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RESEARCH ARTICLE

# Comparative assessment of exopolymer production and chemical characteristics of two environmental biofilm isolates of *Pseudomonas aeruginosa*

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**Abstract:** Two *Pseudomonas aeruginosa* isolates from natural biofilms (marine and freshwater environment) were investigated for exopolymer (EPS) production and chemical characterization. Both the isolates were categorized using molecular tools as *P. aeruginosa* species. The EPS production was distinct with various carbon and nitrogen sources, the average EPS yield by the two *Pseudomonas* strains was 55 $\mu$g.ml$^{-1}$. Modelling was done to establish the relation between observed and predicted EPS yields. The chemical composition, FTIR and Raman spectroscopy analysis of the two EPS showed that carbohydrate content was more in marine strain, while protein content was relatively high in the freshwater strain. Thermo-gravimetric analysis of the two EPS showed endothermic decomposition. Biochemical study by gel permeation chromatography showed that the marine strain EPS is a highly glycosylated biomolecule, while the freshwater EPS is a weakly glycosylated biomolecule with molecular weights 140,000 and 300,000 Daltons respectively. The EPS produced by the two *Pseudomonas* isolates has implications in process and chemical industries.

**Keywords:** bacteria, EPS, carbohydrate, protein, biofilm, biofouling, industries

## 1 Introduction

Microorganisms that colonize abiotic surfaces in nature produce exopolymer (EPS) and form a gel matrix linkage with the substratum to further their propagation. The EPS has numerous influences on the interfacial processes, like immobilizing water at the biofilm/substratum interface, trapping of nutrients and decreasing diffusion[1]. According to Simon *et al.*[2] EPS gel is not a passive matrix but exhibits physical, chemical, and electrical responses to surrounding environment[3]. Although EPS are important for the attachment of cells on natural and engineered surfaces, the physiological control systems responsible for modulating polymer formation are not well understood[4]. Some studies[5] have examined the composition of bacterial EPS and reported the presence of mono-polysaccharides, glycol-proteins and glucose-amines as moieties[6]. In laboratory cultures it is seen that EPS production is not initiated until the late log phase[4]. EPS also provides protection to the microorganisms from biocidal action and predation. Exopolymers (in the form of bacterial capsule, slime, glycol-calyx, diatom mucus and fungal slime) of microbiota are functionally important components of aquatic ecosystem[7]. The copious EPS produced by bacteria also enhances the aggregation of other bacteria and micro algae[8]. EPS have many functions that enhance the survival and propagation of microorganisms in natural environments. Most notably among them is the attachment of cells to surfaces which supports the subsequent biofouling phenomena which has significant industrial implications[9].

Study of the EPS produced by bacteria may reveal important ecological characteristics for the adaptation of bacteria to an aquatic ecosystem. Little is known about the identity of bacterial species and EPS composition in most natural biofilms and their relative concentrations. Although, several investigators have examined the composition of bacterial EPS as well as their structural details[10–13], knowledge gaps exist with respect to the chemical composition of EPS and on the specific role it plays for a bacterial cell and in biofouling process[7, 14, 15].

In this study two exopolymer producing bacteria were

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isolated from natural biofilms formed in marine and freshwater environment in the vicinity of a nuclear power plant. The two strains were studied to assess their cell growth and EPS production. Culture media, carbon and nitrogen sources and growth variables were optimized for maximum EPS yield. The EPS was characterized using IR spectroscopy, Thermo-gravimetry and other biochemical methods. Further, the study highlights the importance of the exopolymers in biofilm formation and biofouling problem in cooling water systems.

## 2 Materials and methods

Plexiglass panels (15 × 10 × 0.3 cm and 7 × 3 × 0.3 cm) were suspended using a stainless steel frame at a depth of 1.5 m for 5 days in a freshwater impoundment (open reservoir), which is used as an intermediate storage facility for ground water collected from a river-bed. The second biofilm isolation site was the coastal waters of Bay of Bengal in the vicinity of a nuclear power plant, at Kalpakkam, East coast of India. (12°33'N and 80°11'E). The panels were exposed at a depth of 1.5 m for 5 days for the growth of natural biofilm. The biofilm was scrapped from the panels, suitably diluted and plated on respective culture media Zobel agar (Hi Media, India) for marine biofilm and nutrient agar (0.5% peptone; 0.3% yeast extract, 0.5 % sodium chloride and 1.5% agar, Hi Media, India), for freshwater bacteria isolation. Two EPS producing bacteria were isolated and purified by sub-culturing and checked for culture purity. The two bacterial strains were then cultivated in King's B medium (Composition - Proteose peptone- 20 gm, Glycerol 10 ml, K2HPO4 1.5 gm, MgSO4 1.5 gm, Agar 15 gm, Water 1 litre, Hi Media, India). The growth in the form of turbidity (O.D at 600nm) was recorded at regular intervals. Bacterial DNA extraction (QIAamp DNA Mini Kit (50) - Cat No./ID: 51304, QIAGEN, India) and 16S rRNA gene amplification was carried out as per the standard procedure[16]. DNA sequencing and the phylogenetic trees were constructed using the neighbour-joining method using Mega 5 software[17].

### 2.1 Exopolymer production and extraction

The production and extraction of EPS depends on the fermentation process. EPS production by the two *Pseudomonas* sp. was standardized for the maximum EPS yield. Batch cultures that had reached stationary phase were centrifuged (20,000 X g for 25 mins) to separate cells from EPS. The supernatant was then precipitated with three volumes of 95% v/v ethanol. The precipitate was collected after centrifugation and dissolved in filter sterilized distilled water and stored overnight at 4°C. The precipitated mass was again dissolved in distilled water, dialyzed with a suitable membrane with cut-off of 8,000 Daltons to remove low molecular weight components and freeze dried[18, 19].

### 2.2 Effect of different carbon and nitrogen sources on exopolymer production

The bacteria were cultivated in the chemically defined medium (composition: Yeast extract- 0.5 gm; Sodium chloride 1.5 gm; Water 1 litre, media components were obtained from Hi Media, India), containing the nitrogen sources; amino nitrogen, ammonium nitrate, peptone and tryptone. The carbon sources studied were fructose, galactose, glucose, lactose, mannose and sucrose. A 100 ml of the chemically defined medium was dispensed into 250 ml conical flasks and sterilized. After adding the filter sterilized carbon and nitrogen sources, the broths were inoculated with overnight grown cultures and incubated at 30°C, for 48 hrs and EPS was extracted and quantified.

### 2.3 Characterization of the exopolymer

The culture media were subjected to elemental (CHNS) analysis in a Perkin Elmer PE 2400 II CHNS/O Analyzer (Perkin Elmer, India), to obtain the C/N ratio. Proteins and carbohydrates (sugars) were analysed following the methods of Lowry *et al.*[20] and Dubois *et al.*[21], respectively. Uronic acid assay was done as described by Dische[22]. EPS separation and molecular weight characterization were carried out using HPLC system [Waters, India]. Isocratic solvent mode was used, and the response was detected at 280nm by a Tuneable UV-Vis detector. Gel permeation chromatography was carried out using Sephadex G 200 column (Pharmacia, Sweden) after standardizing. The various fractions collected were detected at 280nm and the molecular weight of the EPS was obtained from the standard graph. Infra-red spectroscopy (Model: VERTEX 80/80v FTIR spectrometer, Bruker, UK) was carried out to identify the functional groups in the two EPS. The freeze dried and powdered EPS extracted from the *Pseudomonas aeruginosa* strains were analysed in a Thermo-gravimetric unit (TG-DTA, Model: SETSYS 16/18, Setaram Instrumentation, France). The EPS samples were subjected up to a temperature of 550°C at an increment of 50°C in an inert atmosphere of Argon gas, the weight loss was recorded.

### 2.4 Statistical analysis

The data from five experiments in each set for carbon and nitrogen sources along with the control data were used to obtain the mean values and the estimated standard deviation (esd). Origin 8.5 was used for the purpose of

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fitting the data to a cubic polynomial. The mean was obtained by averaging all the experimental data points in each set and the esd was obtained from the mean by $esd(y) = \sqrt{\sum_{i=1}^{n}(y_i - mean(y))^2/(n-1)}$ for the $n$ data points in each set. The mean data points for all the sets for each source were then fitted to a third-degree polynomia $y = p(x) = a_0x^3 + a_1x^2 + a_2x + a_3$ where the $\chi$ implies the concentration of the sources and y implies the EPS yield. The ESDs were taken as the weight assigned to each data point for the least square fitting procedure to obtain the coefficients ($a_0$, $a_1$, $a_2$ and $a_3$) of the polynomial p($\chi$) of degree 3. The fitting procedure minimized the least-square-error of the fit to the points [x, y]. We had five data points for each source and a polynomial with four unknown coefficients, so the values of the polynomial coefficients that are obtained are reliable. The polynomial has been fitted by using the transformed $\chi$ values given by $\chi_t$ = ($\chi$ - mean($\chi$))/esd($\chi$). Minitab version -18 was used for the analysis as well as for modelling the data.

## 3 Results and Discussion

### 3.1 Bacterial identification and exopolymer composition

The two strains were confirmed as *Pseudomonas aeruginosa* species by 16S rRNA gene study. The genomic sequence was submitted to Gen-bank and were assigned the accession No: JQ811768.1 for freshwater and JQ809326 for marine strains. Table 1 provides data on the chemical composition of the two EPS. Hexose sugar content was relatively more 42.5% (per gram EPS). Ribose sugar amounted to 9.3%; hexose-amines 4.2%; uronic acid 4.8% and protein content was 9.8%. HPLC analyses revealed the presence of glucose monomers (30.2%) which indicates that the EPS is by and large a polymer of monosaccharides. In case of freshwater strain, the hexose sugar content of the EPS was 17.8%, uronic acid content was 13.5%; hexose-amines 8.6% and total protein was 42.6%. Nucleic acid content was 1.4%. Monomer units of the sugars are glucose 10.4%; galactose 3.4% and mannose 2.8%.

### 3.2 Growth and exopolymer production by the two Pseudomonas aeruginosa

The growth curve and the corresponding exopolymer yield (gm $l^{-1}$) by the marine strain are given in Figure 1(a). The curve describes a lag phase up to 10 hrs and thereafter the exponential phase, which was observed up to 55 hrs. The stationary phase was observed up to 70 hrs and by 80 hrs the culture was in decline phase. The

**Table 1** Percentage distribution of chemical components of the two EPS produced by *Pseudomonas aeruginosa* strains

| Component | Pseudomonas aeruginosa (marine strain) [%] | Pseudomonas aeruginosa (freshwater strain) [%] |
|---|---|---|
| Hexose sugar | 42.5 | 17.8 |
| Ribose sugar | 9.3 | Not present |
| Uronic acid | 4.8 | 13.5 |
| Hexose-amines | 4.2 | 8.6 |
| Total protein | 9.8 | 42.6 |
| Nucleic acid content | Not present | 1.4 |
| Galactose | Not present | 3.4 |
| Glucose* | 30.2 | 10.4 |
| Mannose | Not present | 2.8 |
| Molecular weight♯ | 140,000 Daltons | 300,000 Daltons |

Note: * By HPLC; ♯ By electrophoresis

EPS production was observed after 14 hrs of growth and the yields were linear after 25 hrs. The maximum EPS yield (4.6 gm $l^{-1}$) was obtained during the log and early stationary phases *i.e.*, up to 60 hrs. The growth and EPS production by freshwater strain are presented in Figure 1(b). The lag phase of the growth curve was up to 10 hrs and the exponential growth was observed till 40 hrs. The EPS yield was observed after 16 hrs of growth and continued till stationary phase. In natural biofilms the irreversible bacterial adhesion / attachment and subsequent biofouling propagation is after EPS production.

Culture conditions such as medium composition and concentration, temperature, pH are important in the production of EPS[23–25]. In the present study EPS was produced in the early log phase and continued till stationary phase. Overall the late log phase and up to mid stationary phase showed maximum EPS production. Phosphate influenced the EPS production of both the strains and the maximum EPS production was obtained when $KH_2PO_4$ was added to the basic medium. This has resulted in 25% higher EPS yield. Mangwani *et al.*[26] have reported that 20 mM $Ca^{2+}$ influenced EPS production of few marine bacteria. However, $Ca^{2+}$ beyond 25 mM did not had any influence on EPS production/biofilm formation in *Staphylococcus aureus* strain[27]. Phosphate concentration in the medium had enhanced EPS yield with increasing concentration. In case of freshwater strain maximum EPS production (6.4 gm $l^{-1}$) was observed at 70 mM concentration, while the marine strain showed a maximum yield (4.6 gm$l^{-1}$). (Table 2)

### 3.3 Effect of pH and phosphate concentration on EPS production

Figure 2 illustrates the effect of pH on EPS production, both the strains have showed simultaneous growth and EPS production with increase in pH from 4 to 8 which was a unique observation. The marine strain showed a

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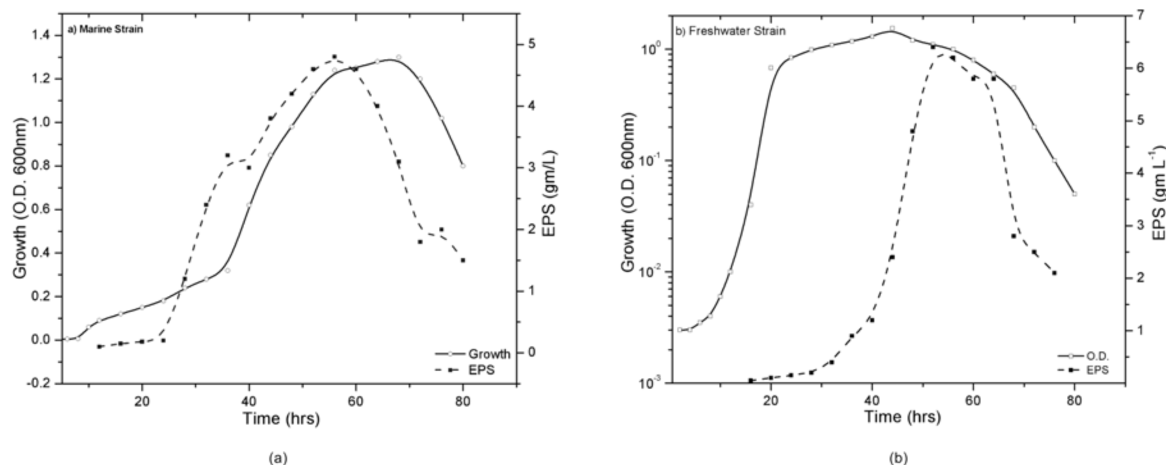


**Figure 1** (a) Growth curve and EPS production by marine Pseudomonas aeruginosa; (b) Growth curve and EPS production by freshwater Pseudomonas aeruginosa

**Table 2** Effect of phosphate concentration on EPS production

| Phosphate (mM) | Pseudomonas aeruginosa [marine] ($gml^{-1}$) | Pseudomonas aeruginosa [freshwater] ($gml^{-1}$) |
|---|---|---|
| 0(control) | 0.01 | 0.1 |
| 10 | 0.4 | 0.5 |
| 20 | 0.6 | 0.7 |
| 30 | 0.9 | 1 |
| 40 | 1.1 | 1.2 |
| 50 | 3.5 | 3.6 |
| 60 | 3.6 | 4.8 |
| 70 | 4.6 | 6.4 |
| 80 | 4.2 | 6.1 |
| 90 | 3.2 | 4.1 |
| 100 | 1.2 | 1.8 |

low growth phase up to pH 5.2, thereafter the growth was linear up to pH 8.2. In case of freshwater strain, the growth was linear from pH 5.0 7.8 and beyond pH 8.0 the growth was retarded. It was noticed that for marine strain the optimum pH for EPS production was 7.9 and for freshwater strain it was 8.0.

## 3.4 Effect of carbon to nitrogen (C/N) ratio on EPS production

Figure 3 describes the C/N ratios and EPS production by both the Pseudomonas strains. The C/N ratio is an important factor affecting the synthesis of EPS[28]. The best EPS production was achieved when the ratio of carbon and nitrogen was 10, however, the present findings were not in accordance with the results reported by Elisashvili *et al.*[28]. In this study, a noticeable observation was that, the maximum EPS yield was achieved with glucose as carbon source followed by sucrose and other mono-saccharides. The C/N ratio of 5-25 was found to be favourable for EPS production by both the Pseudomonas strains. The maximum EPS yield was noticed when the C/N ratio was in between 15-25. Generally, the yield of EPS will be in the range 100-300 mg dry weight per 100 ml of culture medium[24,25]. In the present study the EPS content produced by marine strain was 4.6 $g.ml^{-1}$ (460 mg/100 ml) and the freshwater strain produced 6.4 $gml^{-1}$ (640 mg/100 ml), these EPS values are higher than published reports.

## 3.5 Exopolymer characterization

Generally, EPS are high molecular weight biomolecules with mass of 100 kilodaltons and 25% of which is made up of uronic acids[10]. In the present study the EPS molecular weights were 140,000 ($1.4 \times 10^5$) and 300,000 ($3.0 \times 10^5$ Daltons for marine and freshwater strains respectively. The EPS produced by the marine strain is a highly glycosylated molecule and acidic in nature (pH in aqueous solution was 5.2). The freshwater strain EPS has more protein content and can be designated as a weakly glycosylated protein molecule. CHNS analysis of the EPS showed Carbon - 58.64%, Hydrogen - 26.88%, Nitrogen - 9.50% and Sulphur - 4.42% for marine strain EPS and Carbon - 33.67%, Hydrogen - 16.20%, Nitrogen - 47.43% and Sulphur - 5.38% for freshwater strain EPS.

The Infrared spectra are presented in Figure 4 and Table 3, the data showed a major peak for -OH group. IR spectra of marine *Pseudomonas* EPS showed a broad peak at 3280 $cm^{-1}$ which is characteristic of intermolecular hydrogen bonding for polymeric substances. Peak at 2038 $cm^{-1}$ is characteristic of N-H interaction and is useful in distinguishing protonated tertiary amines which generally have significant band in the region 2200 to 1800 $cm^{-1}$. Aliphatic amines have strong absorption band between

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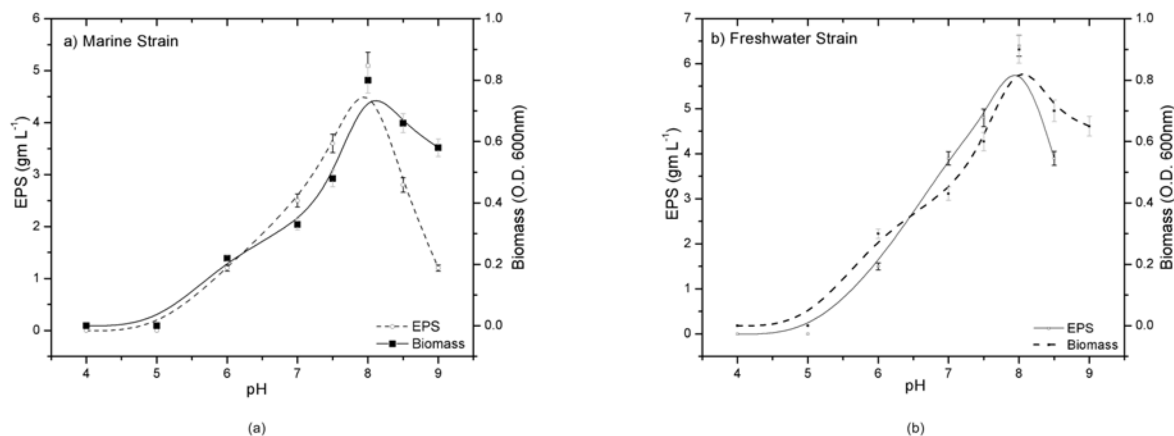


**Figure 2** Effect of pH on growth and exopolysaccharide yield of (a) marine, (b) freshwater strains

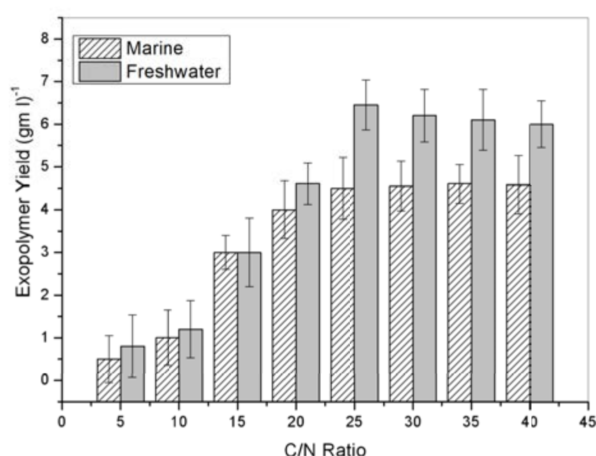


**Figure 3** Effect of C/N ratio of the growth medium on exopolymer production by both the *Pseudomonas strains*

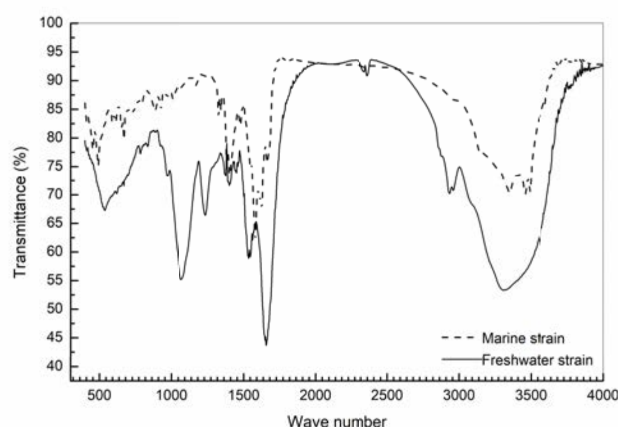


**Figure 4** Infrared spectra of the two exopolymers produced by *Pseudomonas aeruginosa*

1650 to 1560 $cm^{-1}$, the peak at 1610 $cm^{-1}$ indicate the presence of primary amine groups. Peak at 1360 $cm^{-1}$ is characteristic of O-H compounds with OH bending and a peak at 580 $cm^{-1}$ is characteristic of P=S compound. In case of freshwater strain, the EPS showed a peak at 3600 $cm^{-1}$ characteristic of intra and inter molecular hydrogen bonding (OH groups) and also polyvalent group chelation. Peak at 3320 $cm^{-1}$ indicates the presence of aliphatic amine N-H group with a terminal CH stretching. Peak at 3016 $cm^{-1}$ is characteristic of carboxylic acid and OH stretching. A peak at 1742 $cm^{-1}$ is characteristic of saturated aldehydes (CHO). A peak at 1158 $cm^{-1}$ is characteristic of ether linkage and at 1116 $cm^{-1}$ is characteristic of -CO stretch[29]. A confocal Raman spectrum of the two EPS was also recorded (Data not shown). Spectral variation of the two exopolymers indicated that, in case of marine *Pseudomonas* exopolymer the spectrum was a horizontal line suggesting an amorphous property. Freshwater *Pseudomonas aeruginosa* exopolymer showed a very minor peak at 500-600 $cm^{-1}$ indicating (C-C) aliphatic chain vibration. A minor peak at 750 $cm^{-1}$ shows the presence of weak (C-O-C) linkage. A vibration at 1600 $cm^{-1}$, shows the presence of aromatic ring and amines, and a peak at 1750 $cm^{-1}$, indicates the presence of (C=O) group[30].

Thermo-gravimetric analysis measures the amount and rate of change in the mass of a sample as a function of temperature or time in a controlled atmosphere. The first weight change in the TGA was due to moisture loss, and with DTA it is due to endothermic process. Figure 5 illustrates the TG DTA analysis spectra of the two exopolymer samples. TGA thermogram of EPS from freshwater bacterium has showed three step degradation process. In first step, 15% loss was noticed between 25 to 135°C. It can be attributed to loss of water molecules and carboxyl groups. Second phase of de-polymerization

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**Table 3** Infrared spectral analysis of the two exopolymers

| S. No | Wave Number ($cm^{-1}$) | Functional group characteristics) |
|---|---|---|
| (a) Freshwater Pseudomonas aeruginosa exopolymer | | |
| 1 | 3948 | Near IR region peak |
| 2 | 3608 | Inter and intra molecular hydrogen bonding and presence of OH group, indicates polyvalent chelation property |
| 3 | 3320 | Characteristic of N-H group with terminal CH stretching |
| 4 | 3016 | Indicates the presence of COOH and OH stretching |
| 5 | 2264 | Characteristic of tertiary amines and also strong P-H linkage |
| 6 | 2064 | Characteristic of N=C, N=N and N=C=S broad doublet. |
| 7 | 1970 | Characteristic of imine group useful to distinguish from protonated tertiary amines |
| 8 | 1814 | Typical of CO - O - O - CO saturation state |
| 9 | 1742 | Presence of aldehydes -CHO group |
| 10 | 1642 | Characteristic of aliphatic primary amines, amides and keto esters |
| 11 | 1598 | Presence of Nitramines group |
| 12 | 1404 | Typical of sulfonates and sulphates |
| 13 | 1380 | Characteristic of C - NO2 indicating N = O bond stretching |
| 14 | 1204 | Characteristic of - O - C (CH3)3 and -OH stretching |
| 15 | 1158 | Indicative of secondary amine linkage |
| 16 | 1116 | Indicate the presence of CO stretching |
| 17 | 1006 | Presence of f mono-aliphatic group |
| 18 | 788-688, 438 | Presence of primary alkyl group and secondary alkanes |
| 19 | 596 | Characteristic of f C - S and S - S bonds |
| (b) Marine Pseudomonas aeruginosa exopolymer | | |
| 1 | 3308 (3280) | Typical intermolecular hydrogen bonding of polymeric substances |
| 2 | 2048 (2038) | Presence of imine group differentiates from protonated tertiary amines |
| 3 | 1618 (1610) | Primary amines of amide II type with NH bending |
| 4 | 1364 (1360) | Characteristic O-H bending, primary aliphatic alcohols |
| 5 | 1200 | Indicates the presence of C-O |
| 6 | 584(580) | Characteristic of P = S compounds |

was at 260°C wherein 49% weight loss was observed. Third step showed 65% weight loss at 520°C. The EPS of marine bacterium has shown two step degradation processes, first step degradation was observed at 135°C with 8% weight loss. Second step degradation was observed at 380°C with 12% weight loss. At 550°C the EPS has retained 80% weight and it can be termed as thermostable. The D (or d half) temperature was 225°C when 50 wt. % of the exopolymer has decomposed.

## 3.6 Effect of carbon sources on exopolymer yield

The effect of various carbon sources on EPS yield obtained from 5 independent experiments with marine and freshwater *Pseudomonas* species are presented as box plots (Table 4). Figure 6 shows the spread of experimental data obtained for each carbon source and at different concentrations for freshwater and marine *Pseudomonas*. The box plots show that the data is skewed, the centres of the box plot suggests that the median of various carbon sources is different. The Box plots for 0.1 and 0.2% are comparatively short, suggesting that the overall EPS production has good correlation with other concentrations tested. Similarly, glucose box plot is relatively tall suggesting its uniqueness in EPS production. The overall box plot distribution suggests differences between various carbon sources on EPS production. The overall trend in EPS production for different carbon sources remains same for both marine and freshwater strains. The restricted maximum likelihood analysis of ANOVA model at 0.05 p-value indicating significant correlation between the two energy sources (carbon and nitrogen).

## 3.7 Effect of nitrogen sources on exopolymer yield

The effect of various nitrogen sources on EPS yield is presented in the box plots in Figure 7. The plots showed the spread of experiment data obtained for each nitrogen source and at each concentration levels tested both for freshwater and marine *Pseudomonas* species. The box plots show that the data is skewed, a look at the centres of the box plot suggests that the median of various nitrogen sources is different except for amino nitrogen and ammonium nitrate. A 0.3 and 0.5% concentration of nitrogen source showed similarity in the exopolymer production. The box plots are relatively tall suggesting that the overall EPS production is maximum at 0.3% for marine strain. Hsieh *et al*.[31], have reported that EPS yield was highest under nitrogen limitation and glucose as main carbon source.

Figure 8 describe the polynomial fit for EPS yield for carbon and nitrogen sources. For obtaining reliable and accurate fits, the number of data points should be at least twice the number of unknown parameters for generating any fit. That is why the data sets and their fits cannot be expected to predict the yield in a region beyond the fitted points as the esd for those points are very large. Both the carbon and nitrogen sources played a role in EPS yield. The major observation from the polynomial fit of the EPS yield with various carbon and nitrogen sources depicts that EPS yield does not follow an exponential behaviour. Instead, it shows a power law (cubic) behaviour, which implies that the change in the EPS yield as a function of concentration depends proportionally on the concentration of the substrates. Thus in low carbon and nitrogen concentration the EPS yield showed a linear trend. However, at high concentration the yield decreases because of cellular stress and permeability[32]. As per the polynomial fit, the maximum yield for carbon source was noticed at 0.4% and for nitrogen source at 4% concentration. Mod-

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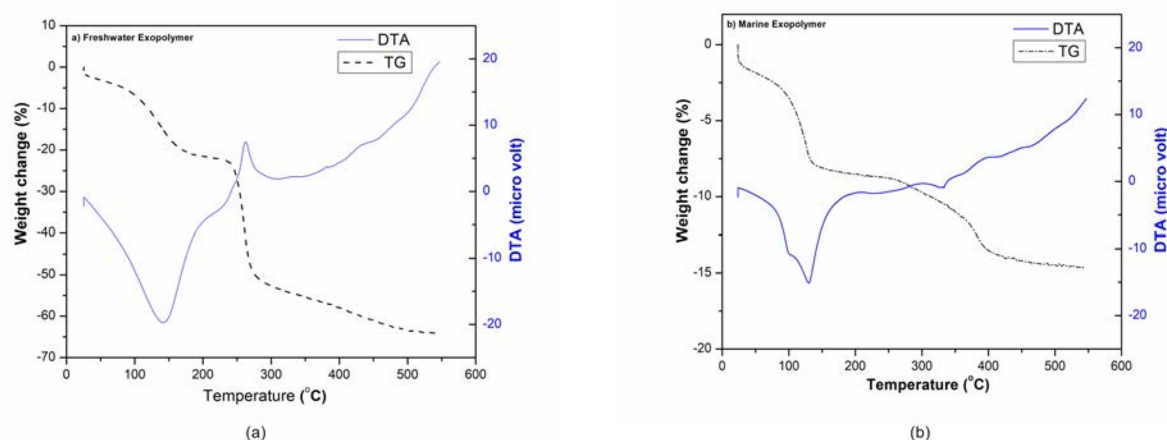


**Figure 5** Thermo-gravimetry analysis spectra of the two EPS produced by Pseudomonas strains. (a) Freshwater Exopolymer; (b) Marine Exopolymer

**Table 4** Effect of various carbon and nitrogen sources on EPS yield ($gml^{-1}$) by the two *Pseudomonas* strains

| | | | 0.10% | 0.20% | 0.30% | 0.50% |
|---|---|---|---|---|---|---|
| Marine strain | Carbon Source | Fructose | 1.32 ± 0.39 | 1.48 ± 0.26 | 1.86 ± 0.10 | 1.72 ± 0.52 |
| | | Galactose | 1.24 ± 0.33 | 1.64 ± 0.09 | 1.83 ± 0.24 | 1.98 ± 0.09 |
| | | Glucose | 1.60 ± 0.07 | 2.44 ± 0.20 | 2.93 ± 0.57 | 4.11 ± 0.37 |
| | | Lactose | 0.69 ± 0.13 | 1.44 ± 0.41 | 2.26 ± 0.33 | 1.44 ± 0.78 |
| | | Mannose | 1.26 ± 0.17 | 1.59 ± 0.15 | 1.82 ± 0.41 | 2.12 ± 0.64 |
| | | Sucrose | 1.21 ± 0.16 | 2.03 ± 0.07 | 2.78 ± 0.19 | 3.67 ± 0.26 |
| | Nitrogen Source | Amino nitrogen | 0.75 ± 0.13 | 0.93 ± 0.06 | 1.37 ± 0.15 | 1.46 ± 0.47 |
| | | Ammonium nitrate | 1.12 ± 0.15 | 1.65 ± 0.16 | 0.92 ± 0.36 | 1.33 ± 0.36 |
| | | Peptone | 1.10 ± 0.22 | 2.07 ± 0.31 | 3.44 ± 0.27 | 3.64 ± 0.40 |
| | | Tryptone | 0.86 ± 0.07 | 1.47 ± 0.06 | 2.51 ± 0.20 | 2.53 ± 0.35 |
| Freshwater strain | Carbon Source | Fructose | 0.77 ± 0.08 | 0.68 ± 0.08 | 1.35 ± 0.19 | 1.24 ± 0.05 |
| | | Galactose | 0.86 ± 0.06 | 1.42 ± 0.08 | 2.16 ± 0.23 | 1.91 ± 0.02 |
| | | Glucose | 2.83 ± 0.09 | 4.24 ± 0.11 | 6.42 ± 0.05 | 5.71 ± 0.73 |
| | | Lactose | 0.58 ± 0.09 | 0.78 ± 0.08 | 0.91 ± 0.03 | 2.37 ± 0.25 |
| | | Mannose | 0.58 ± 0.08 | 1.40 ± 0.24 | 1.78 ± 0.12 | 2.29 ± 0.11 |
| | | Sucrose | 1.85 ± 0.05 | 3.24 ± 0.18 | 4.20 ± 0.14 | 4.12 ± 0.18 |
| | Nitrogen Source | Amino nitrogen | 0.85 ± 0.25 | 1.11 ± 0.25 | 1.71 ± 0.27 | 1.86 ± 0.53 |
| | | Ammonium nitrate | 1.43 ± 0.25 | 2.10 ± 0.30 | 2.77 ± 0.79 | 3.05 ± 0.41 |
| | | Peptone | 1.58 ± 0.08 | 2.30 ± 0.26 | 3.72 ± 0.26 | 4.19 ± 0.12 |
| | | Tryptone | 0.94 ± 0.13 | 1.79 ± 0.51 | 2.76 ± 0.43 | 2.84 ± 0.53 |

elling studies provide valuable information in optimizing the EPS production parameters. In an earlier study[33], biofilm development and EPS production were monitored by modelling the growth and EPS biosynthesis. Figure 9 describes the actual and predicted exopolymer yield by both freshwater and marine *Pseudomonas* bacteria, when grown on Carbon sources. Figure 10 illustrates the comparative account of the actual and predicted yield of the EPS when the marine and freshwater *Pseudomonas* bacteria were grown on Nitrogen sources. There is good agreement between the actual and predicted exopolymer yields of both the strains.

Bacterial EPS represent a diverse range of macromolecules. In the initial stages of biofilm formation, EPS aid in adsorption to solid surfaces in an apparently reversible manner by means of weak bonding. Based on the chemical analysis, the EPS of both marine and freshwater microorganisms are effective cation binding compounds which may act to keep heavy metal concentrations outside the cell. The increased production of EPS will tend to decrease the activity of free metal ion in solution and protect from toxicity[34]. The poly-anionic nature of the EPS makes it function as an ion exchange matrix to concentrate organic nutrients and at the same time limit the penetration of charged molecules such as cationic biocides and antibiotics. Thus, a better understanding of microbial activities in biofilms and EPS can help in designing an effective strategy for biofilm/biofouling control.

The discovery of numerous biopolymers by bacteria has shown to have industrial and medical relevance with

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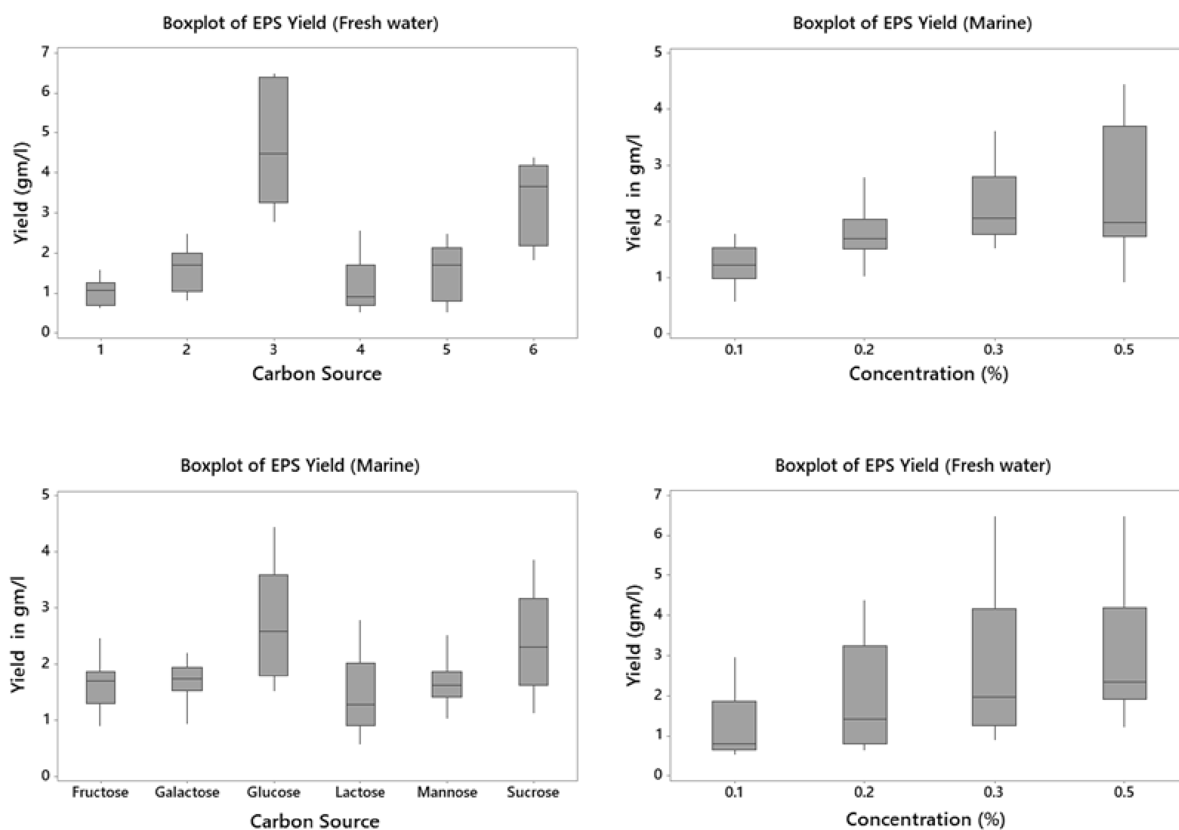


**Figure 6** Box plots of EPS yield with concentration and various carbon sources

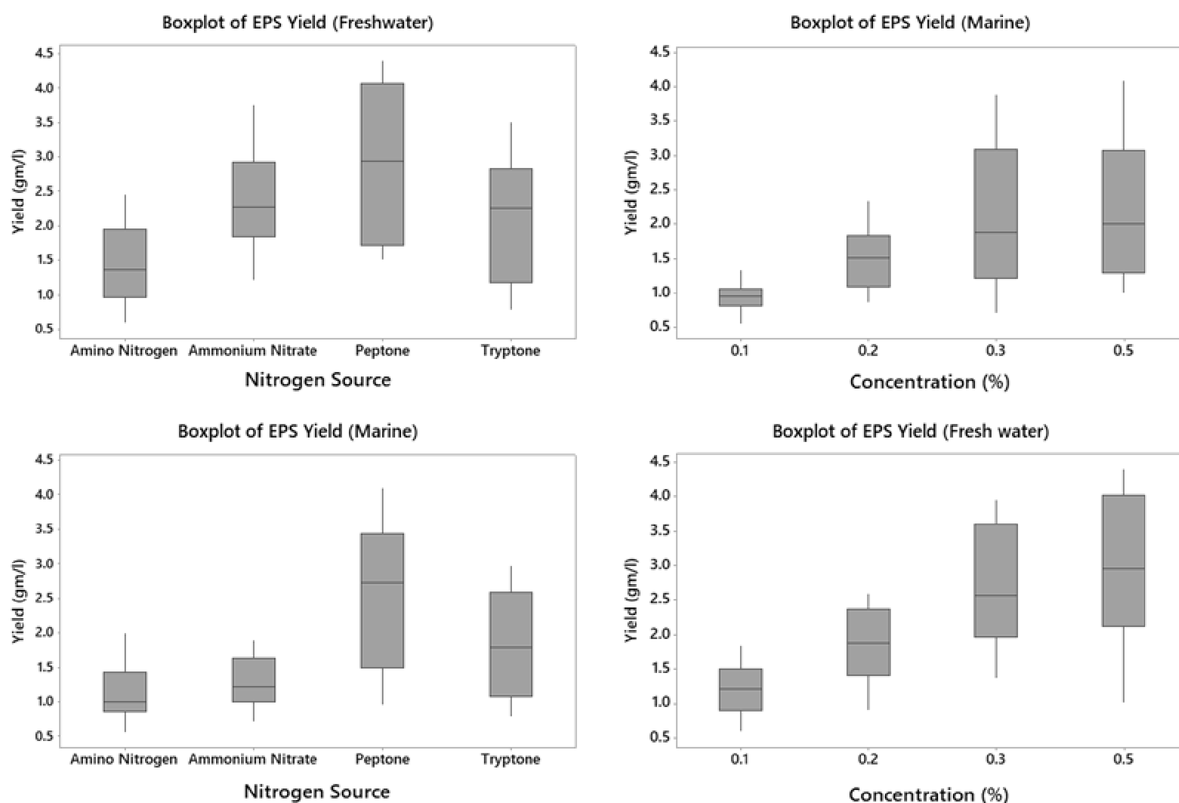


**Figure 7** Box plots of EPS yield with concentration and various nitrogen sources

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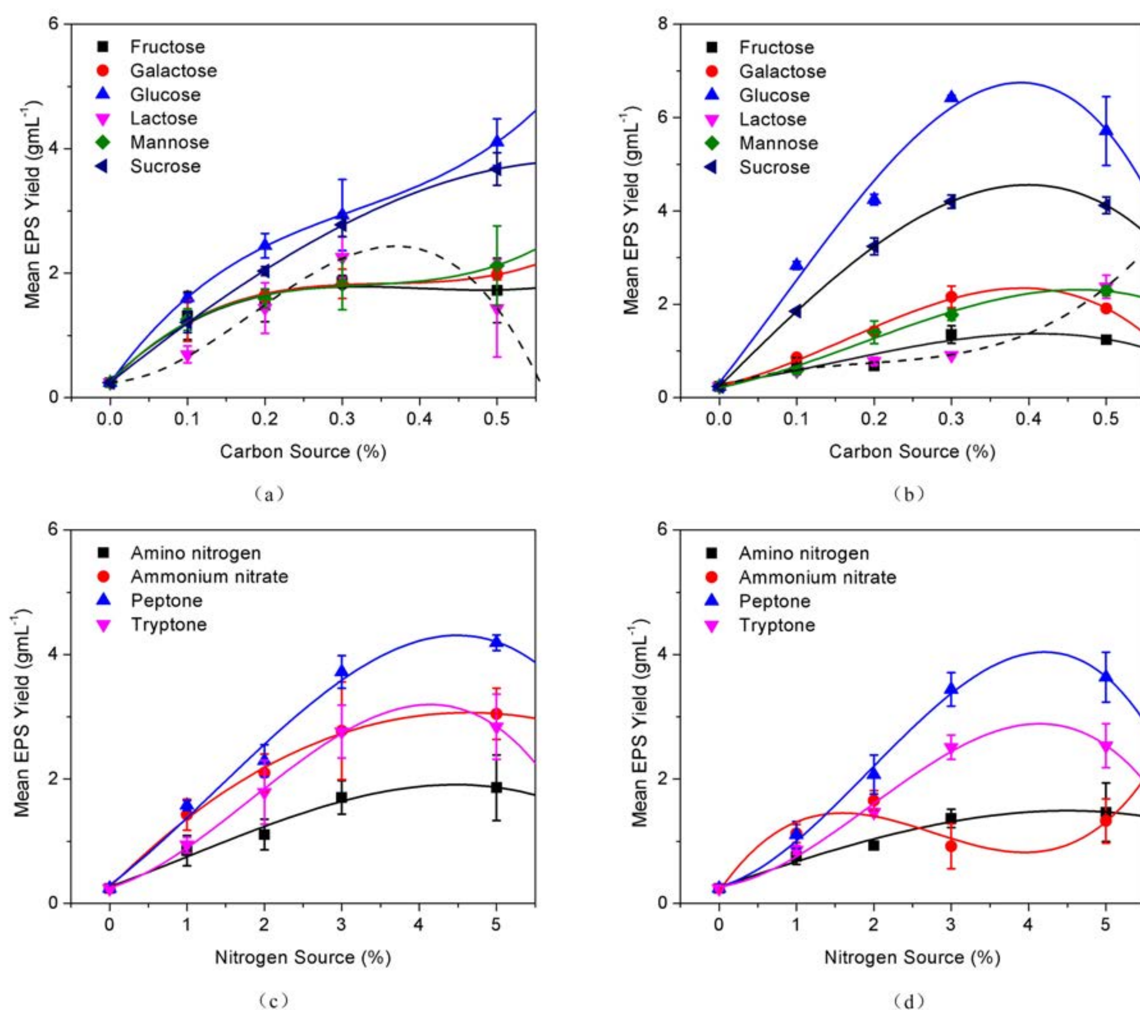


**Figure 8** Polynomial fit plots for EPS yield with various carbon and nitrogen sources. (a) Marine *Pseudomonas* with carbon sources; (b) Marine *Pseudomonas* with nitrogen sources; (c) Freshwater *Pseudomonas* with carbon sources; (d) Freshwater *Pseudomonas* with nitrogen sources

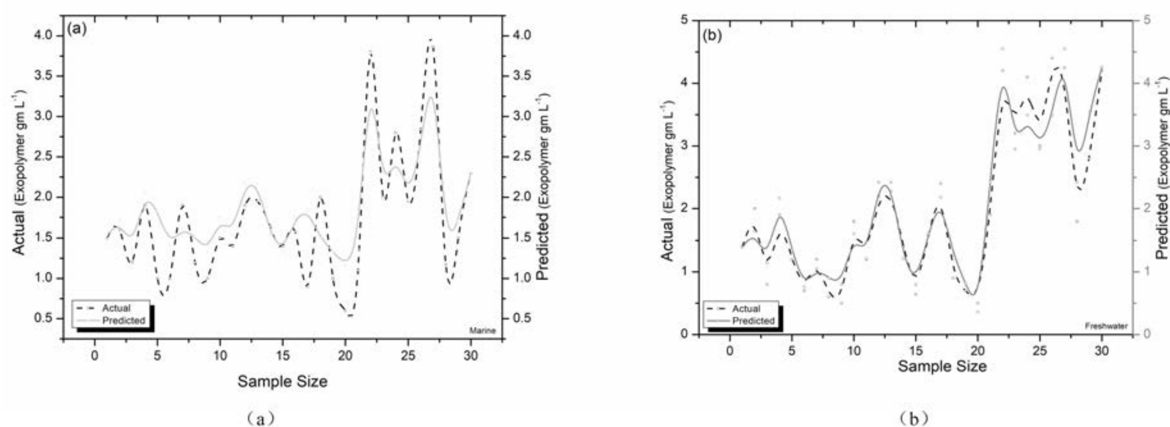


**Figure 9** Predictive vs actual EPS production by *Pseudomonas aeruginosa*. (a) freshwater and (b) marine strains with various carbon sources

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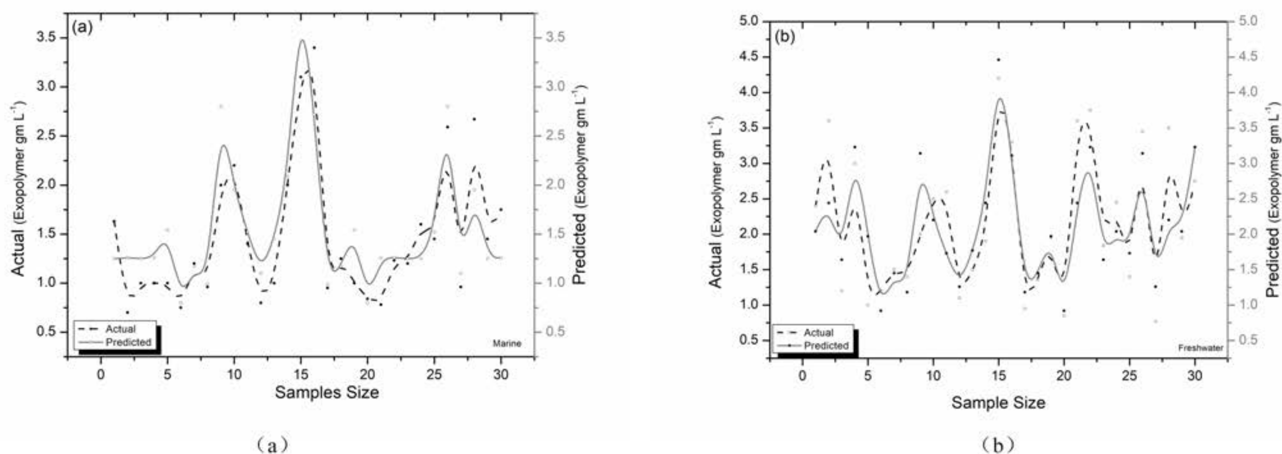


**Figure 10** Predictive vs actual EPS production by *Pseudomonas aeruginosa*. (a) freshwater and (b) marine strains with various nitrogen sources

significant commercial value. Bacterial EPS have wide range of applications such as the industry (textile, dairy, food, cosmetics, *etc*.), health (medicine and pharmaceuticals) and environment (remediation of toxic compounds/metals). The EPS application as a flocculants has a significant role in promotion and eco-friendly usage in wastewater treatment. This implies that EPS can effectively mediate flocculation and thus may be applied in large scale industrial processes. The possession of unique properties by the EPS can be invariably translated to high-value applications wherein the product quality and production cost can be reduced. One of the application of bacterial EPS in medicine and biotechnology include bacterial alginate in cell microencapsulation, such as microsphere vector for drug delivery, making dental impressions, as absorbent dressings, and in anti-reflux therapy. Likewise, dextran, produced by *Leuconostoc mesenteriodes*, is used to prepare plasma substitutes. The sulphated forms of alginate have therapeutic properties such as, anticoagulant, antithrombotic, anti-atherosclerotic, anti-angiogenesis, anti-metastatic and anti-inflammatory. Xanthan gum produced by *Xanthomonas campestris* has broad application in food industry, toiletries, oil recovery, cosmetics[35,36].

## 4 Conclusions

The average EPS yield by the two Pseudomonas strains was 55 $\mu$g ml$^{-1}$. The chemical analysis, FTIR and Raman spectroscopy of the two EPS were carried out. Thermogravimetric analysis of the two EPS showed endothermic decomposition. The marine strain, EPS with a molecular weight of 140,000 Daltons is a highly glycosylated biomolecule. While the freshwater strain with a molecular weight of 300,000 Daltons is a glyco-protein molecule. EPS apart from environmental beneficiation to biofilm formation, also has industrial manifestations such as, biofilm formation and bio-corrosion of heat exchanger materials, they can also be used in sequestration of toxic metals and radionuclides.

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The authors declare that they do not have any conflict of interest. The proposed work was not funded by any funding agency.

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## References

[1] Flemming HC and Wingender J. The biofilm matrix. Nature Reviews Microbiology, 2010, **8**(9): 623-633. https://doi.org/10.1038/nrmicro2415

[2] Simon M, Grossart HP, Schweitzer B, *et al*. Microbial ecology of organic aggregates in aquatic ecosystems. Aquatic Microbial Ecology, 2002, **28**: 75-211. https://doi.org/10.3354/ame028175

[3] Nwodo U, Green E and Okoh A. Bacterial exopolysaccharides: functionality and prospects. International Journal of Molecular Sciences, 2012, **13**(11): 14002-14015. https://doi.org/10.3390/ijms131114002

[4] Ha YW, Stack RJ, Hespell RB, *et al*. Some chemical and physical properties of extracellular polysaccharides produced by Butyrivibrio fibrisolvens strains. Applied and Environmental Microbiology, 1991, **57**(7): 2016-2020. https://doi.org/10.1128/AEM.57.7.2016-2020.1991

Chemical Reports © 2020 by *SyncSci Publishing*. All rights reserved.

[5] Abu GO, Weiner RM, Rice J, *et al*. Properties of an extracellular adhesive polymer from the marine bacteria Shewanella colwelliana. Biofouling, 1991, **3**: 69-84.
https://doi.org/10.1080/08927019109378163

[6] Christensen BE. The role of extracellular polysaccharides in biofilms. Journal of Biotechnology, 1989, **10**(3-4): 181-202.
https://doi.org/10.1016/0168-1656(89)90064-3

[7] Rao TS. Biofouling in Industrial Water Systems: In: Amjad Z, Demadis, KD, editors. Mineral Scales and Deposits, The Netherlands, Elsevier, 2015, 123-140.
https://doi.org/10.1016/B978-0-444-63228-9.00006-1

[8] Evans LR and Linker A. Production and characterization of the slime polysaccharide of Pseudomonas aeruginosa. Journal of Bacteriology, 1973, **116**(2): 915-924.
https://doi.org/10.1128/JB.116.2.915-924.1973

[9] Costerton JW, Lewandowski Z, Caldwell DE, *et al*. Lappinscott, Microbial biofilms. Annu. Rev. Microbiology, 1995, **49**: 711-745.
https://doi.org/10.1146/annurev.mi.49.100195.003431

[10] Sage A, Linker A, Evans LR, *et al*. Hexose phosphate metabolism and exopolysaccharide formation in Pseudomonas cepacia. Current Microbiology, 1990, **20**: 191-198.
https://doi.org/10.1007/BF02091996

[11] Suresh Kumar A, Mody K and Jha B. Bacterial exopolysaccharides-a perception. Journal of Basic Microbiology, 2007, **47**(2): 103-117.
https://doi.org/10.1002/jobm.200610203

[12] Rehm BH. Bacterial polymers: biosynthesis, modifications and applications. Nature Reviews Microbiology, 2010, **8**: 578-592.
https://doi.org/10.1038/nrmicro2354

[13] Freitas F, Alves VD and Reis MA. Advances in bacterial exopolysaccharides: from production to biotechnological applications. Trends in Biotechnology, 2011, **29**(8): 388-398.
https://doi.org/10.1016/j.tibtech.2011.03.008

[14] Nielsen PH, Jahn A and Palmgren R. Conceptual model for production and composition of exopolymers in biofilms. Water Science and Technology,1997, **36**(1): 11-19.
https://doi.org/10.2166/wst.1997.0002

[15] Flemming HC. EPS-then and now. Microorganisms, 2016, **4**(4): 41-47.
https://doi.org/10.3390/microorganisms4040041

[16] Weisburg WG, Barns SM, Pelletier DA, *et al*. 16S ribosomal DNA amplification for phylogenetic study. Journal of Bacteriology, 1991, **173**(2): 697-703.
https://doi.org/10.1128/JB.173.2.697-703.1991

[17] Tamura K, Peterson D, Peterson N, *et al*. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Molecular Biology and Evolution, 2011, **28**(10): 2731-2739.
https://doi.org/10.1093/molbev/msr121

[18] Jiao Y, Cody GD, Harding AK, *et al*. Characterization of extracellular polymeric substances from acidophilic microbial biofilms. Applied and Environmental Microbiology, 2010, **76**(9): 2916-2922.
https://doi.org/10.1128/AEM.02289-09

[19] Cui JD and Zhang B. Comparison of culture methods on exopolysaccharide production in the submerged culture of Cordyceps militaris and process optimization. Letters in Applied Microbiology, 2011, **52**(2): 123-128.
https://doi.org/10.1111/j.1472-765X.2010.02987.x

[20] Lowry OH, Rosebrough NJ, Farr AL, *et al*. Protein measurement with the Folin phenol reagent. The Journal of Biological Chemistry, 1951, **193**(1): 265-275.

[21] Dubois M, Gilles KA, Hamilton JK, *et al*. Colorimetric method for determination of sugars and related substances. Analytical Chemistry, 1956, **28**(3): 350-356.
https://doi.org/10.1021/ac60111a017

[22] Dische Z. A new specific color reaction of hexuronic acids. The Journal of Biological Chemistry, 1947, **167**: 189-198.

[23] Muralidharan J and Jayachandran S. Physicochemical analyses of the exopolysaccharides produced by a marine biofouling bacterium, Vibrio alginolyticus. Process Biochemistry, 2003, **38**: 841-847.
https://doi.org/10.1016/S0032-9592(02)00021-3

[24] Lee WY, Park Y, Ahn JK, *et al*. Factors influencing the roduction of endopolysaccharide and exopolysaccharide from Ganoderma applanatum. Enzyme and Microbial Technology, 2007, **40**(2): 249-254.
https://doi.org/10.1016/j.enzmictec.2006.04.009

[25] Poli A, Anzelmo G and Nicolaus B. Bacterial exopolysaccharides from extreme marine habitats: production, characterization and biological activities. Marine Drugs, 2010, **8**(6): 1779-1802.
https://doi.org/10.3390/md8061779

[26] Mangwani N, Shukla SK, Rao TS, *et al*. Calcium-mediated modulation of Pseudomonas mendocina NR802 biofilm influences the phenanthrene degradation. Colloids and Surfaces B: Biointerfaces. 2014, **114**: 301-309.
https://doi.org/10.1016/j.colsurfb.2013.10.003

[27] Shukla SK and Rao TS. Effect of calcium on Staphylococcus aureus biofilm architecture: a confocal laser scanning microscopic study. Colloids and Surfaces B: Biointerfaces. 2013, **103**: 448-454.
https://doi.org/10.1016/j.colsurfb.2012.11.003

[28] Elisashvili VI, Kachlishvili ET and Wasser SP. Carbon and nitrogen source effects on basidiomycetes exopolysaccharide production. Applied Biochemistry and Microbiology, 2009, **45**(5): 531-535.
https://doi.org/10.1134/S0003683809050135

[29] Nichols PD, Henson JM, Guckert JB, *et al*. Fourier transform infrared spectroscopic methods for microbial ecology: analysis of bacteria, bacteri-polymer mixtures and biofilms. Journal of Microbiological Methods, 1985, **4**: 79-94.
https://doi.org/10.1016/0167-7012(85)90023-5

[30] Nyquist RA. Interpreting infrared, Raman, and nuclear magnetic resonance spectra. Academic Press. New York, 2001.

[31] Hsieh C, Tsai MJ, Hsu TH, *et al*. Medium optimization for polysaccharide production of Cordyceps sinensis. Applied Biochemistry and Biotechnology, 2005, **120**(2): 145-157.
https://doi.org/10.1385/ABAB:120:2:145

[32] Dertli E, Mayer MJ and Narbad A. Impact of the exopolysaccharide layer on biofilms adhesion and resistance to stress in Lactobacillus johnsonii FI9785. BMC Microbiology, 2015, **15**: 8.
https://doi.org/10.1186/s12866-015-0347-2

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[33] Laspidou CS and Rittmann BE. Modeling the development of biofilm density including active bacteria, inert biomass, and extracellular polymeric substances. Water Research, 2004, **38**(14-15): 3349-3361. https://doi.org/10.1016/j.watres.2004.04.037

[34] Lion LW, Shuler ML, Hsieh KM, *et al*. Trace metal interactions with microbial biofilms in natural and engineered systems. Critical Reviews in Environmental Science and Technology, 1988, **17**(4): 273-306. https://doi.org/10.1080/10643388809388338

[35] Nwodo UU, Green E and Okoh AI. Bacterial Exopolysaccharides: Functionality and Prospects. International Journal of Molecular Sciences, 2012, **13**, 14002-14015. https://doi.org/10.3390/ijms131114002

[36] Moscovici M. Present and future medical applications of microbial exopolysaccharides. Frontiers in Microbiology, 2015, **6**(1012): 1-11. https://doi.org/10.3389/fmicb.2015.01012

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