Appl Biochem Biotechnol (2010) 162:1327–1338
DOI 10.1007/s12010-010-8922-3

Isolation and Characterization of Exopolysaccharides Produced by the Cyanobacterium *Limnothrix redekei* PUPCCC 116

J. I. S. Khattar · D. P. Singh · Namita Jindal · N. Kaur · Y. Singh · P. Rahi · A. Gulati

Received: 29 September 2009 / Accepted: 26 January 2010 /
Published online: 20 February 2010

Abstract *Limnothrix redekei* PUPCCC 116, a filamentous cyanobacterium, has been identified through 16S rRNA gene sequencing. Exopolysaccharides (EPS) of this organism have been isolated and characterized chemically, and its rheological properties were compared with commercial xanthan. The organism produced 304 μg EPS/ml culture in 21 days. The rate of EPS production was maximum (313 μg EPS/mg protein/day) during the initial days of growth, and it decreased to 140 μg EPS/mg protein/day during 18-21 days of growth. Chemical analysis of EPS revealed the presence of glucose/mannose, ribose, rhamnose, and uronic acid. Fourier transformed infrared spectrum of EPS further revealed the presence of methyl and carboxyl groups besides C–N groups indicating the presence of peptidyl moieties. Elemental analysis of EPS showed the presence of 4.97% N. The organism under continuous light produced 102% more EPS compared to when grown under a light/dark cycle of 14/10 h. The rheological properties of EPS were comparable with commercial xanthan gum.

Keywords Cyanobacterium · Exopolysaccharides · *Limnothrix redekei* · Monomer composition · Rheology · 16S rRNA gene

Introduction

Many microorganisms produce varying amounts of extracellular organic polymers principally made up of polysaccharides with smaller quantities of proteins, lipids, glycoproteins, etc. Such extracellular polymers are commonly referred to as exopolysaccharides (EPS) [1]. Depending upon the location, EPS may occur as capsular polysaccharides when the polymer is closely associated with cell surface or as slime polysaccharides when these are loosely associated with cell surface [2]. Microbial EPS are produced by both prokaryotes (eubacteria and

J. I. S. Khattar (✉) · D. P. Singh · N. Jindal · Y. Singh
Department of Botany, Punjabi University, Patiala 147 002, India
e-mail: jisk_pbi@rediffmail.com

N. Kaur · P. Rahi · A. Gulati
Plant Pathology and Microbiology, Institute of Himalayan Bioresource Technology, Palampur 176 061, India

archaebacteria) and eukaryotes (phytoplankton, fungi, yeasts, and algae) that form a gel-like matrix around the cell [3–6]. Microbial polysaccharides are known to be involved in the pathogenesis, symbiosis, biofilm formation, and stress resistance in microorganisms [3].

The unique properties of certain microbial EPS as flocculating, gelling, emulsifying, and suspending agents make them suitable for varied applications in pharmaceutical, chemical, and food industries. Microbial EPS are also preferred in these industries owing to their novel functionality, reproducible physicochemical properties, stable cost, and supply [7]. In recent years, attention towards cyanobacterial EPS has increased because a large number of cyanobacteria are characterized by the presence of polysaccharidic outer investments, and these organisms are photosynthetic, easy to culture, some are even N_2 fixers, and are amenable to manipulation of conditions for enhancing growth and/or EPS production [8–10]. Cyanobacterial EPS are complex in nature due to the presence of proteins, uronic acids, pyruvic acid, and O-methyl, O-acetyl, and sulfate groups [2, 11, 12]. It has been suggested that EPS produced by cyanobacteria could be useful in various applications such as water holding capacity of soil and removal of heavy metals and solid materials from water reservoirs [11–14].

Since relatively few microbial polysaccharides, such as xanthan from *Xanthomonas campestris* and gellan gum from *Sphingomonas paucimobilis*, are being widely used as food additives, stabilizers, and suspending agents [15], there is a need to screen and find new sources of novel exopolysaccharides. The present study was undertaken with the aim to identify new sources of novel exopolysaccharides. Thus, EPS of *Limnothrix redekei*, a filamentous cyanobacterium, have been isolated and characterized chemically, and its rheological properties were compared with commercial xanthan.

Materials and Methods

Organism and Culture Conditions

Isolate PUPCCC 116 was isolated from the rice fields of Patiala, India. Isolation and purification were performed by dilution and plating of water samples. Chu-10 medium [16] was slightly modified (calcium nitrate was replaced with equimolar ratio of calcium chloride and KNO_3 was used as a source of nitrogen) and used for the growth of the organism in batch cultures. The nutrient medium contained (in g/L) $CaCl_2 \cdot 2H_2O$, 0.232; K_2HPO_4, 0.01; $MgSO_4 \cdot 7H_2O$, 0.025; Na_2CO_3, 0.02; $Na_2SiO_3 \cdot 5H_2O$, 0.44; KNO_3, 1.01; ferric citrate, 0.0035; and citric acid, 0.0035. The cultures were incubated at 28±2 °C and illuminated with daylight fluorescent tube lights for 14 h a day giving a radiant flux of 9.8 W/m^2. The cultures were manually shaken at least twice a day.

16S rRNA-Based Identification of the Organism

16S rRNA Gene Sequence Analysis

Genomic DNA extraction was done by HiPurA™ plant genomic DNA Miniprep Purification Spin kit (HIMEDIA®, Mumbai, India). 16S rRNA gene was amplified using cyanobacteria specific primers CYA359F and CYA781R [17]. The total volume of the polymerase chain reaction (PCR) reaction mixture was 50 µl, comprising 200 µM dNTPs, 50 µM each primer, 1× PCR buffer, 3 U *Taq* polymerase, and 100 ng genomic DNA. The thermocycling procedure involved an initial denaturation at 94 °C for 4 min, followed by 35 cycles of 94 °C for 1 min, 52 °C for 1 min, and 72 °C for 2 min, and a final extension at

72 °C for 8 min. The gel purified product obtained using Real Genomics™ Gel DNA Extraction kit (Real Biotech Corporation, Taipei Country, Taiwan) was sequenced using a Big-Dye Terminator v.3.1 Cycle Sequencing kit and an ABI Prism 310 Genetic Analyzer (Applied Biosystems, CA, USA). The sequence was analyzed using the gapped BLASTn (http://www.ncbi.nlm.nih.gov) search algorithm and aligned to the nearest neighbors. The evolutionary distances among related taxa were calculated using Kimura's two-parameter model with TREECON software package version 1.3b (Copyright © Yves Van de Peer, University of Antwerp, 1994, 1998) after aligning the sequences with ClustalW. The 16S rRNA gene sequence of *Spirulina major* 0BB 36S18 was used as an out-group.

Growth

Exponentially growing 6-day-old stock cultures were washed with sterilized distilled water and inoculated into nutrient medium to attain initial absorbance λ_{660}, 0.1. Growth was monitored as increase in absorbance of the cultures with time.

Isolation, Purification, and Quantification of EPS

Isolation and extraction of exopolysaccharides were done following the procedure of Reddy et al. [18] with little modifications. Cells were separated from the culture medium by centrifugation at 15,000×*g* for 20 min, and the supernatant (A) was collected for extraction of released polysaccharides (RPS). The pellet (B) was used for the extraction of cell wall polysaccharides (CPS). The pellet was treated with a mixture of 0.6 M NaCl and 0.06 M of EDTA at 50±2 °C for 3 h. The mixture was centrifuged at 15,000×*g* for 20 min and supernatant (B) was saved. The EPS from both supernatants (A containing RPS and B containing CPS) was extracted by vacuum evaporating each separately to half volume and dialyzing (membrane with 3,500 Da cutoff) against distilled water for 72 h. To the dialyzed solutions, equal volumes of chilled isopropanol were added, mixed, and kept in a deep freezer (−20 °C) for 72 h. Precipitates of EPS formed were collected by centrifugation at 15,000×*g*, washed with chilled isopropanol, lyophilized, and stored in air tight vials.

The amount of EPS was quantified with anthrone reagent following the procedure of Seifter et al. [19]. Dried EPS was hydrolyzed with 2 N HCl at 100 °C for 2 h. Appropriately diluted hydrolysate was used for quantification using glucose as standard.

Rate of EPS Production

Growth of the organism and the amount of EPS produced by it were studied simultaneously. To calculate the rate of EPS produced by the organism during a specific time period, an increase in the amount of EPS during that time period was divided by the increase in biomass (in terms of protein content) during that particular time period.

EPS Production Under Nitrogen Limitation and Salt Stress

Nitrogen limitation in the cultures was created by decreasing the concentration of nitrate in the basal nutrient medium from 10 to 5.0 and 1.0 mM. Chu-10 basal medium contained no sodium chloride. Salt stress in the cultures was created by adding sufficient volumes of 1.0 M sodium chloride stock solutions to the basal medium to attain 8.5, 17, and 42.5 mM concentrations. These concentrations were selected after a preliminary experiment, which demonstrated that the organism did not grow in sodium chloride beyond 50 mM. Exponentially growing (6-day-old)

cultures of the test organism were inoculated in these media to attain λ_{660}, 0.1. Growth and EPS production were studied for 6 days as described earlier.

Identification of Monosaccharidal Units of EPS

EPS (3 mg) was hydrolyzed in sealed tubes with 6 ml of 4 N trifluoroacetic acid (TFA) at 121± 2°C for 1 h. The solutions were evaporated to dryness with additions of methanol to remove TFA. The dried hydrolysate was dissolved in 1 ml sterilized distilled water. The monosaccharidal units were firstly separated on TLC using water/ethyl acetate/*n*-butanol in a 1:2:10 ratio as running solvent. Separated sugars on TLC plates were spotted following the procedure of Chaplin [20] using a diphenylamine/aniline/phosphoric acid mixture as spraying reagent. Galacturonic acid was used as the standard for the identification of uronic acid. After preliminary separation/identification of monosaccharides on TLC, the hydrolysate was subjected to high performance liquid chromatography (HPLC) analysis for the exact identification of monosaccharides. The HPLC system used was Waters 410 HPLC System fitted with a differential refractometer, Waters 600 Controller, and NH_2 column. The mobile phase was acetonitrile/water in a 7:3 ratio with 1 ml/min flow rate. Glucose, galactose, ribose, mannose, fucose, arabinose, xylose, and rhamnose were used as standards.

Fourier Transformed Infrared Analysis

The major structural groups of EPS were detected using Fourier transformed infrared (FT-IR) spectroscopy. The pellet for infrared analysis was prepared by carefully grinding 2-mg dry EPS with 200-mg dry KBr and pressing it in a mold. The FT-IR spectra were recorded in transmittance mode in the region of 4,000 to 500/cm with a resolution of 4/cm on a Perkin Elmer spectrum GX FT-IR system (Perkin Elmer, USA).

Determination of N and Protein Content

The amount of N present in EPS was determined by using an elemental analyzer (Perkin Elmer CHN Elemental Analyzer 2400).

The protein content of the cells was determined following the procedure of Lowry et al. [21] after alkaline hydrolysis of the cells using bovine serum albumin as standard.

Rheological Properties

The rheological properties of EPS were studied using a Brookfield R/S Rheometer equipped with a double gap cylinder. The inner and outer radii of the measuring bob (R_2 and R_3) of the instrument are 22.75 and 23.50 mm, whereas the inner and outer radii of the measuring cup (R_1 and R_4) are 22.25 and 24 mm, respectively. All data are average±SD of three independent experiments.

Results and Discussion

16S rRNA Gene Sequence Analysis

As a result of the amplification and sequencing of 16S rRNA gene fragment, a partial nucleotide sequence of 420 bp was obtained, which showed 100% identity with *L. redekei* M2-7 (EF634458) (Fig. 1). Thus, the organism was identified and named as *L. redekei*

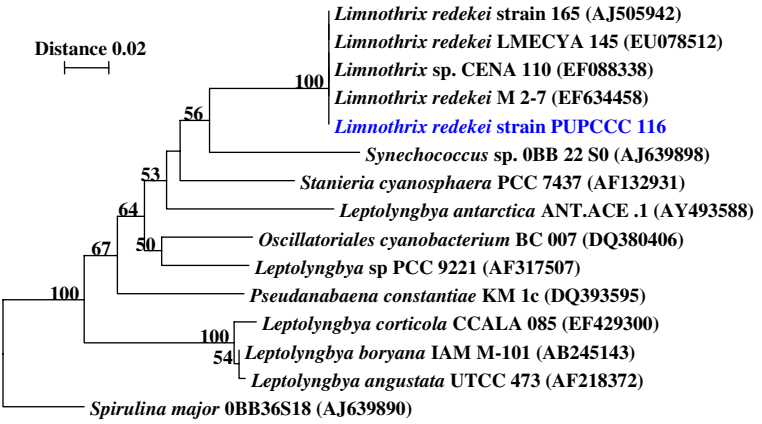

Fig. 1 Phylogenetic tree showing relationship among *Limnothrix redekei* strain PUPCCC 116 and representatives of some related taxa, based on analysis of partial 16S rRNA gene sequence. The *numbers on the nodes* indicate how often (number of times, percent) the species to the right are grouped together in 100 bootstrap samples. *Spirulina major* 0BB36S18 was used as the out-group. *Bar*, 0.02 substitutions per site

PUPCCC 116. The nucleotide sequence determined in this work has been deposited in the NCBI GenBank database (accession number GU552680).

EPS Production

Extracellular polymeric substances are renewable energy sources representing an important class of polymeric materials of biotechnological importance with a wide variety of potential useful applications [9, 22, 23]. Culture conditions affect the productivity of the cyanobacterial extracellular polymeric substances. The results of the present study (Fig. 2) showed that the organism produced EPS throughout the incubation period, and maximum EPS production (304±3.12 μg/ml) was observed after 21 days of incubation. Of the total EPS produced, 68.5% (208 μg/ml) were RPS and 31.5% (96 μg/ml) were CPS. Thus, most of the EPS produced by the organism was released into the medium. Organisms producing large amounts of RPS are biotechnologically important since it is easy to isolate released polysaccharides compared to polysaccharides attached to the cell wall. The production of large amounts of EPS has been suggested during the late exponential or stationary phase of growth [8, 24]. It was observed that the test organism produced maximum EPS (313±30.87 μg/mg protein/day) during the first 3 days of growth and subsequently, the rate of EPS production decreased (140± 10.25 μg/mg protein/day; Fig. 3). This organism is thus a suitable candidate for EPS production in a continuous system. The EPS production in *Cyanothece* sp. ATCC 51142, *Nostoc* sp., and *Oscillatoria* sp. has been shown to be linked to growth [3, 25]. Stage and age of cultures affect the production of cyanobacterial intracellular polymeric substances [8]. On the other hand, Trabelsi et al. [2] demonstrated entirely dissociated kinetics of cellular growth and kinetics of EPS production in *Arthrospira platensis*.

EPS production by cyanobacteria is affected by both nutritional and environmental factors [26]. Of the nutritional factors, sodium chloride stress and nitrogen limitation enhance EPS production [10, 27, 28], and among the environmental factors, duration and intensity of light are important in regulating EPS production [29–31]. During the present study, the growth as well as the EPS production of the organism decreased under sodium chloride stress and nitrogen limitation in the basal medium (Table 1). The organism did

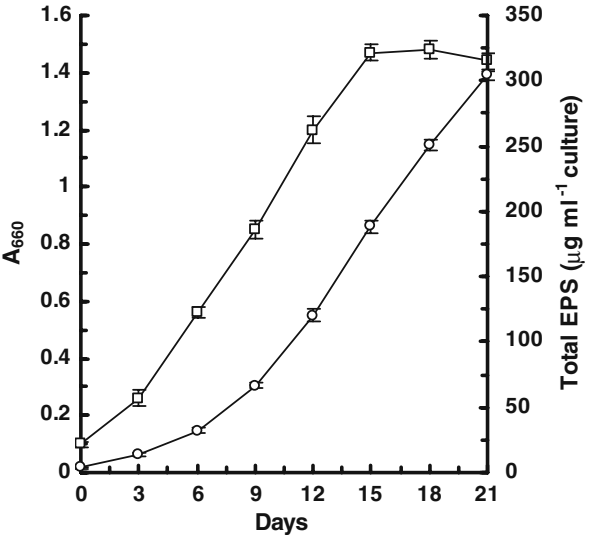

Fig. 2 Growth (*empty square*) and amount of EPS produced (*empty circle*) by *Limnothrix redekei* in basal medium. Amount of EPS includes both RPS and CPS (mean±SD, n=6). On day 0, absorbance of the cultures was set at 0.1 λ_{660}. *Error bars* indicate SD

not survive in 50 mM sodium chloride. This may be due to the fact that the test organism is an isolate from fresh waters. No increase in EPS production by *Cyanothece* sp. was reported when NaCl in the medium was increased in the range of 0–2.0 M [32]. EPS in *Dunalliela salina*, an isolate from high salt environments, increased with salt concentration in the medium and it is suggested that the organism might have adapted itself to survive in high salt concentration [23]. Ozturk and Aslim [10] have shown that not only the amount but also the composition of EPS of cyanobacteria changes with salt

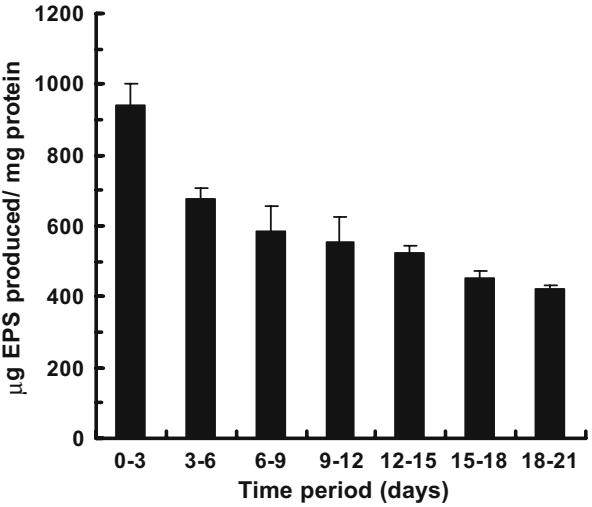

Fig. 3 Rate of EPS production by *Limnothrix redekei* during different periods of growth. *Error bars* indicate SD

Table 1 Growth and EPS production by *Limnothrix redekei* in sodium chloride stress and nitrogen limitation in 6 days.

Condition	λ_{660}	EPS (μg/ml culture)
Control[a]	0.56	32.0±0.28
Medium+8.5 mM NaCl	0.46	32.4±0.25
Medium+17 mM NaCl	0.39	29.6±0.19
Medium+42.5 mM NaCl	0.34	25.6±0.21
Medium with 5.0 mM KNO_3	0.54	28.0±0.16
Medium with 1.0 mM KNO_3	0.50	14.4±0.18

On day 0, absorbance of cultures was set at λ_{660}, 0.1

[a] In control cultures medium contained 10 mM KNO_3 and no sodium chloride

stress. On transfer of the cultures to continuous light conditions from 14:10 light/dark regime, the amount of EPS produced by the organism increased by 102% (614 μg EPS/ml). Thus, optimal conditions for EPS production by *L. redekei* were basal medium cultures kept in continuous light. Trabelsi et al. [31] studied the combined effect of temperature and light intensity on the growth and EPS production of *A. platensis* and concluded that light intensity had a positive linear effect for EPS production. Furthermore, they showed that the optimum conditions for growth of the organism are different from EPS production.

Composition of EPS

The separation of components of EPS hydrolysate through TLC revealed the presence of four monosaccharides and one uronic acid (Fig. 4). HPLC analysis of hydrolyzed samples of EPS showed the presence of the neutral sugars glucose/mannose, ribose, and rhamnose. Although separation through TLC revealed the presence of four monosaccharides, only three monosaccharides were recognized through HPLC. It is suspected that the fourth sugar is galactose as glucose/mannose has different Rf values than galactose when separated on TLC. It is difficult to distinguish glucose/mannose/galactose by the HPLC technique employed during the present study. Since glucose and mannose have the same Rf/retention time when separated through TLC/HPLC, it appears that besides galactose, of the other two, either it is glucose or mannose. The identification of four neutral sugars and uronic acid in EPS of the test organism in the present study confirms earlier reports on complex composition of cyanobacterial EPS [8, 33]. Most of the studies have shown the presence of four to six monosaccharides in cyanobacterial EPS [3, 10, 12, 23] and the composition of EPS changes with culture conditions [10]. The presence of pentose sugar, which is usually absent in polysaccharides of prokaryotic origin and often found in cyanobacterial EPS [3, 30], is remarkable [23]. The FT-IR spectrum of EPS revealed the presence of carboxyl, methyl, and hydroxyl groups and C–N, N–H, and C–H stretching absorption bands (Fig. 5). A peak around wave number 3,779/cm corresponds to N–H stretching frequency; absorption band at 3,405/cm corresponds to O–H stretching; a minor band at 2,928/cm corresponds to C–H stretching; absorption at 1,657/cm corresponds to carboxylate group; and absorptions at 1,384/cm, 1,270/cm, and 1,052/cm correspond to symmetric CH_3 bending, C–N stretching, and C–O stretching, respectively. Besides these groups, sulfate, pyruvate, and acetate groups and peptidic moieties have been reported in EPS of some cyanobacteria [2, 23, 34, 35]. The presence of several peaks less than 1,000/cm may be due to several visible bands and/or to the occurrence of possible linkages between

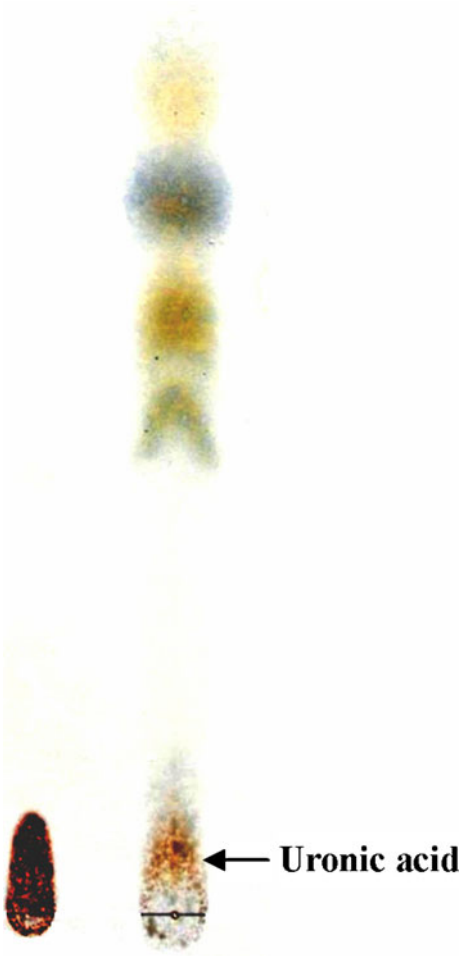

Fig. 4 Thin layer chromatogram of EPS hydrolysate of *Limnothrix redekei* revealing the presence of four monosaccharides and uronic acid. *First lane*: galacturonic acid as standard. *Second lane*: EPS hydrolysate

monosaccharide units [2]. During the present study, sulfate, pyruvate, and acetate groups were not detected while the presence of peptidic moiety was confirmed in the EPS of the test strain. Various workers have ascertained the presence of peptidyl moieties in EPS by determining N content through elemental analysis and then multiplying it with 6.25 to gain a rough estimate of peptidic content [8, 35, 36]. Elemental analysis of EPS of *L. redekei* revealed the presence of 4.97% N. The presence of a variety of sugars, functional groups, and peptidyl moieties in EPS of cyanobacteria may result in their varied functions and biotechnological applications [9, 10]. Cyanobacteria may be regarded as a very abundant source of structurally diverse polysaccharides, some of which may possess unique properties for special applications, not fulfilled by the polymers currently available [10]. The presence of uronic acid imparts anionic character to EPS, and the capacity of charged groups to bind water molecules can be exploited by the cosmetic industry for product formulations [37]. Peptidic moieties and deoxy sugar rhamnose contribute hydrophobic behavior to otherwise hydrophilic macromolecules. This property of cyanobacterial EPS having both hydrophilic and hydrophobic groups makes them suitable for the stabilization of emulsions or as

Fig. 5 FT-IR spectrum of EPS of *Limnothrix redekei*

bioflocculants [35, 38]. Thus, EPS of *L. redekei* has a good potential to be used in cosmetic and food industries.

Rheological Properties

Flow curves of 0.2% (*w*/*v*) and 0.4% (*w*/*v*) aqueous solutions of *L. redekei* EPS are shown in Fig. 6. Aqueous dispersions of EPS exhibited non-Newtonian shear thinning behavior. Shear

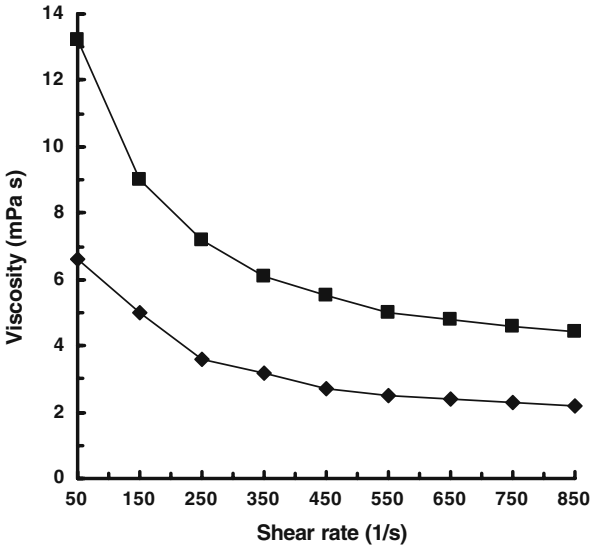

Fig. 6 Effect of shear rate on viscosity of EPS of *Limnothrix redekei*; 0.4% (*w*/*v*; *filled square*) and 0.2% (*w*/*v*; *filled diamond*) EPS. Temperature 25 °C

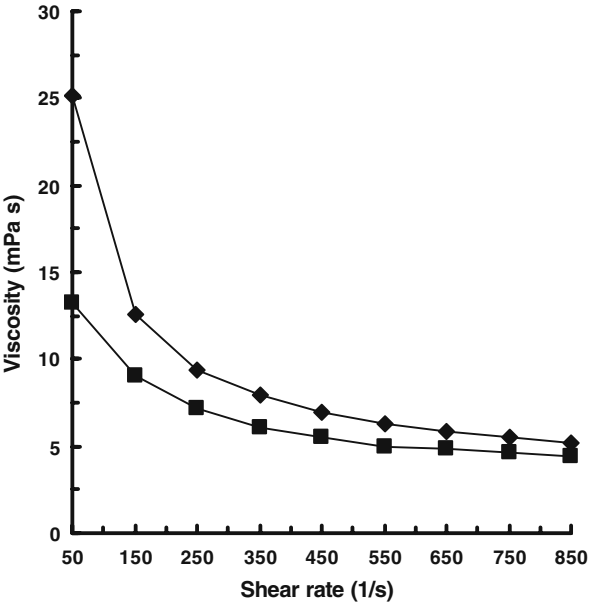

Fig. 7 Effect of shear rate on viscosity of EPS of *Limnothrix redekei* and xanthan (Sigma); 0.4% (*w*/*v*) EPS (*filled square*) and 0.2% (*w*/*v*) xanthan (*filled diamond*). Other conditions were the same as in Fig. 6

thinning was more pronounced up to a shear rate of 350/s. Shear thinning is mainly due to breakdown of structural units in EPS due to hydrodynamic forces generated during shear. Polysaccharides displaying non-Newtonian pseudoplastic behavior can be useful in industrial applications [31]. Shear thinning properties help in providing good suspending and good

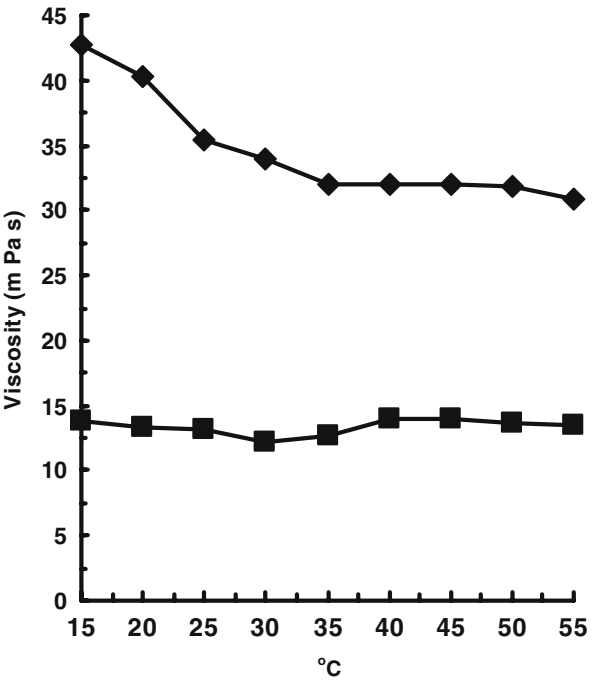

Fig. 8 Effect of temperature on viscosity of EPS of *Limnothrix redekei* and xanthan (Sigma); 0.4% (*w*/*v*) EPS (*filled square*) and 0.2% (*w*/*v*) xanthan (*filled diamond*)

sensory qualities to food products [39]. Viscosity of EPS increased with an increase in EPS concentration from 0.2% (*w*/*v*) to 0.4% (*w*/*v*), and flow curves of both concentrations were almost parallel indicating that no major conformational changes occurred from 0.2% (*w*/*v*) to 0.4% (*w*/*v*) EPS. Comparison of shear rate dependence of viscosity for 0.4% (*w*/*v*) aqueous dispersion of EPS with xanthan (0.2%, *w*/*v*) showed that both had different viscosities but similar shear thinning properties with EPS comparatively less shear sensitive than xanthan (Fig. 7). Recently, it has been demonstrated that drying methods affected the rheological properties of flaxseed gum, which otherwise has rheological properties similar to those of guar gum [40]. Thus, it will be interesting to find whether drying methods affect the viscosity of EPS of cyanobacterial origin as most of the studies pertained to oven-dried or freeze-dried EPS. The rheological properties of EPS of *Cyanospira capsulata* and *Cyanothece* strain CA3 [8] and of *Cyanothece* sp., *Oscillatoria* sp., and *Nostoc* sp. [3] have been compared with and found to be similar to commercial xanthan, which suggested that EPS of these cyanobacteria have potential commercial exploitation. The comparison of viscosities of EPS and xanthan at different temperatures revealed a slight decrease in viscosity of xanthan aqueous solution with increase in temperature from 15 to 55 °C but the viscosity of EPS solution was temperature insensitive (Fig. 8). The commercial applicability of polysaccharides largely depends on the thermal and viscometric behaviors under different conditions [10]. Similarity in the shear thinning properties of EPS and xanthan and insensitivity of viscosity of EPS to temperature increase appear promising as EPS of *L. redekei* may find applications similar to xanthan. Although exopolysaccharides of a number of cyanobacteria have been studied and characterized, to the best of our knowledge this is the first report on the characterization of exopolysaccharides produced by *L. redekei.*

Conclusions

The exopolysaccharides produced by *L. redekei* were found to be heteropolysaccharides with protein moieties. The polysaccharide had a complex composition that contained four neutral sugars and uronic acid. FT-IR spectra demonstrated that EPS was a type of polyanionic polysaccharide that contained carboxyl and carbonyl groups. Rheological studies showed that EPS of *L. redekei* showed non-Newtonian, pseudoplastic shear thinning properties similar to commercial xanthan, which suggests that these may be valuable for use in pharmaceutical and food industries. This is the first report on the characterization of polysaccharides of *L. redekei.* However, much work is still required to elucidate the structure and the relationship between the function and structure of EPS.

Acknowledgments The authors are thankful to the Head & Coordinator DRS and ASIST, Department of Botany, Punjabi University, Patiala and the Director, IHBT, Palampur for laboratory facilities. Financial assistance from the Council of Scientific & Industrial Research, New Delhi with Research Scheme no. 38 (1189)/08/EMR-II is also acknowledged.

References

1. Wingender, J., Neu, T. R., & Flemming, H. C. (1999). In J. Wingender, T. R. Neu, & H. C. Flemming (Eds.), *Microbial extracellular polymeric substances* (pp. 1–19). Berlin: Springer.
2. Trabelsi, L., M'sakni, N. H., Ouada, H. B., Bacha, H., & Roudesli, S. (2009). *Biotechnology and Bioprocess Engineering, 14*, 27–31.

3. Parikh, A., & Madamwar, D. (2006). *Bioresource Technology, 97*, 1822–1827.
4. Zou, X., Sun, M., & Guo, X. (2006). *International Journal of Biodeterioration and Biodegradation, 58*, 89–93.
5. Chi, Z., Su, C. D., & Lu, W. D. (2007). *Bioresource Technology, 98*, 1329–1332.
6. Duan, X., Chi, Z., Wang, L., & Wang, X. (2008). *Carbohydrate Polymers, 73*, 587–593.
7. Selbmann, L., Onofri, S., Fenice, M., Federici, F., & Petruccioli, M. (2002). *Research in Microbiology, 153*, 585–592.
8. De Philippis, R., & Vincenzini, M. (1998). *FEMS Microbiology Reviews, 22*, 151–175.
9. De Philippis, R., Sili, C., Paperi, R., & Vincenzini, M. (2001). *Journal of Applied Phycology, 13*, 293–299.
10. Ozturk, S., Aslim, B. (2009). *Environmental Science and Pollution Research.* doi:10:1007/S11356-009-0233-2.
11. Freire-Nordi, C. S., Vieira, A. A. H., & Nascimento, O. R. (2005). *Process Biochemistry, 40*, 2215–2224.
12. Ozturk, S., Aslim, B., & Suludere, Z. (2009). *Bioresource Technology, 100*, 5588–5593.
13. Bender, J., & Phillips, P. (2004). *Bioresource Technology, 94*, 229–238.
14. Ozturk, S., & Aslim, B. (2008). *Environmental Science and Pollution Research, 15*, 478–480.
15. Sutherland, I. W. (1998). *Trends in Biotechnology, 16*, 41–46.
16. Safferman, R. S., & Morris, M. E. (1964). *Journal of Bacteriology, 88*, 771–775.
17. Nubel, U., Garcia-Pichel, F., & Muyzer, G. (1997). *Applied and Environmental Microbiology, 63*, 3327–3332.
18. Reddy, K. J., Soper, B. W., Tang, J., & Bradley, R. L. (1996). *World Journal of Microbiology and Biotechnology, 12*, 311–318.
19. Seifter, S., Dayton, S., Novic, B., & Muntwyler, E. (1950). *Archives of Biochemistry, 25*, 191–200.
20. Chaplin, M. F. (1986). In M. F. Chaplin & J. F. Kennedy (Eds.), *Carbohydrate analysis: A practical approach* (pp. 1–36). Oxford: IRL.
21. Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951). *Journal of Biological Chemistry, 193*, 265–275.
22. Kumar, A. S., Mody, K., & Jha, B. (2007). *Journal of Basic Microbiology, 47*, 103–117.
23. Mishra, A., & Jha, B. (2009). *Bioresource Technology, 100*, 3382–3386.
24. Kumar, C. G., Joo, H., Kavali, R., Choi, J., & Chang, C. (2004). *World Journal of Microbiology and Biotechnology, 20*, 833–836.
25. Shah, V., Garg, N., & Madamwar, D. (1999). *Applied Biochemistry and Biotechnology, 82*, 81–90.
26. De Philippis, R., Sili, C., Tassinato, G., Vincenzini, M., & Materassi, R. (1991). *Bioresource Technology, 38*, 101–104.
27. Staats, N., Stal, L. J., & Mur, L. R. (2000). *Journal of Experimental Marine Biology and Ecology, 249*, 13–27.
28. Giroldo, D., & Vieira, A. A. H. (2002). *Journal of Applied Phycology, 14*, 185–191.
29. Lupi, F. M., Fernandes, H. M. L., Tome, M. M., Sa-Correia, I., & Novais, J. M. (1994). *Enzyme and Microbial Technology, 16*, 546–550.
30. Otero, A., & Vincenzini, M. (2003). *Journal of Biotechnology, 102*, 143–152.
31. Trabelsi, L., Ouada, H. B., Bacha, H., & Ghoul, M. (2009). *Journal of Applied Phycology, 21*, 405–412.
32. De Philippis, R., Margheri, M. C., Pelosi, E., & Ventura, S. (1993). *Journal of Applied Phycology, 5*, 387–394.
33. Hu, C., Liu, Y., Paulsen, B. S., Petersen, D., & Klavenses, D. (2003). *Carbohydrate Polymers, 54*, 33–42.
34. Sudo, H., Grant Burgess, J., Takemasa, H., Nakamura, N., & Matsunaga, T. (1995). *Current Microbiology, 30*, 219–222.
35. De Philippis, R., Ena, A., Paperi, R., Sili, C., & Vincenzini, M. (2000). *Journal of Applied Phycology, 12*, 401–407.
36. Vicente-Garcia, V., Rios-Leal, E., Calderon-Dominguez, G., Canizares-Villanueva, R. O., & Olvera-Ramirez, R. (2004). *Biotechnology and Bioengineering, 85*, 306–310.
37. Sutherland, I. W. (1994). *Biotechnological Advances, 12*, 393–448.
38. Shepherd, R., Rockey, J., Sutherland, I. W., & Roller, S. (1995). *Journal of Biotechnology, 40*, 207–217.
39. Enriquez, L. G., Hwang, J. W., Hong, G. P., Bati, N. A., & Flick, G. J. (1989). In G. Charalambons & G. Doxastakis (Eds.), *Plant and microbial food gums* (pp. 335–416). Amsterdam: Elsevier.
40. Wang, Y., Wang, L. J., Li, D., Xue, J., & Mao, Z. H. (2009). *Carbohydrate Polymers, 78*, 213–219.