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Characterization and optimization of production of exopolysaccharide from *Chlamydomonas reinhardtii*

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ABSTRACT

Chlamydomonas reinhardtii strain RAC was isolated based on its ability to secrete large amount of exopolysaccharide (EPS). The purified EPS had a molecular weight of 2.25×10^5 Da, and showed fibrillar structure with surfaces having sheet-like appearance. Chemical analysis showed the presence of galacturonic acid, ribose, arabinose, xylose, glucose, galactose and rhamnose sugars. The production of EPS was optimized by the classical one-at-a-time approach and Plackett–Burman design, followed by response surface methodology. The resulting response surface model was statistically significant ($p < 0.5$) and predicted maximum EPS production of 628 mg/L. The optimum production medium consisted of $CaCl_2$ – 74, $NaNO_3$ – 422, K_2HPO_4 – 10 and $MgSO_4$ – 200 mg/L with a pH 7. The EPS showed significant antioxidant activity, which can have several industrial applications. This is the first report on characterization and production of EPS from a *Chlamydomonas* strain isolated from India. Its differences from the earlier reported EPS are discussed.

1. Introduction

Polysaccharides are linear or branched carbohydrate polymers, often made of heterogeneous monomers which are linked together by glycosidic bonds. Depending on the structure, they possess interesting physico-chemical properties, which are distinct from the constituent monosaccharides. Some of the useful properties include stabilizing, suspending, thickening, flocculating, encapsulating, emulsifying and water retention activities. Due to such properties, the polysaccharides find a wide range of industrial applications in textiles, pharmaceuticals, adhesives, detergents, cosmetics, food additives, brewing and wastewater treatment (Raza et al., 2012).

Microbial exopolysaccharides (EPS) are receiving increasing attention because they are biodegradable and generally non-toxic, and hence, do not cause secondary pollution. Algae are a rich source of structurally diverse polysaccharides exhibiting important activities, like immunomodulating, anticoagulant, antiviral, antioxidant and antitumor activities (Lordan, Ross, & Stanton, 2011). Algal polysaccharides also constitute significant proportion of primary production, and therefore, have important ecological effect in the environment (Anastyuk, Imbs, Shevchenko, Dmitrenok, & Zvyagintseva, 2012). There is a rapidly growing interest in the production of biofuel and other value-added products from algae. EPS can be obtained as by-product, increasing the economy of such processes (Sui, Gizaw, & BeMiller, 2012). Since several algal species secrete polysaccharides into the medium, its isolation and purification is very easy (Gerbersdorf, Westrich, & Paterson, 2009). Furthermore, algae can be easily and economically grown in non-cultivable land and non-potable water using sunlight and cheap growth medium. Algal growth and EPS production generates less wastewater due to the absence of organic material in the growth medium. It also results in sequestration of atmospheric CO_2, addressing the problem of greenhouse gas emission too.

The type and the amount of EPS produced depend on several factors such as species employed, cultivation conditions and age of the cultures. Hence, the design of fermentation conditions is very vital (Allard & Tazi, 1993). Statistical design of experiments provides an economic and efficient method of optimizing several conditions at a time. Further, the production of EPS is not species specific and each strain of the same species may produce different kinds of EPS with different biotechnology properties. Hence, there is interest in screening algal strains for new polysaccharides that may compete with traditional polysaccharides for bioactivities. Until now there is no report about the characterization and optimization of production of EPS from *Chlamydomonas* strains from India. The antioxidant potential of *Chlamydomonas* EPS has also not been investigated yet. Antioxidants are widely used in food, medicine

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and personal care industries. Although synthetic antioxidants are effective and cheap, their safety and toxicity is of great concern. Recently polysaccharides have been reported as effective natural antioxidants both *in vitro* and *in vivo* (Gao, Wang, Su, Zhang, & Yang, 2012).

In this study a new strain of *Chlamydomonas reinhardtii* was isolated from Palampur, India, and its EPS was purified and characterized. The production of EPS was optimized by classical method followed by statistical experimental design, and its antioxidation potential was also investigated.

2. Materials and methods

2.1. Chemicals

Sepharose CL-4B and dextran standards for gel filtration chromatography, and monosaccharide standards for gas chromatography–mass spectrometry (GC–MS) were obtained from Sigma–Aldrich, USA. Other AR-grade chemicals for characterization of EPS were purchased from Merck, USA. Culture media components were from Himedia, India.

2.2. Isolation of algae

Samples were collected by scrapping green-colored growth over rock surfaces partially/completely submerged in rivers in and around Palampur, Himachal Pradesh, India. The samples were centrifuged and resulting pellet was washed twice with sterile saline. It was plated on algal growth medium M1 ($CaCl_2$ – 100, K_2HPO_4 – 100, $MgSO_4$ – 100, $NaNO_3$ – 100, $FeCl_3$ – 5 and Na_2MoO_3 – 10 mg/L, pH – 7) solidified with 15 g/L of agar. Algae were purified by repeated colony isolation, membrane filtration and treatment with antibiotics like penicillin (100 μg/mL) and streptomycin (50 μg/mL). The resulting algal cultures were maintained in both liquid and agarified M1 medium at ambient temperature under cool-white fluorescent light (20 $\mu mol/m^2/s$) with a light to dark cycle of 12:12 h. The cultures were transferred to fresh medium every 15 days. They were regularly checked for contaminants like bacteria and fungi using nutrient agar and Saboraud's agar media, respectively.

2.3. Screening for EPS production

Primary screening was done in 2 different growth media, M1 (composition as above) and M2 ($CaCl_2$ – 200, K_2HPO_4 – 200, $MgSO_4$ – 200, $NaNO_3$ – 500, $FeCl_3$ – 5 and Na_2MoO_3 – 10 mg/L, pH – 7), solidified with 15 g/L of agar. Algal colonies producing large amount of EPS were selected from these plates by their mucoid appearance. Further screening was done by growing the selected algae in liquid M1 and M2 media. The algae were grown in both the media for 9 days in shake flasks at 100 rpm. Then the cells were separated by centrifugation at 12,000 × *g* for 15 min at 4 °C, and the cell-free supernatant was analyzed for EPS by Anthrone method using glucose as standard (Beeley, 1985). The algal culture producing maximum EPS was selected for further characterization and optimization studies.

2.4. Identification of the culture

The selected alga was identified by sequencing of partial 18S rRNA gene. The gene was amplified with 18SCOMF (TGCATGGCCGTTCTTAGTTGGTGG) and 18SCOMR (CACCTACGGAAACCTTGTTACGAC) primers as described earlier (Zhang & Lin, 2002). The PCR product was visualized by agarose gel electrophoresis and purified by gel extraction. The complete PCR product was then sequenced and the sequence was submitted to GenBank (accession no. KC310450). Identification was performed by BLAST search against the nr database (Altschul et al., 1997). 16S rRNA gene was also amplified using universal primers to check for bacterial contaminants (Bafana, 2013).

Table 1
Coded values of factors and their effects from Plackett–Burman design.

Factor (mg/L)	Coded values		Effect (+) to (−)
	+	−	
$CaCl_2$	200	10	−75.250
K_2HPO_4	200	10	−52.500
$MgSO_4$	200	10	63.875
$FeCl_3$	5	0	−73.500
$NaNO_3$	200	10	86.625
$NaMoO_4$	5	0	−49.875

Table 2
Plackett–Burman experimental design.

Run	$CaCl_2$	K_2HPO_4	$MgSO_4$	$FeCl_3$	$NaNO_3$	$NaMoO_3$	EPS yield (mg/L)
1	–	–	–	–	–	–	386.75
2	+	+	+	–	+	–	409.50
3	–	+	+	+	–	+	274.75
4	–	–	+	+	+	–	374.50
5	+	–	–	+	+	+	274.75
6	–	+	–	–	+	+	281.75
7	+	–	+	–	–	+	236.25
8	+	+	–	+	–	–	96.25

2.5. Optimization of growth medium for EPS production

The strain was grown in liquid M1 growth medium and effect of replacing key medium components with equimolar amount of alternative salts was studied. Specifically, the effect of source of chloride ($CaCl_2$ vs. NaCl), sulphur ($MgSO_4$ vs. Na_2SO_4) and nitrogen ($NaNO_3$ vs. NH_4Cl) was studied by changing one salt at a time and keeping rest of the composition as M1. Similarly, the effect of growth medium pH was studied at pH values of 6, 7 and 8. The pH was maintained constant by addition of 1 N NaOH or HCl through out the experiment. EPS productivity after addition of 10 mg/L of EDTA to M1 medium was also determined. The culture was grown in shake flasks at 100 rpm under respective conditions, and EPS yield was estimated after 9 days as above.

2.6. Plackett–Burman design

The optimum medium components were identified qualitatively by the above experiment. The components were then optimized quantitatively by two-level orthogonal Plackett–Burman design (Plackett & Burman, 1946). All 6 medium components were selected as the experimental factors and tested at 2 levels (Table 1). pH of the medium was kept at 7, as optimized above. The design consisted of 8 experimental runs (Table 2). Each run involved growing the culture in shake flask at 100 rpm and determining the EPS yield after 9 days, and was carried out in triplicate.

2.7. RSM (response surface methodology) design

Plackett–Burman design was used to pick the factors that influenced EPS production most significantly and required to be further optimized. The 2 most significant factors (amount of $CaCl_2$ and $NaNO_3$) were optimized by central composite design (CCD) (Box & Draper, 1987). Rest of the medium components were kept at their optimized values from the Plackett–Burman design (K_2HPO_4 – 10 and $MgSO_4$ – 200 mg/L, $FeCl_3$ and Na_2MoO_3 were omitted, pH – 7). The selected factors were evaluated at 4 different levels (−1.414, −1, +1, +1.414) and the design contained 8 experimental (4 factorial and 4 axial with $\alpha = \sqrt{2}$) and 1 center point runs (Tables 3 and 4).

Table 3
Coded values of factors in response surface design.

Factor (mg/L)	Coded values				
	−1.414	−1	0	1	1.414
$CaCl_2$	17.2	100	300	500	582.8
$NaNO_3$	17.2	100	300	500	582.8

Table 4
Response surface experimental design.

Run	$CaCl_2$	$NaNO_3$	EPS yield (mg/L)	
			Observed	Predicted
1	1	−1	405.6	407.9
2	1	1	485.0	471.2
3	−1	−1	496.9	499.2
4	−1	1	632.6	618.9
5	−1.414	0	598.5	604.2
6	1.414	0	429.4	435.2
7	0	−1.414	419.9	414.3
8	0	1.414	526.6	543.7
9	0	0	580.2	580.2

Each run consisted of growing the culture in shake flask at 100 rpm and determining the EPS yield after 9 days, and was carried out in triplicate. Multiple linear regression of the result was carried out using the software Design-Expert 7.1.3 (Stat-Ease Inc., USA) to get a second-degree polynomial model:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_{11} {X_1}^2 + \beta_{22} {X_2}^2 + \beta_{12} X_1 X_2$$

where Y = predicted response, β_0, β_1, β_2, β_{11}, β_{22} and β_{12} = regression coefficients for intercept, linear, quadratic, and interaction terms, and X_1, X_2 = levels of the independent factors. Statistical significance of the model was tested by the coefficient of determination R^2 and analysis of variance (ANOVA). The medium composition for maximum EPS productions was determined by plotting response curve.

2.8. Isolation of EPS

The strain was grown in 1 L of optimized medium for 9 days in shake flask at 100 rpm. The cells were separated by centrifugation at 12,000 × *g* for 15 min at 4 °C and the cell-free supernatant was used as the EPS fraction. It was concentrated and dialyzed by tangential-flow filtration (50 kDa cut off pellicon membrane, Merck, USA) to a final volume of 100 mL. The process removed small molecules and resulted in concentration of only high-molecular weight EPS. It was then lyophilized to white powder using freeze dryer (FreeZone 1, Labconco, USA).

The cell-bound capsular polysaccharide (CPS) was also isolated from the cell pellet. The cell pellet was resuspended in 1% NaCl and heated for 4 h at 80 °C under agitation. The cells were then eliminated by centrifugation to obtain the CPS fraction. It was also concentrated and lyophilized as above.

2.9. Characterization of physical properties

Scanning electron microscopy (SEM) of algal cells, and lyophilized EPS and CPS was done after putting them on carbon tape-coated aluminum stubs and carbon sputtering. High resolution images were taken on Hitachi S-3400N microscope (Japan). Viscosity of the EPS was determined by using Ostwald viscometer (80–100 s) at 20 °C. EPS was diluted to a final concentration of 1 mg/mL, 15 mL of the sample was poured in the viscometer and time required for the solution flow was determined. Water was used as the reference. Viscosity was calculated using the following formula:

$$\eta_s = \frac{\eta_w \times \rho_s \times t_s}{\rho_w \times t_w}$$

where η = viscosity, ρ = density, t = time, and subscripts s, w represent sample and water respectively.

The molecular weight (MW) of EPS was determined by gel filtration chromatography on AKTAprime system (GE, USA). Sepharose CL-4B column was calibrated using dextran standards of 5, 10, 20, 50 and 80 kDa, and void volume was determined with blue dextran (MW 2000 kDa). Elution was carried out with 50 mM phosphate buffer (pH 7) containing 200 mM NaCl. MW of the EPS was determined from the calibration curve of log(MW) against V_e/V_0, where V_e and V_0 are elution volume and void volume respectively.

2.10. Analysis of chemical composition

The EPS was hydrolyzed in 2 M TFA at 120 °C for 2 h, and the resulting sugars were derivatized to corresponding alditol acetates by reduction with $NaBH_4$, and acetylation with pyridine and acetic anhydride (Beeley, 1985). The sample was then analyzed by GC–MS (Varian 450 GC and MS 240, USA) using DB-5 MS column (30 m × 0.25 mm × 0.25 μm). Helium was used as carrier gas at a flow rate of 1 mL/min. Oven temperature was held at 40 °C for 2 min, increased to 130 °C at the rate of 25 °C/min, again increased to 180 °C at a rate of 12 °C/min, finally heated to 280 °C at 3 °C/min and held for 7 min. Injector and ion trap temperatures were set at 270 °C and 240 °C respectively, and acquisition mass range was from 50 to 1000 Da. 1 μL sample was injected into GC and fragmentation pattern was observed. Standard monosaccharides were also derivatized and analyzed using the same procedure.

The EPS acid hydrolysate was analyzed for various functional groups using suitable colorimetric methods (Beeley, 1985). Total sugar was measured with Anthrone method using glucose as standard. Uronic acid and amino sugars were detected by carbazole–sulphuric acid and Elson–Morgan methods respectively. The sulfate group was determined by $BaSO_4$ turbidimetry. Pyruvate group was estimated by converting it into a colored product with 2,4-dinitrophenylhydrazine. The acetyl group was determined by reaction with hydroxylamine in alkali to form hydroxamic acid, which forms a colored complex with Fe^{3+}. Protein content in the EPS was assayed with Bradford method (Bradford, 1976). The diffuse reflectance-Fourier transform infrared (DR-FTIR) spectrum of EPS was recorded with a Vetex 70 spectrometer (Bruker, USA) in the range of 3000–1000 cm^{-1}. The sample was analyzed as KBr pellets.

2.11. Antioxidant assay

Measurement of the total antioxidant capacity was based on reduction of Mo^{6+} to Mo^{5+} by EPS and subsequent formation of phosphomolybdate complex at acidic pH, which was read at 695 nm (Prieto, Pineda, & Aguilar, 1999). Reducing power of the EPS was determined by its ability to reduce $K_3Fe(CN)_6$ to $K_4Fe(CN)_6$, which then reacts with $FeCl_3$ to form Prussian blue that can be measured at 700 nm (Oyaizu, 1986). Ascorbic acid was used as standard in both the assays and results were expressed as ascorbic acid equivalents.

3. Results

Several algal cultures were isolated from various rivers in Palampur, India. A non-carbon mineral medium M1 was used for this purpose. The cultures were purified and their axenicity was verified by microscopic observation, inoculation in nutrient broth and

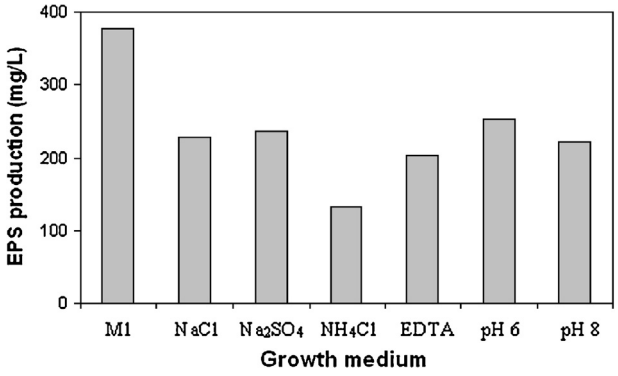

Fig. 1. Effect of growth medium on EPS production. M1 – M1 medium, NaCl – M1 medium with NaCl in place of $CaCl_2$, Na_2SO_4 – M1 medium with Na_2SO_4 in place of $MgSO_4$, NH_4Cl – M1 medium with NH_4Cl in place of $NaNO_3$, EDTA – M1 medium with EDTA, pH 6 – M1 medium with pH 6, pH 8 – M1 medium with pH 8.

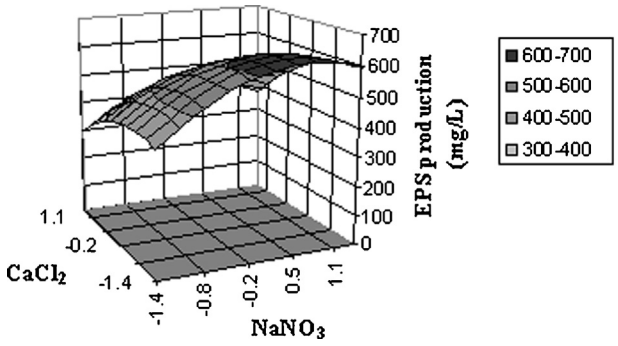

Fig. 2. Response surface plot showing the effect of $CaCl_2$ and $NaNO_3$ concentration on EPS production.

Saboraud's broth, and 16S rRNA gene amplification. The cultures were then screened on M1 and M2 media agar plates, where EPS-producing colonies showed mucoid appearance. This provided a quick primary screening method and resulted in selection of 6 cultures. On microscopic observation, all the 6 cultures were found to be microalgae. These cultures were further screened in M1 and M2 broth media, as full EPS production potential can be demonstrated in liquid medium. M1 medium showed higher productivity for majority of the cultures. The culture showing highest EPS yield (culture no. 4) was selected for further study (Supplementary Table S1). The partial 18S rRNA gene was amplified and sequenced from this culture (GenBank accession no. KC310450). By similarity search, it was identified as *C. reinhardtii* strain RAC.

Medium composition is known to significantly affect algal EPS production. Hence, the growth medium was optimized in a stepwise manner. Firstly, it was optimized qualitatively *i.e.* different salts were screened for maximum EPS production by the classical one-at-a-time approach. Since M1 medium showed higher productivity, it was used as the base medium, and the source of nitrogen, sulphur and chloride, EDTA, and pH were varied individually. $CaCl_2$, $MgSO_4$ and $NaNO_3$ supported greater EPS production as compared to NaCl, Na_2SO_4 and NH_4Cl respectively. Addition of EDTA had negative effect, and a neutral pH of 7 was found to optimum (Fig. 1). Thus, the medium composition of M1 was optimum by itself and was used for further experimentation. Once the medium components were finalized, they were optimized quantitatively *i.e.* amount of each component required for optimum EPS production was determined. For this, Plackett–Burman experimental design was used, which is a two-level orthogonal screening design. Being a saturated design, it can determine only the main effect of experimental factors, but not their interactions. From the results, the most important factors can be identified and used for further optimization. The medium components and their concentrations used in the design are given in Tables 1 and 2. EPS production from each run of the design and the calculated effect of each variable are also shown. Addition of $FeCl_3$ and $NaMoO_4$, and increase in concentration of $CaCl_2$ and K_2HPO_4 showed negative effect. Increase in the level of $MgSO_4$ and $NaNO_3$, on the other hand, showed positive effect. Hence, $FeCl_3$ and $NaMoO_4$ were removed, amount of K_2HPO_4 was reduced, and $MgSO_4$ was increased for further experimentation. $CaCl_2$ and $NaNO_3$ had the most significant effect on EPS production. Hence, the concentration of these 2 nutrients was further optimized by response surface modeling. CCD was used because it is a rotatable design, which gives quadratic and interaction effects of the variables under study (Tables 3 and 4). The modified M1 medium for this purpose contained 10 mg/L of K_2HPO_4 and 200 mg/L of $MgSO_4$ with varying amounts of $CaCl_2$ and $NaNO_3$. Response surface equation was obtained by multiple regression analysis of the CCD design data. The equation was found to be statistically significant ($p < 0.5$) by ANOVA analysis (Supplementary Table S2). Coefficient of determination $R^2 = 0.986$ and adjusted $R^2 = 0.962$ also indicated a good fit.

$$Y = 580.23 - 59.75 \times [CaCl_2] + 45.75 \times [NaNO_3] - 30.28 \times [CaCl_2]^2 - 50.64 \times [NaNO_3]^2 - 14.09 \times [CaCl_2] \times [NaNO_3]$$

where Y = EPS production (mg/L), and $[CaCl_2]$ and $[NaNO_3]$ = concentration of $CaCl_2$ and $NaNO_3$ respectively (mg/L). Response surface curve was generated from the above equation and solved for maximum EPS production (Fig. 2). Optimum EPS production was 628 mg/L and occurred at 74 mg/L of $CaCl_2$ and 422 mg/L of $NaNO_3$. The final optimized growth medium composition was $CaCl_2$ – 74, $NaNO_3$ – 422, K_2HPO_4 – 10 and $MgSO_4$ – 200 mg/L with pH 7. EPS productivity in the initially used M1 and M2 media was 382.5 and 231.7 mg/L respectively. Thus, the optimization process led to a significant increase in the EPS yield.

Soluble EPS released in the growth medium was isolated and purified by ultrafiltration for further characterization. The cell-bound capsular polysaccharide (CPS) was also isolated by heating algal cells in NaCl solution. Both the fractions were lyophilized and visualized by SEM. Both showed a similar fibrillar structure with surfaces having sheet-like appearance, indicating that they might have the same composition. SEM photographs of algal cells showed round and oval cells covered with small amount of CPS (Fig. 3). The yield of both soluble and capsular fractions was determined in the optimized growth medium, and was found to be 628 and 60 mg/L respectively. Since the yield of CPS was very low, further study was concentrated on the soluble EPS. The viscosity of 1 mg/mL of EPS solution was determined with Ostwald viscometer using water as reference. It was found to be 11.1 mPa s. Gel filtration chromatography showed that the EPS was homogeneous with respect to chain length and had a monodisperse MW of about 2.25×10^5 Da.

EPS was then characterized for the chemical composition. GC–MS analysis showed the presence of galacturonic acid, ribose, arabinose, xylose, glucose, galactose and rhamnose sugars. Specific colorimetric assays for various functional groups detected pyruvate group and uronic acid. This confirmed the presence of galacturonic acid identified from GC–MS. The FTIR spectrum of EPS (Fig. 4) showed characteristic peaks at 2900, 1700, 1400, and 1200–1000 cm^{-1}. The peaks at positions 2958 and 2893 cm^{-1} indicate the C–H stretching vibration of methyl and methylene groups. These peaks along with the one at 1136 cm^{-1} display the characteristic absorption of polysaccharides. The peak at 1728 cm^{-1} is assigned to the C=O stretching vibration of esters, which might arise from pyruvate group identified by colorimetric assay. In addition, the peak at 1400 cm^{-1} indicates stretching vibration of

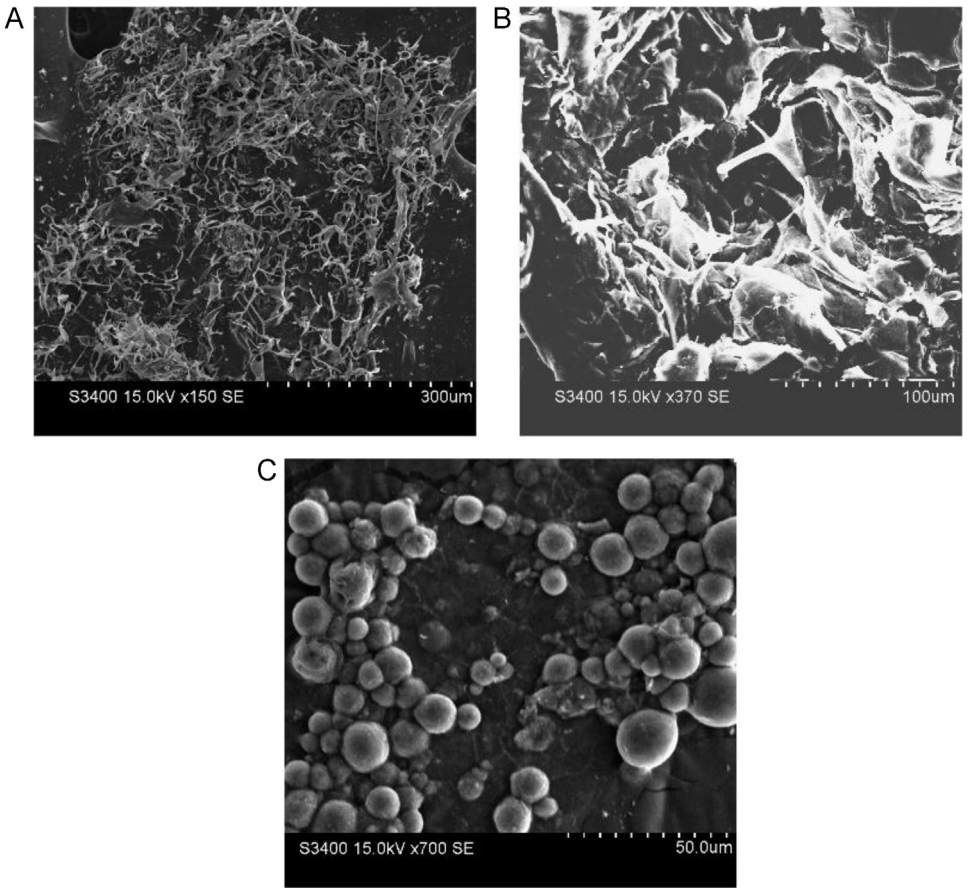

Fig. 3. (A) SEM of CPS showing fibrillar structure, (B) SEM of EPS at higher magnification revealing the sheet-like surfaces within the fibrillar structure, (C) SEM of algal cells showing round cells with small amount of associated polysaccharide.

COO^- ion, which might be caused by galacturonic acid. The weak peaks appearing in the range of 1200–1000 cm^{-1} are mainly attributed to the stretching vibration of C—O. Thus, results from all chemical analyses supported a common pattern. The purified EPS did not contain any protein as tested by Bradford reagent.

The EPS was also tested for antioxidant activities to check its potential as a natural antioxidant. Two different assays were employed. Total antioxidant activity based on the reduction of Mo^{6+} was found to be 5.7 μg ascorbic acid equivalent/mg EPS, while reducing power based on the ability to reduce Fe^{3+} was 2.3 μg ascorbic acid equivalent/mg EPS.

4. Discussion

Intense research is going on toward utilization of algae for manufacturing value-added products. Although bacteria generally have

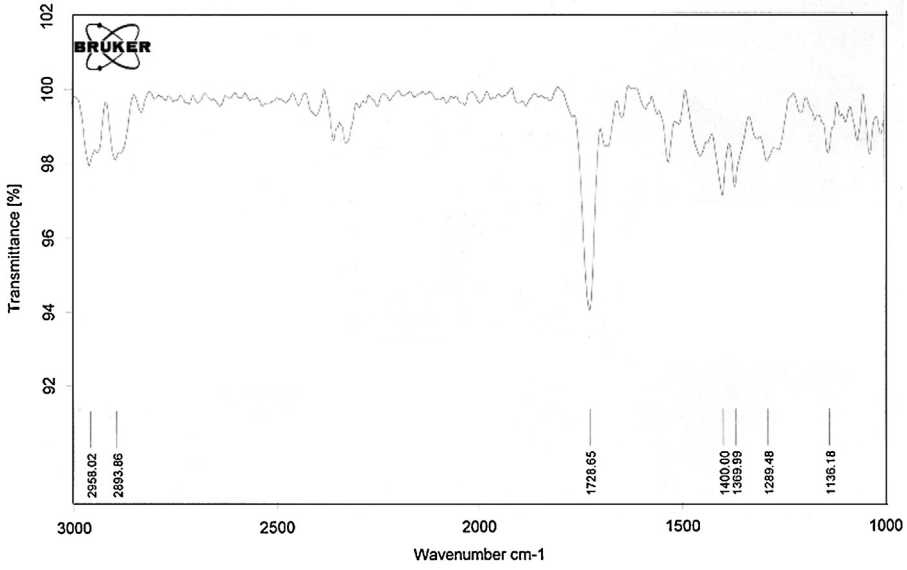

Fig. 4. FTIR spectrum of the EPS.

superior productivity, the increasing environmental awareness and regulations give the algal green technology a distinct advantage. Economic viability of the algal technology can be further improved if multiple products are extracted from the algal biomass. Algal polysaccharides exhibit many industrially and pharmaceutically important activities, and get secreted into the growth medium, which leaves the biomass available for extraction of other products. Present study describes screening of algae for production of EPS. Flowing river water was selected as the sampling site because algae thriving in such environment produce large amount of EPS, which helps them in anchoring to solid surfaces. Screening was done in 2 growth media (M1 and M2) differing in the concentration of constituent salts. This was done to avoid missing any potential strain, as medium composition has significant effect on the EPS productivity. Both media contained only few inorganic salts and no growth factors or organic constituents. This allowed for the selection of algae with high EPS productivity in simple mineral medium. Use of mineral medium has several advantages like reduced chances of contamination, improved process economy and easy waste handling. Six algal strains were selected by screening on solidified media agar plates. They were purified to get axenic cultures. It avoided the potential contribution of contaminating bacteria under non-axenic conditions (Zhu et al., 2012).

The actual EPS yield of all 6 cultures was determined in liquid M1 and M2 media. One of the cultures showed highest EPS yield in both the media, and was selected for further study. Its culture flask showed lot of foaming during growth indicating production of extracellular polymeric material. It was identified as *C. reinhardtii* strain RAC, which is a very common unicellular flagellated green alga belonging to the phylum *Chlorophyta*. *C. reinhardtii* has been successfully used for expression of several pharmaceutical proteins, indicating that it can be used for simultaneous production of multiple value-added products (Rosales-Mendoza, Paz-Maldonado, & Soria-Guerra, 2012). Hence, it was a preferred candidate for the current study.

The EPS yield and composition may vary depending on the growth medium, age of the culture, and even among different strains of same species. Few data exist about nutritional factors which influence EPS production in *C. reinhardtii*. In general sources of nitrogen, sulphur and phosphorus have significant effect on algal EPS production. Stationary phase caused by nitrogen or phosphorus depletion has been reported to induce EPS production in *Chlamydomonas mexicana* (Kroen & Rayburn, 1984). The growth medium was optimized in current study through various statistical designs. Key nutrients like nitrogen, sulphur, phosphorus and chloride were varied, and they had significant effect on EPS production. $CaCl_2$ and $NaNO_3$ were found to be the most influential medium constituents. Optimization process resulted in a simple medium containing only 4 inorganic salts, and a 1.6-fold enhancement in EPS yield from 382.5 to 628 mg/L.

Characterization of EPS from Indian strains of *Chlamydomonas* is not reported in the literature, and there are very few reports from other geographical regions. *Chlamydomonas* sp. MIC-G24 recorded highest value of cellular carbohydrates during bioprospection of microalgae in India, although the EPS was not separately analyzed (Ratha, Prasanna, Gupta, Dhar, & Saxena, 2012). Besides EPS, the algal cell wall also contains covalently linked polysaccharides. Bollig et al. (2007) reported that the hydroxyproline-bound O-glycans of the outer cell wall glycoproteins from *C. reinhardtii* consisted exclusively of arabinose and galactose. Some studies have reported that EPS composition in *Chlamydomonas* may vary depending on the culture age. In a study, the EPS released by *Chlamydomonas augustae* contained mainly arabinose, glucose and galactose at the beginning of growth, and glucose and glucuronic acid in the stationary phase. In sharp contrast, the EPS from *Chlamydomonas corrosa* was independent of growth status and contained arabinose and galactose as major sugars (Allard & Tazi, 1993). EPS composition was determined in current study through various analytical techniques. It contained galacturonic acid, ribose, arabinose, xylose, glucose, galactose and rhamnose. Thus, present EPS was more heterogeneous and did not contain glucuronic acid, unlike earlier reports from *Chlamydomonas* sp. It also contained ester-linked pyruvate group, although its exact location could not be determined. Extracellular polymers in some strains contain non-carbohydrate macromolecules. Extracellular bioflocculant produced in a *C. reinhardtii* strain was found to contain 42.1% proteins, 48.3% carbohydrates, 8.7% lipids, and 0.01% nucleic acid (Zhu et al., 2012). Some strains have also been reported to simultaneously produce multiple polysaccharides of different compositions (Shuxiu, Yuanying, & Changxu, 1995). When the extracellular polymeric material was purified in the current study by ultrafiltration, it contained only carbohydrate. On gel filtration analysis, it showed a monodisperse peak with a MW of 2.25×10^5 Da. Thus, the current strain produced only a single type of EPS. There are very few reports on the MW of *Chlamydomonas* EPS. Zhu et al. (2012) reported MW of 7×10^5 Da for bioflocculant produced in a *C. reinhardtii* strain, although it contained significant proportion of proteins and lipids along with carbohydrates. Due to the high MW and presence of charged uronic acid residues, the current EPS may also have significant flocculating activity. Several algal strains possess a fraction of polysaccharide associated with the cells wall (CPS). CPS was also isolated and purified in this study. SEM photographs showed that both CPS and the soluble EPS had similar appearance. However, the yield of CPS was very low, and hence, it was not investigated further.

EPS from *Chlamydomonas* sp has been reported for several important activities. Metting (1986) demonstrated soil stabilization with EPS from *Chlamydomonas sajao*, while Zhu et al. (2012) reported flocculating activity of *C. reinhardtii* EPS. However, antioxidant activity has not been demonstrated so far. Currently there is a growing demand for natural antioxidants for food, cosmetic and pharmaceutical applications. 1 mg of the current EPS showed total antioxidant activity and reducing power equivalent to 5.7 and 2.3 μg of ascorbic acid respectively.

The marine microalga *Porphyridium* can synthesize and secrete high amount of polysaccharides in the culture medium, and hence, is extensively studied for EPS production. Díaz et al. (2012) reported the EPS yield from *Porphyridium cruentum* to be 360 mg/L with negligible antioxidant activity. The current strain produced much higher amount of EPS with significant antioxidant activity. The high MW of EPS makes it a potential candidate for several applications. Further, this is the first study on characterization and optimization of production of EPS from a *Chlamydomonas* strain isolated in India.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carbpol.2013.02.016.

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