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Effect of blue light on growth and exopolysaccharides production in phototrophic *Rhodobacter* sp. BT18 isolated from brackish water

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ABSTRACT

Rhodobacter sp. BT18, a phototrophic salt-resistant bacterium, was isolated from brackish water and screened for the production of exopolysaccharides (EPS). The effect of different light sources on the growth of *Rhodobacter* sp. BT18 was investigated. The effect on the growth order was found to be blue > white > green > red > yellow > dark. Based on Box-Behnken design, the studied variables (pH 7.0, 35 °C, and 30% of sucrose concentration under 60 h of incubation with blue light illumination) were found to be ideal for the maximum production of EPS (582.5 mg/L). Scanning electron microscopy images revealed the porous nature of EPS. Fourier transform spectroscopy and X-ray diffraction were applied to study the functional groups and the crystalline nature of the EPS, respectively. The emulsification index of the EPS was >75% and the maximum flocculating activity was about 75.4% at 30 mg/L concentration of EPS. In addition, EPS showed effective arsenic (64%) and lead (51%) chelating activities in liquid solutions. The multiple environmental applications of the EPS produced by *Rhodobacter* sp. BT18 make it be a promising alternative for emulsification, flocculation and metal removal in various industries.

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1. Introduction

Bacterial exopolysaccharides (EPS) are extracellular biopolymers that are linear and/or branched sugar molecules composed of heterogeneous monomers coupled with glycosidic bonds [1]. Due to their viscosity, high emulsification, gelling nature [2], they find extensive applications in food, pharmaceutical and agricultural areas [3,4]. Owing to the wide range of industrial applications and unique structural properties, bacterial EPS are gaining increased attention and are gradually becoming economically competitive with synthetic and other biopolymers produced by plants and algae [5–7]. Bacterial EPS have been found to be highly water soluble, stable, and rheological. They have emulsifying properties and are effective across a wide range of pH and temperature [8].

Several studies have reported the production of EPS by bacteria including *Pseudomonas* sp. [5], *Bacillus* sp. [9,10], *Rhodothermus marinus* [11], *Leuconostoc mesenteroides* [12], and *Halomonas* sp. [1,4]. However, Bacteria from extreme environments have gained increased attention due to their special metabolic pathways and defensive mechanisms that enable them to withstand the harsh conditions [5]. Numerous studies have reported the EPS production by bacteria isolated from a variety of soil and water matrixes; however, there is no report on EPS produced by bacteria from brackish water environment, a buffering zone of sea and inland water.

Rhodobacter sp. is a gram-negative, phototrophic non-sulphur purple bacterium widely distributed across seas, rivers and lakes [13]. It grows under both anaerobic and aerobic conditions with several metabolic pathways depending on the growth environment [14]. Several studies have reported the environmental applications of *Rhodobacter* sp. including bioremediation of heavy metals and polyaromatic hydrocarbons [15–17]. It has been reported that the growth of the phototrophic bacterium is greatly influenced by several physico-chemical parameters, such as light source, light intensity, temperature, pH, and available carbon sources present in the cultivation medium. Among all other parameters, light source greatly affects the cell growth, and metabolic pathways of the phototrophic bacterium [18]. Therefore, the present study investigated the effect of different light on the growth and metabolic activity of the phototrophic bacterium. From this perspective, this study was designed (i) to isolate and identify the phototrophic *Rhodobacter* sp. from brackish water, (ii) to evaluate the influence of different light sources on the growth of *Rhodobacter* sp., (iii) to screen for EPS production, and analyze the effect of different sources of light and carbon, pH, and temperature of the growth medium on EPS production (iv) and to assess the flocculating, emulsifying and the metal chelation activity of the *Rhodobacter* sp. BT18 EPS.

2. Materials and methods

2.1. Isolation of EPS-producing phototrophic bacteria

Brackish water samples (~1 to 2 m depth) was collected from Pichavaram mangrove (11°23′–11°30′ and 79°45′–79°50′), Tamil

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Nadu, India, using sterile serum bottles and transported on ice to the laboratory. The enrichment cultures were prepared by adding 10 mL of water sample into a 250 mL Erlenmeyer flask containing 100 mL of Biebel and Pfennig's (PNB) medium supplemented with 3 g/L pyruvate. The flasks were incubated in a shaking incubator (250 rpm) at 30 ± 2 °C for 2 d. The subsequent enrichment cultures were serially diluted and plated on the PNB agar medium. Isolated colonies were purified and stored at 4 °C for further EPS production.

2.2. Identification of EPS-producing phototrophic bacteria

Genomic DNA was extracted from the pure culture according to the method described by Sambrook and Russell [19] and the isolated DNA was used as a template for PCR. The 16s rRNA gene of the isolate was amplified by using universal primer-27f (5′-AGAGTTTGATCCTGGCTCAG-3′) and -1492r (5′-CCCCGTCAATTCATTTGAGTTT-3′). The amplicons were purified using QIAGEN PCR (Valencia, CA, USA) purification kit and sequenced using ABI PRISM 3700 sequencer (Foster City, CA, USA). The obtained 16S rRNA sequence was compared with the available sequences in the database using NCBI BLAST program (http://www.ncbi.nlm.nih.gov/BLAST). A phylogenetic tree was constructed using the neighbor-joining distance method by Mega software version 6.0.

2.3. Effect of different light source on growth of Rhodobacter sp. BT18

Effect of different light sources on *Rhodobacter* sp. BT18 growth was investigated by carrying out batch experiments. Briefly, the flasks were covered with blue (~470 nm), green (~500 nm), yellow (~550 nm), red (~600 nm), and white papers (light source was not covered by any color paper). Flasks covered with black color cloth served as the control. All the flasks were kept in an incubator installed with an illuminated light source (Philips T8 Master TL-D T8 16 Watt 830) (Warm White) for 24 h at 30 ± 2 °C. After appropriate incubation, the optical density of the cell suspension was measured at 805 nm [18] using a UV–Vis spectrophotometer. Based on the effect of lights on the growth of *Rhodobacter* sp. BT18, blue light was used for further incubation for the enhanced production of EPS in the optimization study.

2.4. EPS production

The production of EPS by the isolate *Rhodobacter* sp. BT18 was carried out by flask fermentation with a slight modification of the simple culture medium (SCM) (pH 7) composed of KH_2PO_4, 0.5 g/L; ammonium acetate, 0.5 g/L; $MgSO_4.7H_2O$, 9.4 g/L; NaC1, 0.4 g/L; $CaC1_2.2H_2O$, 0.1 g/L; yeast extract, 1 g/L; ferrous citrate, 0.005 g/L; glucose, 5 g/L [20,21]. The isolate BT18 (10^8 cells/mL (0.8 OD) at 600 nm) was inoculated into 100 mL of SCM and incubated in a shaking incubator at 150 rpm at 30 ± 2 °C for 24 h. After the incubation, the cells were separated from SCM by centrifuging at 10,000 rpm for 15 min at 4 °C. The resulting supernatant was precipitated overnight at 4 °C by the addition of ethanol (6 volumes of 100% ethanol). The precipitated EPS were recovered by centrifuging at 10,000 rpm for 15 min at 4 °C. The crude EPS were dissolved in sterile distilled water and dialysed with Millipore membranes to remove excess salts and other compounds of the culture medium to obtain pure EPS. The purified EPS were freeze-dried for further characterization and application studies.

2.5. Optimization of EPS production

Four independent variables, pH (5–8), temperature (30–40 °C), sucrose concentration (10–50%), and blue light exposure time (24–96 h), were employed in this study and the experiments were designed by using a design expert software (Design Expert, version 10.0). The subsequent EPS production rate was evaluated by coefficient of determination (R^2), ANOVA and response surface plots. A second-order polynomial equation was developed to fit the data from the experimental investigations:

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i X_j$$

where Y is the predicted response; β_0, β_i, β_{ii}, β_{ij} are the fixed regression coefficients of the model; and X_i and X_j represent the independent variables.

2.6. Characterization of EPS produced by Rhodobacter sp. BT18

The functional groups present in the EPS were determined by using Fourier transformed infrared (FT-IR) spectroscopy (Perkin-Elmer, Norwalk, VA, USA). An infrared spectrum of the EPS was recorded using the KBr method in the range of 4000–400/cm. The porous nature and surface morphology of the EPS were examined by scanning electron microscopy (SEM; JEOL-64000, JEOL, Tokyo, Japan). The crystalline nature of the EPS was determined by using an X-ray diffractometer (Rigaku, Tokyo, Japan).

2.7. Emulsification activity of EPS

The emulsifying activity of EPS produced by *Rhodobacter* sp. BT18 was analyzed according to the method described by Cooper and Goldenberg [22]. Briefly, 5 mL of EPS solution and 5 mL of selected hydrophobic substrates (xylene, vaseline, coconut oil, and peanut oil) were vigorously mixed with a vortex mixer and incubated at room temperature for 24 h. Tween 80 was used as the control in this experiment. The emulsifying activity of the EPS was expressed as a percentage of total height occupied by the emulsification after 1 h and 24 h [4].

2.8. Flocculating activity of EPS

To determine the flocculating nature of the EPS, the batch assay was performed according to Prasertsan et al. [23] with a minor modification. Briefly, 0.5 ml of EPS solution (10, 20, 30, 50 and 50 mg/L) was mixed with 1.5 ml of $CaCl_2$ solution (pH 7.0) and 8 ml of kaolin clay suspension (pH 7.0). The assay mixture was stirred with a magnetic stirrer for 2 min and incubated at a room temperature for 5 min. Sterile distilled water was used as a control for this experiment. Flocculating activity was measured by optical absorbance at 550 nm and calculated as the % of activity.

$$\text{Flocculating activity} = [(OD_{control} - OD_{test})/OD_{control}] \times 100$$

2.9. Heavy metal chelating activity of EPS

The heavy metal chelating property of EPS was estimated against different concentrations (50, 100, 150 and 200 mg/L) of arsenic and lead. Briefly, 1% of EPS was mixed with metal solutions, incubated at room temperature for 1 h, and filtered using a glass filter. The precipitates present in the glass filter were carefully digested with nitric acid and the metal concentration in the sample was measured using inductively coupled plasma mass spectrometry (Agilent 4500, Agilent Technologies, Palo Alto, CA, USA).

3. Results and discussion

3.1. Identification of isolate BT18

Morphologically, three different bacterial colonies were observed in the PNB agar plates and the isolates were designated as BT17, BT18, and BT19. Of the three isolates, only BT18 produced EPS. Thus, the isolate BT18 was chosen for further studies. Based on 16S rDNA sequencing, the isolate was identified as *Rhodobacter* sp. (96.0% identity with *Rhodobacter* sp. YS18 (Accession No: LN879886)). The partial 16S rDNA of the isolate BT18 was deposited in GenBank (Accession Number: MK116466). Fig. 1 shows the phylogenetic relationship of the *Rhodobacter* sp. BT18. Several studies reported that the EPS producer

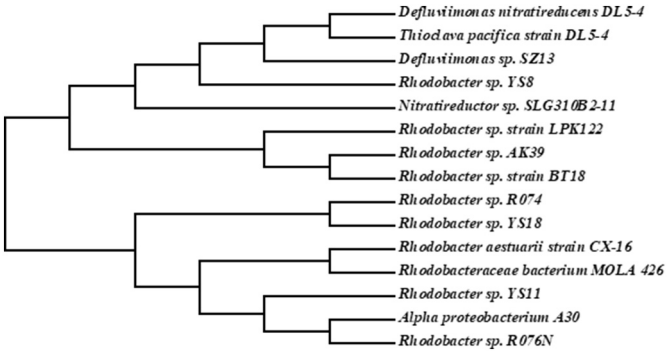

Fig. 1. 16S rDNA based phylogenetic analysis of *Rhodobacter* sp. BT18. The phylogenetic tree constructed by the neighbor-joining method showing the position of isolate *Rhodobacter* sp. BT18.

belongs to the genera *Bacillus*, *Pseudomonas*, *Halomonas* etc. [1,5,9]. However, EPS producing phototrophic *Rhodobacter* sp. has not been reported. To the best of our knowledge, this is the first report on the EPS-producing *Rhodobacter* sp. BT18.

3.2. Growth of Rhodobacter sp. BT18 under different light conditions

Light is an important factor for the growth of photosynthetic microorganisms. Thus, the growth of *Rhodobacter* sp. BT18 under different lights was investigated and the results are reported in Fig. 2. The batch experiments were performed under different light sources (blue (~470 nm), green (~500 nm), yellow (~550 nm), red (~600 nm), white, and darkness). The results clearly indicated that the best light source for the growth of *Rhodobacter* sp. BT18 was blue light at 470 nm. The order of the bacterial growth under the different light sources was blue > white > green > red > yellow > dark. The minimum growth of the isolate BT18 was observed in the flasks exposed to the yellow light. Kuo et al. [18] reported that the enhanced growth of photosynthetic *Rhodopseudomonas palustris* was found to be under the blue light emitting diode (LED-Blue) at approximate wavelength of 470 nm. Katsuda et al. [24] reported that an enhanced cell growth was observed under the blue light with short wavelength of 380–470 nm. Based on the growth studies, the blue color light was used for the optimization of the EPS production.

3.3. Response surface optimization of EPS production and ANNOVA

The effect of abiotic factors, pH (5–8), temperature (30–40 °C), sucrose concentration (10–50%), light (blue) exposure time (24–96 h),

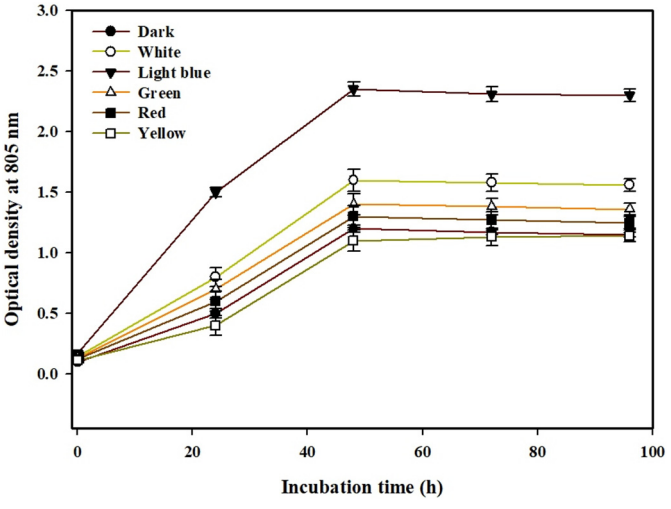

Fig. 2. Effect of different light sources on the growth of *Rhodobacter* sp. BT18.

Table 1
Box-Behnken design for the variables and the experimental observed responses.

Experiment	pH	Temp. (°C)	Sucrose (%)	Incubation with LB (h)	EPS yield (mg/L)
1	7.0	35	10	96	420.0
2	7.0	35	30	60	474.5
3	7.5	30	30	60	392.3
4	6.5	35	30	24	110.1
5	7.0	35	30	60	473.5
6	7.0	30	30	24	128.9
7	7.5	35	30	24	134.5
8	7.5	35	30	96	169.5
9	7.5	35	50	60	375.6
10	7.0	35	30	60	475.5
11	6.5	35	30	96	135.7
12	7.0	35	10	60	129.9
13	7.0	35	30	60	580.8
14	7.0	40	10	60	320.5
15	7.0	40	30	96	360.6
16	7.0	35	50	24	148.9
17	7.0	30	30	96	420.5
18	7.5	35	10	60	440.5
19	7.0	35	50	96	410.0
20	7.0	40	50	60	245.5
21	7.0	35	10	24	120.6
22	7.0	30	50	60	142.0
23	7.0	35	30	60	582.5
24	6.5	30	30	60	229.8
25	7.5	40	30	60	386.5
26	7.0	30	10	60	312.5
27	6.5	40	30	60	100.2
28	6.5	35	50	60	102.4
29	7.0	40	30	24	200.5

and EPS yield was investigated using BBD. Based on the experimental design, the maximum EPS yield (582.5 mg/L) was obtained at pH 7.0, 35 °C, and 30% of sucrose concentration under 60 h of incubation with blue light illumination. Xing et al. [12] reported that the addition of sucrose into the growth medium enhanced the production of EPS by *Leuconostoc mesenteroides*. The optimization study revealed that the blue color light and sucrose concentration in the growth medium significantly affect the EPS production in the fermentation system.

The BBD experimental design and the experimental results of the EPS production are presented in Table 1. An ANOVA test for the EPS production was performed to make sure the significance of the model terms. The test result for the quadratic model (Eq. (1)) was highly significant, as evident from the Fisher's *F*-test ($p < 0.005$). The predicted

Table 2
Analysis of Variance (ANOVA) for the response surface quadratic model.

Source of variation	Sum of squares	Degrees of freedom	Mean squares	*F* value	*p*-Value
Model	5.445E+05	14	38,892.44	4.08	0.0064[a]
A	99,144.63	1	99,144.63	10.39	0.0061
B	12.40	1	12.40	0.0013	0.9717
C	8512.01	1	8512.01	0.8921	0.3609
D	95,899.38	1	95,899.38	10.05	0.0068
AB	3831.61	1	3831.61	0.4016	0.5365
AC	349.69	1	349.69	0.0366	0.8509
AD	22.33	1	22.33	0.0023	0.9621
BC	2280.06	1	2280.06	0.2390	0.6325
BD	4323.06	1	4323.06	0.4531	0.5118
CD	366.72	1	366.72	0.0384	0.8474
A^2	1.824E+05	1	1.824E+05	19.12	0.0006
B^2	66,303.57	1	66,303.57	6.95	0.0196
C^2	78,526.33	1	78,526.33	8.23	0.0124
D^2	1.683E+05	1	1.683E+05	17.64	0.0009
Residual	1.336E+05	14	9541.93	–	–
Lack of fit	1.198E+05	10	11.980.62	3.48	0.1205
Pure error	13,780.79	4	3445.20	–	–
Core total	6.781E+05	28	–	–	–

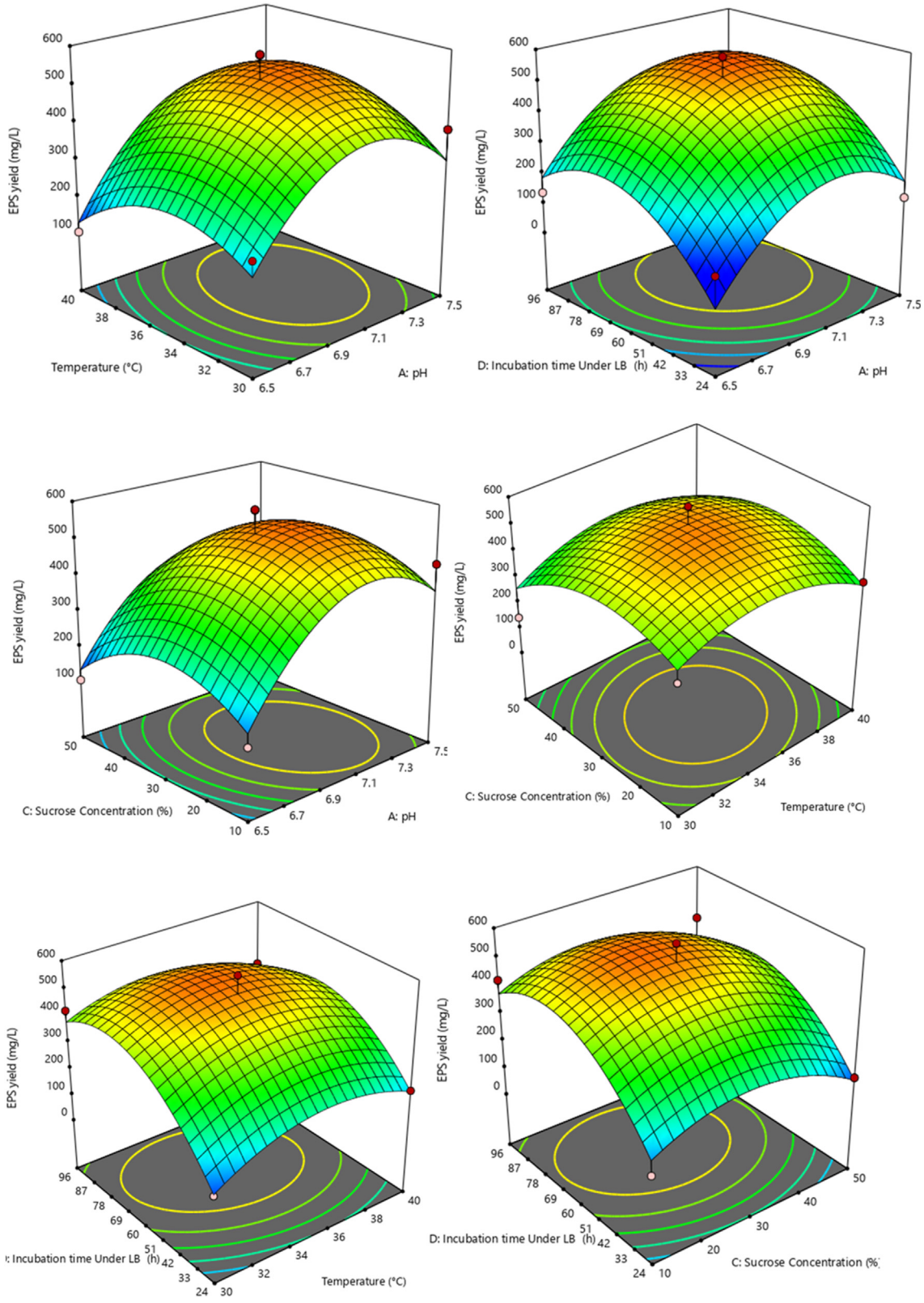

Fig. 3. Response surface 3-D plots of EPS yield under the BBD optimized conditions.

R^2 and adjusted R^2 values were close to 1.0, indicating the model well fitted the experimental data. In addition, sequential model sum of squares, lack of fit tests and model summary statistics further supported the significance and adequacy of the model (Table 2). Three dimensional plots provided in Fig. 3 graphically represent the regression equations and were used to visualize the relationship between the response and experimental levels of each variable to determine optimum EPS production.

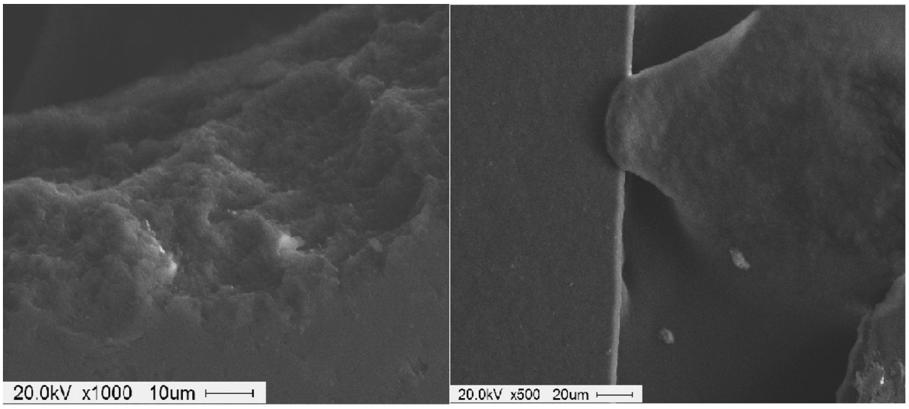

Fig. 4. SEM micrographs of the EPS produced by *Rhodobacter* sp. BT18.

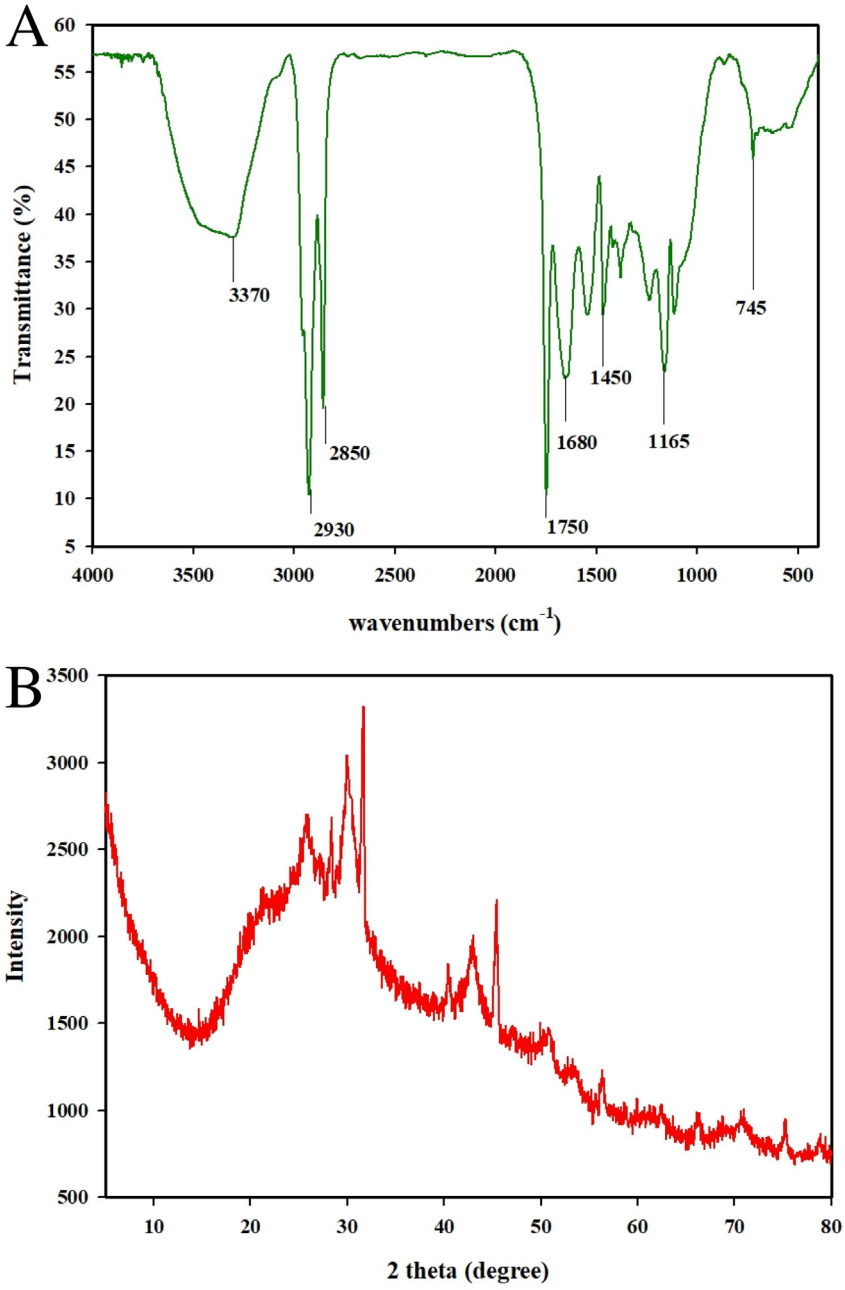

Fig. 5. (a) FT-IR and (b) XRD of EPS.

Table 3
Emulsifying activity of EPS produced by *Rhodobacter* sp. BT18 against the tested hydrophobic substrates.

Hydrophobic substrates	Emulsifying activity (%)	
	Rhodobacter sp. BT18	Control (Tween 80)
Coconut oil	87.3 ± 3.0	54.4 ± 2.0
Peanut oil	84.6 ± 2.6	49.9 ± 3.3
Xylene	75.5 ± 4.2	25.4 ± 2.4
Vaseline	77.3 ± 3.2	31.4 ± 1.9
Sunflower oil	92.3 ± 1.7	57.5 ± 2.5

3.4. Characterization of EPS

SEM images showed the smooth and porous structure of the EPS (Fig. 4). It has been reported that the porous nature of EPS is important for the water holding capacity [25]. The FT-IR spectra of the EPS showed many peaks from 3370 to 745/cm (Fig. 5a). A broad absorption peak at 3370/cm indicated the presence of a high level of O—H stretching of hydroxyls confirming the presence of polysaccharides. The sharp peaks at 2930 and 2850/cm was assigned to the C—H stretching vibrations of the polysaccharide materials. The absorption peaks at 1750 and 1680/cm indicate the amide and carboxyl groups, respectively. The intensity peaks at 1450 and 1165/cm are assigned to be the C—O, C-O-C stretching of carbohydrates. The adoption peak at 745/cm indicates the glycosidic linkage of exopolysaccharides. The results are consistent with previous studies reporting the FT-IR characterization of EPS [26–29].

XRD analysis was carried out to identify the amorphous nature of the EPS (Fig. 5b). The XRD spectra showed diffraction peaks at 31.6, 43.5, 45.3 and 50.8 °C, which indicate the crystalline structure of the EPS. The results are consistent with the previous studies reporting the crystalline nature of the EPS produced from *Bacillus licheniformis* [30], micro-algae [31], and *Leuconostoc lactis* [29].

3.5. Emulsifying activity of EPS

The emulsifying activity is the most important feature of bacterial EPS. In our study, the EPS produced by the isolate *Rhodobacter* sp. BT18 showed a high emulsification activity against tested hydrophobic substrates compared to the Tween 80 (Table 3). The emulsification index of the EPS was >75% for all the substrates (xylene, vaseline, coconut oil, peanut oil, and sunflower oil). Sunflower oil showed the maximum emulsification of 92.3%. Priyanka et al. [2] reported that the EPS produced by *Rhizobium* sp. PRIM-18 emulsified 96% of sunflower oil. Lower emulsifications were seen in the case of xylene (75.5%) and vaseline (77.3%). Coconut oil (87.3%) and peanut oil (84.6%) too were considerably emulsified. The results indicated that the EPS was more efficient than the control Tween 80, which is an alternate to the chemical surfactants, and could be used in drug delivery systems for lipophilic bioactive systems. The highest emulsification activity of the oils (coconut, peanut and sunflower oil) suggests that the EPS can be used as a potential surfactant for oil spill remediation. In addition, the high xylene emulsification ability of EPS could be utilized for enhanced removal of xylene from the ecosystem.

3.6. Flocculating activity of EPS

The flocculating activity of the EPS produced by *Rhodobacter* sp. BT18 was studied with various concentrations of the EPS (Fig. 6). The results showed that the maximum flocculating activity of about 75.4% was observed at 30 mg/L of the EPS. The flocculating activity gradually increased with increase in the concentration of EPS from 10 to 30 mg/L and decreased to 25.5 and 20.2% at 40 mg/L and 50 mg/L, respectively. It has been reported that the dosage of EPS and the size of floc were related to each other. The kaolin particles in the reaction mixture around the EPS might aid in the increasing flocculation at lower concentrations. At higher concentrations that excessive amount of EPS may oversaturate the binding sites of the kaolin particles resulting in a lower flocculating activity [23,32]. Lee et al. [33] observed that both deficiency and abundance of EPS and kaolin clay negatively affected the flocculating activity.

3.7. Metal chelation property of EPS

The EPS showed effective As and Pb chelation activities. About 64% of As and 51% of Pb chelation were observed for 100 mg/L of As and Pb, respectively (Fig. 7). Increase in As and Pb concentration confirmed the chelation activity. It has been reported that the hydroxyl and carbonyl groups of polysaccharides could be responsible for As and Pb chelation [34]. The metal-chelating property of the EPS could open new avenues for the bioremediation of heavy metals from water.

4. Conclusion

In this study, a new phototrophic bacterium, *Rhodobacter* sp. BT18 was isolated from brackish water and the optimal conditions for its EPS production and the structural characteristics of the produced EPS were investigated. The maximum EPS yield (582.5 mg/L) was obtained

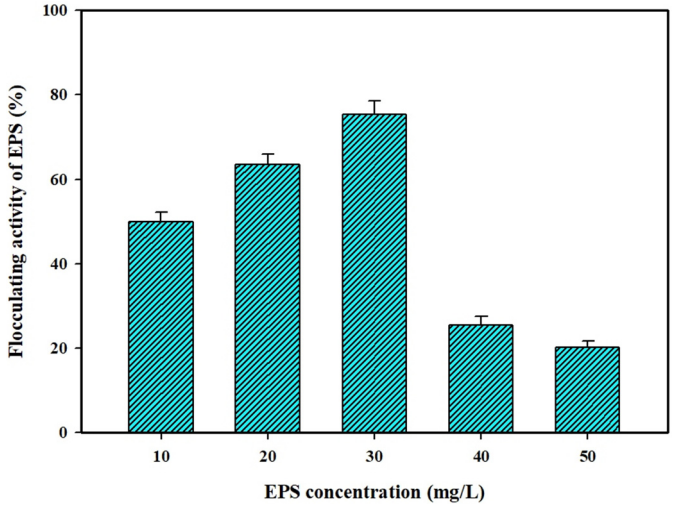

Fig. 6. Flocculating activity of EPS.

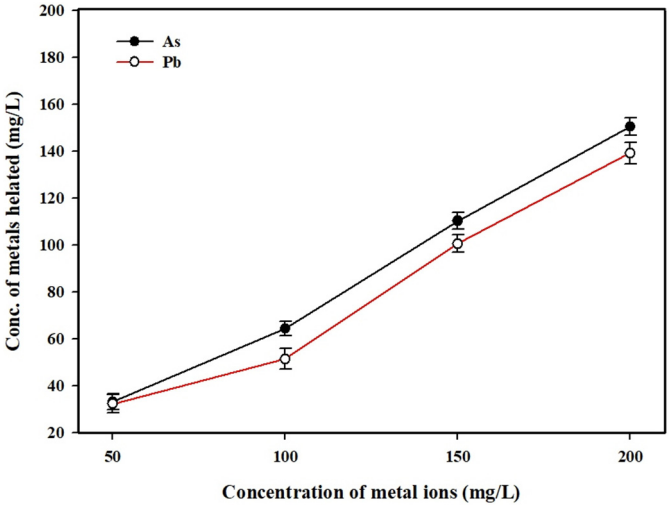

Fig. 7. Metal chelating property of EPS.

under the condition of pH 7.0, 35 °C, and 30% of sucrose concentration, and 60 h of incubation under the blue light illumination. To the best of our knowledge, the EPS production by *Rhodobacter* sp. under different light sources has never been reported in the literature. The EPS showed potential emulsification, flocculation and metal chelating activities.

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