



Flocculating, emulsification and metal sorption properties of a partial characterized novel exopolysaccharide produced by *Rhizobium tropici* SRA1 isolated from *Psophocarpus tetragonolobus* (L) D.C.

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Abstract

A novel exopolysaccharide (EPS) was produced by a bacterium which was isolated from *Psophocarpus tetragonolobus* (L) D.C. and identified as 99% *Rhizobium tropici* SRA1 by 16S rDNA sequencing. The flocculating performances along with emulsifying activity began simultaneously with the growth and the production of EPS and reached its utmost at 28 h. EPS was purified via chilled ethanol precipitation followed by dialysis and lyophilization. The existence of hydroxyl, methoxyl, and carboxylic functional groups were confirmed by Fourier transform infrared (FT-IR) spectrum. EPS was found to be composed of 82.44% neutral sugar and 15.93% uronic acid. The average molecular weight of the exopolysaccharide was estimated as $\sim 1.8 \times 10^5$. Gas–liquid chromatography indicated the presence of glucose and galactose at a molar ratio of 3:1 in EPS. In the pH range of 3–5 with EPS dosage of 15 mg/l at 30 °C, cation-independent flocculation greater than 90% was observed. Emulsification indices (E_{24}) of EPS were observed as 86.66%, 83.33%, 76.66%, and 73.33% with olive oil, kerosene, toluene, and n-hexane respectively. Biosorption of Cu K [45.69 wt%], Cu L [05.67 wt%], Co K [15.58 wt%], and Co L [11.72 wt%] by EPS was confirmed by energy-dispersive X-ray spectroscopy (EDS). This report on the flocculating, emulsifying, and metal sorption properties of EPS produced by *R. tropici* SRA1 is unique in the literature.

Keywords Exopolysaccharide · *Rhizobium tropici* SRA1 · *Psophocarpus tetragonolobus* · Flocculation · Emulsification · Energy-dispersive X-ray spectroscopy

Introduction

Leguminous plants are ecologically and agriculturally significant as they are a major source of biological nitrogen fixation

through legume-rhizobia symbiosis and are a rich source of vegetative protein, especially pulse crops (Bhattacharyya et al. 2017). Rhizobia bacteria belong to the diverse non-pathogenic α and β subclass of the gram-negative proteobacteria and

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belong to the genera of *Rhizobium*, *Azorhizobium*, *Allorhizobium*, *Ensifer* (syn. *Sinorhizobium*), *Mesorhizobium*, and *Methylobacterium* of α -proteobacteria as well as members of β -proteobacteria like *Burkholderia* and *Cupriavidus* (Dresler-Nurmi et al. 2009; Mousavi et al. 2014). Research has shown that rhizobial exopolysaccharides (EPSs) play a vital role in the invasion process and the formation of infection threads and nodules and a role in plant defense responses. These EPSs also confer protection against various environmental stresses (Bomfeti et al. 2011; Hoang et al. 2004; Mousavi et al. 2014). Although they are a major source of biomass, only a very small percentage of polysaccharides are currently utilized and represent an industrially unexploited market (Sharma and Dhingra 2013; Donot et al. 2012). The bulk of EPSs are strain-specific heteropolysaccharides which differ according to sugar composition, linkages, repeating unit size, and extent of polymerization. For the interaction between bacteria and their environment, the chemical diversity of the bacterial exopolymers plays an important role (Bramhachari and Dubey 2006; Mandal et al. 2013). Studies focusing on the composition, structure, and biosynthesis and functional roles of several bacterial EPSs have been reported. However, in spite of the great diversity in the molecular structures of bacterial EPSs, industrial development has only occurred using a small number. Constraints to large-scale industrial production result from high production costs arising from substrate costs and downstream processing (Castellane et al. 2014; Freitas et al. 2011; Prajapati et al. 2013). As reported earlier, consortium of *Micrococcus* sp. Leo and *Halomonas* sp. Okoh produces a biofloculant when grown in a nutrient-rich media composed of 20 g/l glucose as a carbon source (Okaiyeto et al. 2013). Thus, strain which produces EPS in low carbon source medium could decrease the cost of downstream processing. EPSs are significant in producing biofilms and in protecting bacterial cells from desiccation, toxic metal penetration, presence of antibiotics, phagocytosis, and phage attack (Sivakumar et al. 2012). EPSs possessing negatively charged binding sites serve essentially as cation trappers, rendering protection from heavy metal toxicity (Bhattacharyya et al. 2017; Yadav et al. 2012). Microbial EPSs and their properties are applicable in a great number of field applications, such as gelling, stabilizing, suspending, thickening, coagulating, film-forming, and water-retention in the production of textiles, detergents, paints, paper and adhesives, food and beverage, oil recovery, mining, and petroleum, and in the biomedical field (Han et al. 2014; Huang et al. 2012; Roberts 1995; Sutherland 1996; Sutherland 1998). Researchers are gaining interest in the isolation and characterization of microbial EPSs due to their various roles in nutrient sequestration, adhesion, heavy metal chelation, toxic compound detoxification, and protection against hypoosmotic shock (Bhattacharyya et al. 2017; Decho 1990; Hoagland et al. 1993; Ivashina and Ksenzenko 2012).

Rhizobia are unexplored sources of microbial EPSs with promising industrial applications due to their morphological, physiological, and phylogenetic diversity. Moreover, they are non-pathogenic and produce large amounts of EPSs (Bhattacharyya et al. 2017; Bomfeti et al. 2011). The superior properties of the novel rhizobial EPS may lead to further industrial applications and resultant increased demand (Bhattacharyya et al. 2017; Castellane et al. 2015; Hoagland et al. 1993; Huang et al. 2012). However, the majority of work erstwhile done on heavy metals effects on the rhizospheric population of rhizobia (Chaudri et al. 2000), nitrogen fixation (Ibekwe et al. 1996), nodulation (Paudyal et al. 2007), and the production of rhizobial indole acetic acid (IAA) (Bhattacharyya 2006).

The above led us to conduct the present study on the flocculating, emulsifying, and metal sorption capacities and physico-chemical characterization of rhizobial EPS produced by *Rhizobium tropici* SRA1 isolated from the root nodules of *Psophocarpus tetragonolobus* (L.) D.C.

Materials and methods

Plant material

A pulse crop, *Psophocarpus tetragonolobus* (L.) D.C., was selected for the present research work and was grown in controlled environment. The efficacy of *R. tropici* SRA1 (KP205042) was evaluated in renodulating the host *P. tetragonolobus* (L.) D.C. by open soil pot system. The certified seeds of the experimental plant were purchased from reputed seed selling center at Kolkata. Seeds were properly surface sterilized with 0.1% HgCl_2 solution followed by 75% ethanol, washed with sterile distilled water for at least three times, and then soaked in sterile distilled water for several hours. After that, the seeds were kept on water agar (1%) and incubated at 25 °C for germination. The germinated seeds were transferred into sterile pot and the inoculum of the test strain (10 ml) was added. Control pots devoid of any inoculation were also set. Growth of the plants and root systems was evaluated for nodulation after 45 days. The experiments were done in triplicate in the experimental garden of the Department of Life Sciences, Presidency University.

Isolation and characterization of the test strain

For the isolation of microsymbiont, the fresh, healthy, and pink-colored mature nodules of *P. tetragonolobus* (L.) D.C. were harvested. The nodules were properly washed and surface sterilized by 0.1% HgCl_2 and 70% ethanol followed by washing with sterile distilled water. The nodules were cut and crushed between two sterile glass slides and the root nodule sap was streaked aseptically on yeast extract mannitol agar

(YEMA) medium containing Congo red [composition (g/l): yeast extract 1.000; mannitol 10.000; dipotassium phosphate 0.500; magnesium sulfate 0.200; sodium chloride 0.100; agar 20.000; and Congo red 0.025] (Eissa et al. 2009; Skerman 1959). The bacterial growth obtained from ten times serial dilution method. 0.1-ml aliquots were plated on Congo red containing YEMA plates. Twelve individual colonies (SRA1-SRA12) were isolated from the dilution plate and separated on YEMA slants and all the isolates were identified as *Rhizobium* sp. following the methods given earlier (Conn 1957; Jordan 1984). The bacteria were also routinely checked in a microscope and the purified cultures were maintained on YEMA slants. Among the isolates, SRA1 showed the highest EPS production in the culture filtrate and hence identified by 16S rRNA gene sequencing. Isolation of DNA of SRA1 cells, amplification of 16S rDNA sequence, and purification of PCR product and its cloning were done as described earlier (Mandal et al. 2013). The universal primers 27F and 1492R was used to sequence PCR product via using BDT v3.1 cycle sequencing kit on ABI 3730x1 Genetic Analyzer. 16S rDNA sequence was used to carry out BLAST using the database of National Center for Biotechnology Information (NCBI, <http://blast.ncbi.nlm.nih.gov/Blast.cgi>). The 16S ribosomal gene sequences of all the known species were recovered from GenBank and multiple alignments were performed with the 16S rDNA sequence of *R. tropici* SRA1 using CLUSTALW (Thompson et al. 1994). The obtained multiply aligned sequence was checked and edited, for further analysis; approximately 1422-bp-long nucleotide stretch was selected. The alignment ambiguities of the nucleotide sequence from both the ends were omitted. The phylogenetic analysis was conducted in the software package MEGA4 (Tamura et al. 2007).

Scanning electron microscopic studies

Overnight grown culture of SRA1 cells (10^7 cells/ml) were rinsed and re-suspended in PBS. The cells were fixed using 2% glutaraldehyde, dehydrated with ethanol treatments (10%, 30%, 50%, 70%, 90%, and 100%), and examined using scanning electron microscopy (SEM) (FEI Quanta-200 MK2) with an accelerating voltage of 20 kV.

Extracellular polymeric substance production medium and growth conditions

Rhizobium tropici SRA1 cells were grown in YEM broth to study the growth and extracellular polymeric substance (EPS) production. The composition is as follows: yeast extract 1 g/l; mannitol 10 g/l; dipotassium phosphate 0.50 g/l; magnesium sulfate 0.20 g/l; sodium chloride 0.10 g/l; pH 7.0. The bacterial cells were incubated in 100-ml Erlenmeyer flasks containing 20 ml medium with three replicates at 30 ± 1 °C on a rotary shaker at 150 rpm for 28 h (the optimum time for growth and

EPS production). Samples were harvested during various time intervals to monitor bacterial growth using a spectrophotometer at λ_{600} nm and EPS yield. Moreover, the variation in flocculating rate and emulsifying activity of EPS was also monitored from the batch culture during various phases of growth.

Extraction and purification of EPS

For the extraction of EPS from the bacterial culture, the solvent extraction method of Yadav et al. (2012) was followed. Briefly, 28-h grown culture broth was centrifuged at $9587.5 \times g$ for 10 min; supernatant was collected and mixed with double volume of chilled ethanol (95%) and kept overnight at 6 °C. The precipitate was collected via centrifugation, re-dissolved in deionized double distilled water, and dialysed through a cellulose membrane (Sigma-Aldrich, retaining MW > 12,400 Da) against deionized double-distilled water for 24 h to get rid of low molecular weight materials. The dialyzed material was lyophilized to obtain purified EPS and kept for further study.

Optimization of carbon source and pH for EPS production

To study the consequence of carbon source on EPS production of *Rhizobium tropici* SRA1, the basal yeast extract mineral medium was supplemented singly with varied carbon sources (1% v/v). Basal yeast extract mineral medium without any supplementation was served as a control set. Stock solutions of various carbon sources were sterilized via passing through a membrane filter (0.2 μ m) and then aseptically amended to the sterile medium before inoculating with bacterial suspension. For optimization of pH, most preferred carbon source containing medium of different pH was also checked. The pH of the medium was adjusted using 0.1 M HCl or 0.1 M NaOH.

Chemical analysis of EPS

Prior to the determination of sugar composition and the apparent molecular weight of the EPS, it was checked for any protein contamination by recording the UV–vis spectra of the solution in a 1-cm path length quartz cuvette using the Shimadzu UV-visible 1601 spectrophotometer. The content of neutral sugar and uronic acid was measured using anthrone reaction and carbazole-sulfuric acid reaction respectively. Further, using water as the eluant with a flow rate of 0.4 ml/min, the EPS was passed through Sepharose 6B gel permeation column (90 \times 2.1 cm). In whole, 95 test tubes (2 ml each) were collected using Redifrac fraction collector and monitored spectrophotometrically (Shimadzu UV–vis spectrophotometer 1601) at λ_{490} nm with phenol–sulfuric acid reagent (Dubois et al. 1956; Mandal et al. 2013). A single fraction of

exopolysaccharide (EPS) was obtained and the apparent molecular weight was estimated using standard dextrans T-200, T-70, and T-40. The monosaccharide contents was estimated by hydrolyzing the EPS with 2 M CF_3COOH (2 ml) at 100 °C in boiling water bath for 18 h. The excess acid was completely removed by co-distillation with water. Thereafter, the hydrolyzed product was reduced using NaBH_4 (9 mg), followed by acidification using dilute CH_3COOH , and further co-distillation to remove excess boric acid with pure CH_3OH . The reduced sugars (alditol) were acetylated with 1:1 pyridine– Ac_2O in a boiling water bath for 1 h to obtain alditol acetates, which were analyzed by gas–liquid chromatography (GLC). Quantization (in terms of peak area) was carried out from the response factors from standard monosaccharides. A gas–liquid chromatography Hewlett-Packard model 5730A was used with flame ionization detector and glass columns (1.8 m \times 6 mm) packed with 3% ECNSS-M (A) on Gas Chrom Q (100–120 mesh) and 1% OV-225 (B) on Gas Chrom Q (100–120 mesh). GLC analysis was performed at 170 °C. Moreover, the solubility of EPS was tested in distilled water and other solvents like ethanol, methanol, isopropanol, acetone, and carbon tetrachloride. Briefly, 20 mg of EPS was suspended separately in 10 ml of different solvents. The mixture was kept at ambient temperature for 30 min and then vortexed for 5 min.

Fourier -transform infrared spectroscopy

Using Fourier transform infrared (FT-IR) spectroscopy, functional groups of EPS were determined on PerkinElmer spectrum GX FT-IR system (PerkinElmer, USA) with a resolution of 4 cm^{-1} in the $4000\text{--}450\text{ cm}^{-1}$ region under ambient conditions (Mandal et al. 2013).

Effect of EPS dosage, CaCl_2 concentration, pH, temperature, and various metal ions on flocculating rate

The flocculating rates of the purified EPS were measured using the Yadav et al. (2012) method with slide modification, in which activated charcoal (4 g/l) was used as the solid-phase suspension. After the adjustment of pH of the suspension to 7.0, 0.1 ml of the test sample was added to the activated charcoal suspension, vortexed for 2 min, and allowed to settle for 5 min. After that, the absorbance of upper phase was determined via digital spectrophotometer at $\lambda_{550\text{ nm}}$ (A). In the control set, 0.1 ml of water instead of test sample was added and the absorbance was determined (B).

The flocculating rate (%) was calculated according to the following equation:

$$\text{Flocculating rate (\%)} = \frac{[B-A]}{B} \times 100$$

Flocculating rate of the activated charcoal was also determined in order to get more insight in the flocculation efficiency of the extracted EPS. For calculating the flocculating rate of activated carbon, as a control set, activated carbon suspension in the presence of CaCl_2 was taken (B), whereas activated carbon dissolved in deionized water without any CaCl_2 was taken as an experiment set (A). The flocculating rate is then calculated using the formulae mentioned earlier.

Both the EPS dosage and CaCl_2 concentration were varied from 5 to 25 mg/l and from 0 to 2 mM respectively, to ascertain cost-effective dosages. The reasons for choosing CaCl_2 for the flocculation experiment are its solubility; it does not elevate the pH, it produces dense floc, and it does not inherently add contaminants. The pH of the activated charcoal suspension was varied from 1 to 10 using 0.1 M HCl or 0.1 M NaOH, and flocculating rate was measured with or without using optimum CaCl_2 concentration. To determine the effect of temperature on flocculating rate, the temperature of the suspension (activated charcoal) was varied in water bath in the range of 10–100 °C. Finally, different salts like KCl, NaCl, $\text{K}_2\text{Cr}_2\text{O}_7$, MnCl_2 , CoCl_2 , MgCl_2 , ZnSO_4 , HgCl_2 , and FeCl_3 (final concentration 1.3 mM) were added as a substitute of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ in order to determine their effects on flocculating rate.

Emulsification indices (E_{24})

Toluene (Sigma), n-hexadecane (Sigma), n-hexane (Sigma), kerosene oil (commercial brands), and olive oil (commercial brands) were used as hydrophobic substrate to study the emulsification indices (E_{24}) of EPS as mentioned earlier (Yadav et al. 2012). To 5 ml aqueous solution of EPS (0.5% w/v), 5 ml of hydrocarbon or oil was added and vortexed for 2 min. As controls, Tween 20 and Tween 80 (Sigma) were used. The emulsion and aqueous layers were measured after 24 h and emulsification indices (E_{24}) was calculated according to the following formula:

$$E_{24} = \left[\frac{\text{height of the emulsion layer}}{\text{total height}} \right] \times 100$$

The results obtained were the mean of the results of three independent experiments.

Metal biosorption by SEM-energy-dispersive X-ray spectroscopy

Biosorption of toxic heavy metals Cu (II) and Co(II) by EPS was detected by using SEM-energy-dispersive X-ray spectroscopy (EDS) [FEI Quanta-200 MK2]. Briefly, 0.4 ml of 100 mM stock solutions of each metal salt ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ and $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$) was mixed separately with EPS solution (1 mg/ml) in a test tube and adjusted to a final volume of 4 ml with Millipore water. The mixture was then incubated

for equilibration at room temperature for 30 min. A double volume of ice-cold ethanol (95%) was added to precipitate the EPS. The precipitate was re-suspended in deionized double-distilled water and dialyzed overnight (Sigma-Aldrich, retaining MW > 12,400 Da) at 4 °C against Millipore water to remove unbound metal if any and thereafter freeze-dried. All the stock solutions of toxic heavy metals were prepared in Millipore water.

Statistical Analysis

All the experiments were performed in triplicates and the error bars in the figures represent the standard deviations of the data. The experiments were statistically evaluated using ANOVA. In cases where the null hypothesis was rejected at the $\alpha = 0.05$ level, Tukey's HSD (honestly significant difference) test was applied. Significant differences among the treatments were considered at the level of p values < 0.05.

Results

Phylogenetic characterization of the test strain and time course of EPS production

Microsymbionts from the root nodules of *P. tetragonolobus* were isolated in a YEMA medium with Congo red (Supplementary Fig. 1A). The renodulation experiment confirmed the host. Using a YEM medium, EPS production was tested and all the strains were found to be able to produce EPS in culture filtrate. The highest EPS production (550 $\mu\text{g/ml}$) in the medium was in strain number SRA1 and it was selected for further experiments. It was reported earlier that the EPS produced by a *Rhizobium* sp. was found to be 116 $\mu\text{g/ml}$ (Mukherjee et al. 2011). In a separate study, *Rhizobium* sp. VMA301 produced 350 $\mu\text{g/ml}$ EPS (Mandal et al. 2007).

The SRA1 strain has similar morphology with class α -proteobacteria of family Rhizobiaceae. It is short rod 2 μm , aerobic, and gram-negative (Supplementary Fig. 1B). According to the BlastN search results, the SRA1 (GenBank accession no. KP205042) gene sequence showed maximum identity of 99% with the 16S rRNA gene of *Rhizobium tropici* strain CIAT 899 (NR102511), *Rhizobium tropici* RHM54 (JQ085251), and *Rhizobium tropici* RHM14 (JQ085247). Among the cluster of three different strains of *Rhizobium tropici* (NR102511, HQ394213, and JN208906), the strain SRA1 settled in the *Rhizobium tropici* JQ085251 (99% 16S rRNA gene sequence similarity).

Based on 16S rRNA gene sequences, the neighbor joining method was used to find the evolutionary history. The bootstrap test (1000 replicates) results were shown next to the branches (the percentage of replicate trees). The Kimura two-parameter method was used to compute the evolutionary

distances which are in the units of the number of base substitutions per site. The analysis involved 25 nucleotide sequences. The final dataset was containing total of 1310 positions. The MEGA7 was used for evolutionary analyses (Supplementary Fig. 2).

The growth and EPS production of the tested strain began very shortly following inoculation (Fig. 1a). EPS production, flocculating rate, and emulsifying activity decreased following the completion of the stationary phase (Fig. 1a inset). Similar kind of decrease in EPS production after 120 h was noted by the earlier researcher in the case of soil bacterium *Flavobacterium* sp. ASB 3-3 isolated from Arctic glacier (Sathiyarayanan et al. 2015). Then, the effect of individual carbon sources on the growth and EPS production of *Rhizobium tropici* SRA1 was assessed. While the isolate was able to utilize all carbon sources tested for EPS production and growth, mannitol resulted in maximum growth and EPS production at pH 7.0 (Fig. 1b, c). The results showed that the optimum pH for the EPS production was found to be 7.0.

Characterization of purified EPS

The detail isolation and purification steps involved in the purification of EPS are illustrated via flow diagram in supplementary Fig. 3A. EPS was also checked for any protein contamination. Results showed that there is no absorption peak around 280 nm. Thus, we concluded the absence of any protein contamination in the EPS. The content of neutral sugar and uronic acid was estimated to be 82.44% and 15.93% respectively. After passing purified EPS through a Sepharose 6B column, a single fraction was obtained. The fraction (test tubes 7–17) was collected and freeze-dried, yielding purified polysaccharide (EPS) (Supplementary Fig. 3B). The apparent molecular weight of the PS was estimated as $\sim 1.8 \times 10^5$. According to the results from GC analysis, the EPS was composed of glucose and galactose in a molar ratio of 3:1 (Fig. 2a). FT-IR study showed the existence of hydroxyl (3460 cm^{-1}), carboxyl (1636 cm^{-1} and 1415 cm^{-1}), and methoxyl (1076 cm^{-1}) groups (Mandal et al. 2013; Sardari et al. 2017). Absorption peaks of approximately $1000\text{--}1100 \text{ cm}^{-1}$ are considered characteristic of sugar derivatives (Fig. 2b.) The carboxyl groups may have some contribution to the flocculation as they act as binding sites for divalent cations (Mandal et al. 2013).

Effect of EPS dosage, CaCl_2 concentration, pH, temperature, and various metal ions on flocculating rate

EPS (15 mg/l) showed a flocculating rate of 97.41% at pH 7.0 in the presence of 1.3 mM CaCl_2 (Fig. 3a, Supplementary Fig. S4A). Flocculating rate of activated charcoal (without any EPS or CaCl_2) was only 4% (Fig. 3a inset). The flocculating rate of EPS concentrations (between 5 and 25 mg/l) was

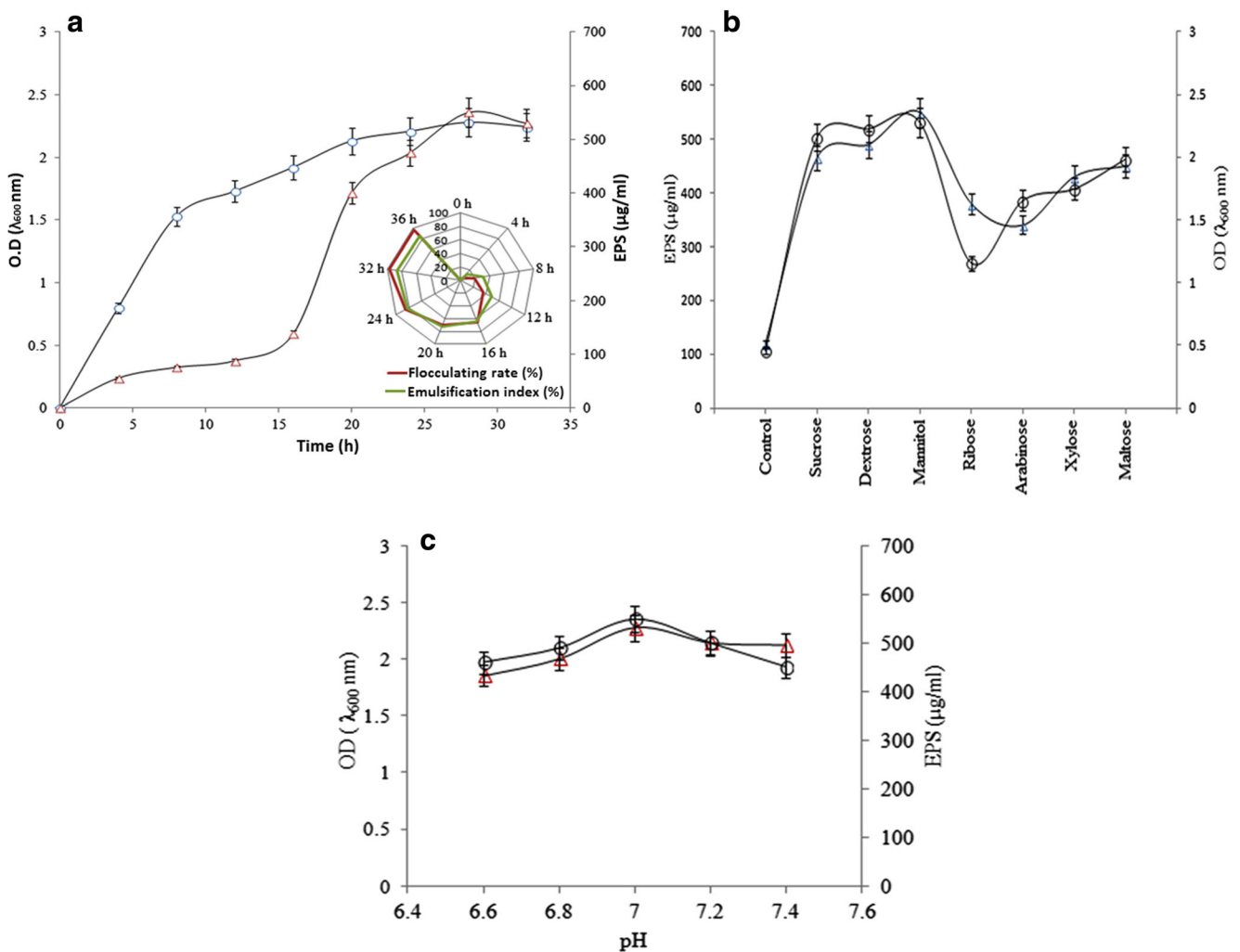


Fig. 1 **a** Growth (Δ) and EPS production (\circ) of *R. tropici* SRA1 grown in YEM medium. Radar plot (in inset) showing the flocculating rate and emulsifying activity of SRA1 EPS during different time intervals. **b**

Effect of various carbon sources on growth (\circ) and EPS production (Δ) of *R. tropici* SRA1. **c** Effect of different pH on growth (\circ) and EPS production (Δ) of *R. tropici* SRA1

studied. Maximum flocculation was achieved by means of an optimum EPS dosage of 15 mg/l (Fig. 3a).

While observing the effect of CaCl_2 concentration on flocculation at pH 7.0, it was noted that 1.3 mM CaCl_2 concentration was optimum for achieving the maximum flocculating rate of 95% and higher or lower salt concentration reduced the flocculating rate to 80.46% and 62.28%, respectively (Supplementary Fig. S4A). The flocculating rate was 77.12% without CaCl_2 (1.3 mM) at pH 7.0.

The consequence of pH on the flocculating rate was investigated both in the presence and absence of CaCl_2 . Results showed that the flocculating rate was above 90% in the pH range of 3–10 (Supplementary Fig. S4B). While observing the flocculating rate of EPS at varied temperatures ranging from 10 to 100 °C, maximum flocculation was observed at 30 °C. When the temperature was dropped down to 10 °C or raised up to 100 °C, a decrease in flocculating rate by 68.49% and 55.25% respectively was observed (Supplementary Fig. S4C).

The effects of different metal salts (KCl, NaCl, $\text{K}_2\text{Cr}_2\text{O}_7$, MnCl_2 , CoCl_2 , MgCl_2 , ZnSO_4 , HgCl_2 , and FeCl_3) on flocculation were observed (Fig. 3b). The results showed that divalent cations (Ca^{2+} , Zn^{2+} , Hg^{2+} , Co^{2+} , and Mn^{2+}) showed better flocculation compared to the other tested metal ions.

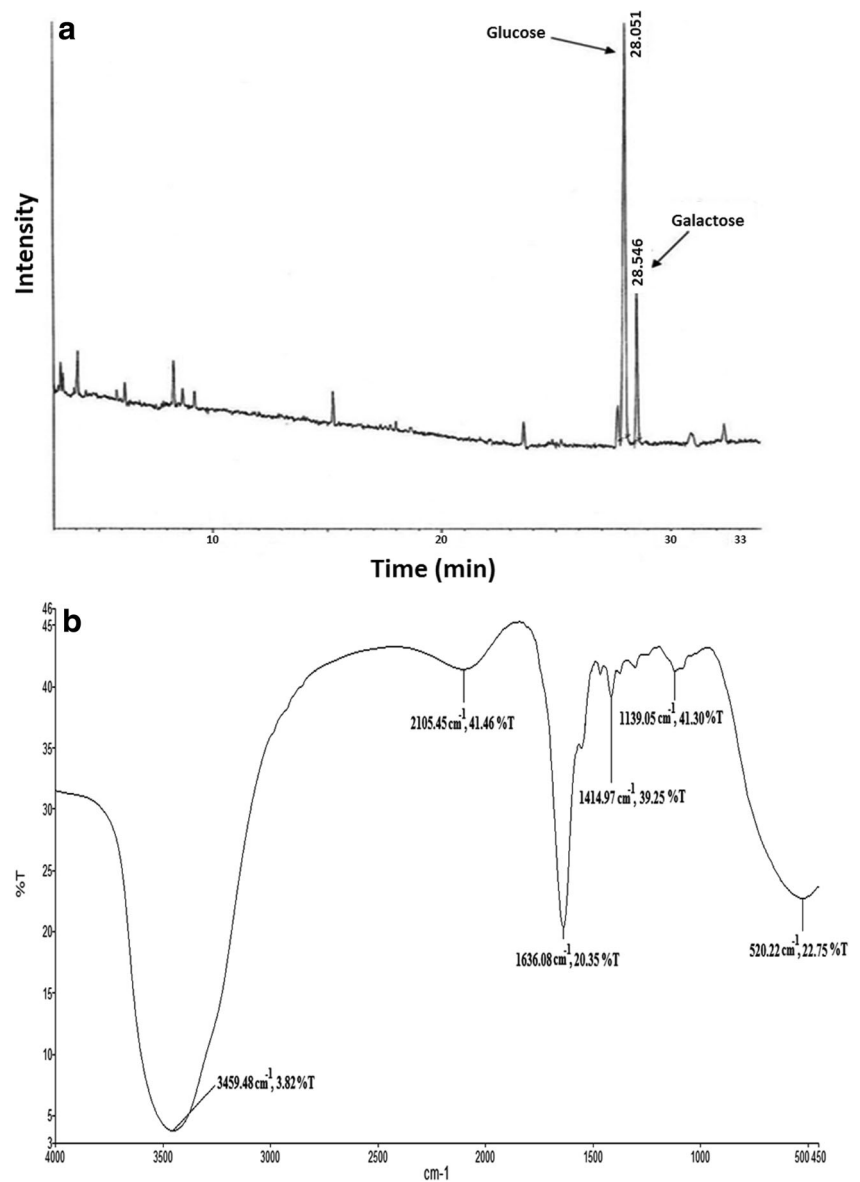
Emulsification indices (E_{24})

Results showed that SRA1 EPS performs superior emulsifying activity (86.66% with olive oil, 83.33% with kerosene, 76.66% with toluene, and 73.33% with n-hexane) when compared with that of commercial available surfactant like Tween 20 and Tween 80 (Table 1).

Metal sorption study by EDS

EDS relies on an interaction of some source of X-ray excitation and a sample could be a powerful technique for the

Fig. 2 a GC chromatogram of the PS fraction of *R. tropici* SRA1 EPS. b FT-IR spectra of *R. tropici* SRA1 EPS



elemental analysis. It detects X-rays emitted from the sample during the bombardment by an electron beam. EPS treated with Cu(II) and Co(II) showed the presence of characteristic peaks at 8.048 and 6.930 keV, respectively, as revealed by EDS (Fig. 4). SEM micrograph showed the sorption of Cu(II) by the EPS produced by *R. tropici* SRA1 which further supports our claim.

Discussion

Time course of EPS production and its relationship with flocculating and emulsifying performances

The yield of EPS production in *Rhizobium tropici* SRA1 decreases after 28 h of incubation which is probably due to

utilization of the synthesized EPS by the test strain. Similar type of decrease in the EPS yield was noted by earlier researchers (Bhattacharyya et al. 2017; Mandal et al. 2013). In another study, *Flavobacterium* sp. ASB 3-3 also showed decrease in EPS production after 120 h. Growth and production of EPS in YEM broth were found to be maximum when supplemented with 1% mannitol. In an earlier study, it was noted that when R2A medium supplemented with glucose as a carbon source was found to be the best for EPS production by *Klebsiella* sp. PB12 (Mandal et al. 2013).

Characterization of purified EPS

According to GLC analysis of the PS, two main sugar residues, glucose and galactose, were shown in a 3:1 molar ratio.

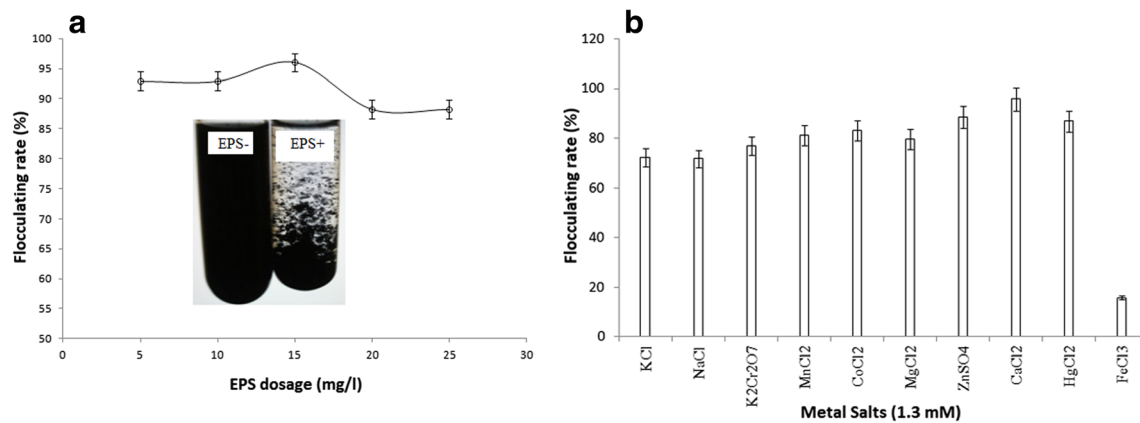


Fig. 3 **a** Effect of different EPS dosages on the flocculating rate. Initiation in flocculation of the activated charcoal after EPS addition (in inset). **b** Effect of different metal salts on flocculating rate

It was reported earlier that the EPS produced by a type strain of *Rhizobium* from arid earth comprised of glucose, galactose, and mannuronic acid in the molar proportion of 2:1:1 (Kaci et al. 2005). In a separate study, the EPS produced by *R. undicola* strain N37 comprised of galactose and mannose (Ghosh et al. 2015). EPS extracted from *R. radiobacter* S10 isolated from kefir was found to be galactose, glucose, glucosamine, and mannose (Zhou et al. 2014). In common, fast-growing rhizobial EPSs (e.g., *S. meliloti* and *R. leguminosarum*) are composed of octasaccharide as repeating units, in which glucose is a predominant sugar component. Interestingly, in *R. leguminosarum* *bv. trifolii*, an EPS subunit is composed of seven sugars, none of which is galactose (Amemura et al. 1983). EPS extracted from the test strain SRA1 was found to be soluble in water but insoluble in organic solvents, such as acetone, carbon tetrachloride, ethanol, isopropanol, and methanol. Thus, these organic solvents can be used for the extraction of EPS from *Rhizobium tropici* SRA1. The carboxyl group as revealed from IR spectra may serve as binding site for divalent metal ions. Therefore, we predicted that the process of flocculation might be due to bridging and charge neutralization. Similar kind of explanation was also provided by the earlier researcher (Mandal et al. 2013).

Effect of EPS dosage, CaCl₂ concentration, pH, temperature, and various metal ions on flocculating rate and study of emulsification indices (E_{24})

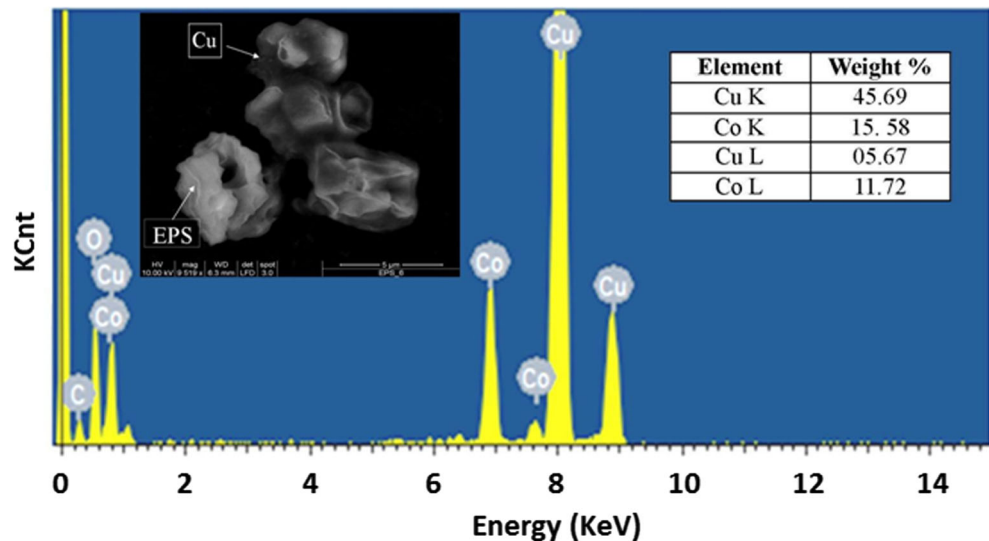
The flocculating rate (> 90% at 15 mg/l dose) of SRA1 EPS revealed its promise as a better alternative in future applications as a bioflocculant when compared with the earlier published data using some commercial flocculants like alginate (11%), Al₂(SO₄)₃ (30%), xanthan (24%), guar gum (8%), and chitosan (6%) at 100 mg/l dosage (Yadav et al. 2012). Higher or lower dosage of EPS showed a reduction in flocculating rate. The possible explanation could be that until a particular concentration of EPS was attained, the desired aggregation by bridging and charge neutralization could not occur to its optimum value. At 15 mg/l EPS, it extends from the surface to obtain an optimum distance greater than the distance over which the inter-particle repulsion acts. With further increase in the concentrations of EPS (i.e., > 15 mg/l), the viscosity of the solution also increases, which leads to a gradual decrease in the sedimentation rate of the particles. At lower dosages of EPS (i.e., < 15 mg/l), the effective bridging phenomenon gets hindered, thus may exhibit a reduction in the flocculation rate.

An increase in the positive charge density over the activated charcoal particles due to the over-addition of

Table 1 Emulsifying activity (%) of *R. tropici* SRA1 EPS and T-20 and T-80 (as control) on different hydrophobic substances

Experimental sets	Hydrophobic substrate						
	Toluene (%)	n-Hexadecane (%)	n-Hexane (%)	Kerosene (%)	Mustard oil (%)	Olive oil (%)	
EPS	76.66	70	73.33	83.33	60	86.66	
Control	T-20	56.66	56.66	60	63.33	43.33	43.33
	T-80	56.66	60	66.66	76.66	53.33	56.66

Fig. 4 Energy-dispersive X-ray spectroscopy (EDS) spectra of *R. tropici* SRA1 EPS treated with Cu(II) and Co(II). Scanning electron micrograph showing Cu(II) sorption by *R. tropici* SRA1 EPS (in inset)



calcium salt also inhibits the flocculating efficiency. The reduction in the flocculating rate at a higher temperature may be explained by the increase in the kinetic energy or the molecular vibration of activated charcoal particles. Cations have been reported to enhance flocculation by neutralizing the negative charges on both bioflocculant and on the suspended activated charcoal particles via forming bridges that hold activated charcoal particles to each other (Sharma and Dhingra 2013). Our results confirmed that the divalent cations of Ca^{2+} , Zn^{2+} , Hg^{2+} , Mn^{2+} , Mg^{2+} , and Co^{2+} resulted in better flocculating rates than those of the other tested cations. The carboxyl groups of uronic acids as revealed by FT-IR analysis in the EPS may be binding sites for the divalent cation, causing the bridging between EPS and cations. Whereas the monovalent cations K^+ and Na^+ were not as effective as the divalent cations, the trivalent cation Fe^{3+} showed the least activity (flocculating rate of 15.7%). Monovalent cations such as Na^+ and K^+ were not as much of effective for flocculation because of their weaker electrostatic attraction with EPS (Mandal et al. 2013). Trivalent cation robustly inhibits flocculation, which may be due to the effect on the surface charge of activated charcoal particles along with the coverage of adsorbing sites on the EPS (Yadav et al. 2012). In the pH range of 1–10, flocculating rates of SRA1 EPS were > 80% showing maximum value of 97.41%, at pH 7 in the presence of 1.3 mM CaCl_2 (Supplementary Fig. S4 B). There is slight decrease in the flocculating rate, in alkaline condition, demonstrating that augment in OH^- resulting in rise in negative charge over the activated carbon particles and as a result neutralizing the bridging effect of CaCl_2 . In the lack of Ca^{2+} ion, no efficient flocculation was observed at pH 7 which say aloud the need of CaCl_2 for flocculation by means of initiating Ca^{2+} -mediated

complexes of the EPS and activated carbon (Mandal et al. 2013).

Emulsification indices (E_{24}) suggest rhizobial EPS can be an attractive green alternative to synthetic surfactant. E_{24} of SRA1 EPS against the entire tested hydrophobic compound is quite satisfactory and well comparable with the previous report (Yadav et al. 2012). This study may open up an avenue for using SRA1 EPS as a promising emulsifier for various different environmental and industrial applications. The E_{24} of SRA1 against hydrophobic compounds like n-hexadecane is well comparable with the emulsification indices reported earlier for the EPS produced by *Rhizobium tropici* SEMIA 4080 (Castellane et al. 2017).

Metal biosorption study

EDS investigates the interactions between matter and electromagnetic radiation and analyzes the X-rays emitted in response to being hit with charged particles (Sathiyarayanan et al. 2015). The biosorption of Cu K [45.69 wt%], Cu L [05.67 wt%], Co K [15.58 wt%], and Co L [11.72 wt%] by EPS was confirmed using both energy-dispersive X-ray spectroscopy (EDS) and SEM (Fig. 4). The exopolysaccharide (EPS) produced by *Pseudomonas* sp. PAMC 28620 exhibited metal complexing property (Sathiyarayanan et al. 2016). In a separate study, the extracellular polymeric substances (EPS) of the *Acidiphilium* 3.2Sup (5) bacterium showed biosorption of 12.7 wt% Fe(III) (Tapia et al. 2011). Biosorption of Pb(II) from aqueous solution by extracellular polymeric substances extracted from *Klebsiella* sp. J1 was also reported by the earlier researcher (Wei et al. 2016). Thus, this study may open up an avenue of using SRA1 EPS for the bioremediations of various toxic metals.

Conclusion

The non-pathogenic rhizobial EPS obtained from *Rhizobium tropici* SRA1 could be an effective alternative for waste water treatment flocculating properties as well as for the biosorption of heavy metals. These findings may make notable contributions to the understanding of rhizobial EPS composition and applications in environmental biotechnology and bioremediation.

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Compliance with ethical standards

This article does not contain any studies with human participants or animals performed by any of the authors.

Conflict of interest The authors declare that they have no competing interests.

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