medium was not much different from that of the nitrogen-containing medium, as shown above. More peaks were observed in the region of 1.0–2.0 δ and peak around 4.2 δ that was not well defined in above spectrum was found to be distinct.

The calcium content in the polysaccharide was found to be 3.2g% (w/w). The reason for such a high concentration of calcium may be traced to the presence of anionic groups in the EPS, which binds to the cations present in the medium.

The DSC and DTA results show three endothermic peaks in the trace. One peak in DSC thermogram is seen at ~100°C and the corresponding one in DTA at around same temperature is quite sharp. Following Reich's method [12], the energy of activation was found out to be 149.6 kcal/mole. Two minor peaks are observed at 160°C and 230°C in DSC and corresponding peaks are at 140°C

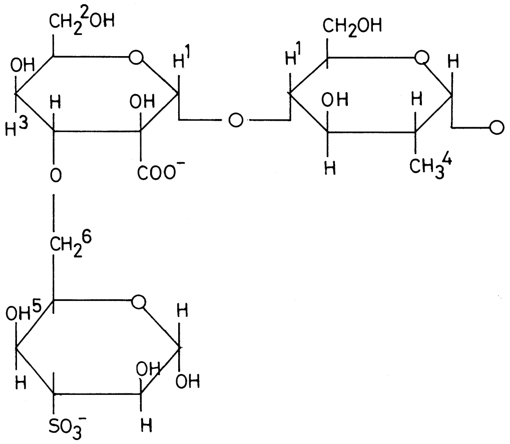

Fig. 4. Putative structure of the exopolysaccharide with the numbering system used in the interpretation of results.

and 205°C in DTA. Reedy et al. [11] have indicated the possibility of the EPS being made of seven different types of sugars, including glucuronic acid. All the above peaks may be attributed to the change in conformation of different sugar molecules at different temperature. The evidence of this fact was obtained from the IR spectrum of the EPS, which was heated up to 100°C for 1 h and then cooled in a desiccator immediately. The three vibrations coupled around 1,160 cm^{-1} (Fig. 2) became distinct. Three peaks were now seen at 1,159, 1,120, and 1,100 cm^{-1}. It may be possible that after the conformation change at 100°C, the vibrations of S = O (sulfite moiety), OH bending, and > C–O–C < streching may not be in a position to couple.

Conformation change probably finds support from the TGA data. In TGA the weight loss of polysaccharide was found to be just 10% till 400°C. The EPS appears to be highly stable, and no major decomposition is taking place. This is supported by the fact that the IR spectrum taken after heating the EPS at 250°C for 30 min followed by immediate cooling shows no appreciable change from the original. The stability of the compound may be attributed to the presence of cations, which act as a bridge between different charged sugar moieties.

The successful application of EPS largely depends on their physicochemical properties rather than on yield alone. To gain insight about the viscometric properties and behavior, relative viscosity (η_r) and specific viscosity (η_{sp}) were studied under different concentrations of EPS in water, 1% $CaCl_2$, 1% $MgSO_4$, and 1% NaCl. It was observed that with increasing concentration of polysaccharide, there was an increase in η_r and η_{sp} in all the solutions. Intrinsic viscosity, which is considered as a characteristic parameter governing many physicochemical aspects of the polymers, was found to be maximum of 1.06 dl/g in 1% $MgSO_4$. It was determined from the intercept of the plot of ln η_r/C versus concentration and η_{sp}/C versus concentration. This will enable us to predict the molecular weight of the polysaccharide upon further characterization.

The EPS produced by *Cyanothece* has a characteristic phenomenon of gel formation. The phenomenon of gel formation is supposed to be caused due to precepitation of the EPS. It has been seen that concentrated suspensions of polysaccharide gelled at divalent cation concentration similar to those that yielded maximal viscosity with diluted EPS suspensions [2, 9]. The medium in which the culture is grown contains cations whose concentration may be sufficient for the polysaccharide to precipitate and form a gel.

The gelation property of EPS is seen to be highly effective for metal removal from solutions. More than 90% of Ni^{2+}, Cu^{2+}, and Co^{2+} could be removed from the solutions having total metal ion concentrations of 22.5, 17.5, and 5.0 mg, respectively.

Conclusion

Thus, with partial physical and chemical characterization of the exopolysaccharide attained, *Cyanothece* ATCC 51142 seems to be promising for industrial exploitation. The gelling property can be useful in food industries and also to remove various metals from industrial wastes. The thermal stability of the polymer in inert atmosphere opens the possibility of its application in packaging and related industry. In view of the stability of the polysaccharide and presence of functional groups, it may be inferred that appropriate grafting will improve the processibility and properties of this polymeric material. This might pave the way to find many more applications of polymeric material.

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