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Microbial exopolysaccharides: Effect on corrosion and partial chemical characterization

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Abstract

Test panels of mild steel were deployed in the surface waters of the Dona Paula Bay over a period of 28 days. Biofilm material on mild steel panels was analyzed for biofilm biomass (as organic carbon), exopolysaccharide (EPS) production and corrosion rates. Concentrations of biofilm carbon and EPS increased whereas corrosion rate of mild steel decreased over the period of immersion. Corrosion of mild steel showed significant inverse relationships with biofilm carbon and EPS. This implies that biofilm organic matter inhibits the corrosion of mild steel. A few bacterial cultures isolated from the microfouling material were screened for EPS production. One of the cultures identified as *Vibrio* sp produced the highest amount of exopolysaccharides. Production, isolation and characterization of the exopolysaccharides produced by the bacterium were evaluated. Exopolysaccharide was produced in all the phases of growth and with all the substrates tested. Production of the exopolysaccharide was influenced by source and concentration of carbon and nitrogen as well as concentration of phosphate in the growth medium. Exopolysaccharide is a heteropolysaccharide-containing glucose as the most abundant monosaccharide and mannose, xylose, galactose and fucose as minor sugars. The presence of uronic acid and pyruvate indicates the acidic nature of the polysaccharide

Keywords: Mild steel, corrosion, microorganisms, exopolysaccharides.

1. Introduction

Soon after their immersion, materials immersed in an aquatic environment adsorb dissolved organic matter onto their surfaces thereby conditioning the surface.[1–3] Conditioned surfaces are then colonized by microorganisms including bacteria, diatoms, fungi and protozoa. While colonizing the surface microorganisms may produce exopolysaccharides (EPS) and/or other cellular components.[4–6] Several functions have been ascribed to EPS in the adherent biofilm; however, protection and maintenance appear to be the most important.[5, 7] Furthermore, EPS may also influence the corrosion of metals.[7, 8]

Growth phase and nutrient status of surface-associated bacteria may influence the quality and chemical composition of EPS produced.[9–11] Further, each adherent bacterial species may produce chemically and structurally unique EPS. The EPS are usually acidic heteropolysaccharide. Functional groups (e.g. hydroxyl, carboxyl and phosphoric acid) associated with EPS exhibit a high affinity towards certain metal ions.[12] Calcium ions and the pH of the medium appear to play an important role in the adhesion of EPS to surfaces.[13] The involvement of functional groups of EPS in biofouling and biocorrosion processes has been demonstrated by using surface analytical techniques.[14, 15]

Numerous studies have been carried out on microfouling of surfaces immersed in temperate and tropical waters.[16–22, 41] Despite these studies little is known about synthesis, production and chemical characterization of EPS produced by the fouling microorganisms.[10–11, 23–24]

As part of our ongoing programme on microfouling of surfaces and its effect on the corrosion of metals and alloys immersed in tropical sea water, bacterial isolates were collected. These cultures were purified and identified. In this paper, data on EPS production by the biofilm and the effect of the biofilm EPS on corrosion of mild steel, and isolation and characterization of EPS produced by a bacterium, *Vibrio* sp., are presented and discussed.

2. Experimental

2.1. *Deployment of test panels*

Studies on the corrosion of mild steel were carried out at a station in the Dona Paula Bay during October/November, 1995. Mild steel used for the study contains C–0.73, Mn–0.58, Si–0.45, S–0.04, P–0.52 % and Fe the rest. Before use, mild steel panels ($10 \times 15 \times 0.3$ cm) were treated with 20 % HCl, washed with water and dried in an oven, cooled in a desiccator, weighed on a balance and kept in a desiccator until used. Panels were immersed in surface waters (~1 m) of the Dona Paula Bay (15.31°N, 73.59°E), the Arabian Sea. Before immersion replicate panels were fixed vertically on a PVC frame using PVC nuts and bolts. A float was attached at about 1 m above the frame. Due to their weight and attached float, panels remained suspended at about 1 m depth. Panels were deployed in November 1995 and were retrieved after 24 hours and thereafter at weekly intervals over a four-week period.

2.2. *Corrosion rate*

After retrieval, the corrosion products were removed using a stainless-steel knife. They were dried in an oven, powdered in a pestle and mortar, transferred to acid-cleaned vials and stored at 4°C until analysis. After the removal of corrosion, product panels were cleaned and reweighed as above to estimate weight loss. The corrosion rates (mg. Dm^{-2} d^{-1}) were than calculated using the formula:

$$C = W1 - W2/At$$

where C is the corrosion rate (mg. dm^{-2} d^{-1}), $W1$ and $W2$ are weights in gram of mild steel panels before and after immersion, A is the area of the panels (cm^2) and t the duration of immersion in days. Precision of the method based on six replicates was ± 7.67 %.

2.3. *Biofilm biomass and exopolysaccharide analysis*

In order to estimate biofilm biomass, a known amount of the corrosion products was analyzed for organic carbon following the method of Parsons *et al.*[25] Precision of the analytical method based on six replicates was ± 8 %. Exopolysaccharides associated with the corrosion products of mild steel were extracted using 10-mM EDTA and were quantified using phenol–sulphuric acid method.[26] D-glucose was used as a standard. Precision of the analytical method based on replicate samples was ± 10 %.

2.4. *Isolation of bacteria*

In order to isolate bacterial cultures, corrosion products were removed using a brush and sterile sea water. Samples were serially diluted. A known aliquot was plated onto ZoBell marine agar plates. The plates were incubated at room temperature (28 ± 2°C) for 24 hours. Colonies were randomly selected, purified and stored on ZoBell marine agar slants. Eight cultures from these were randomly selected to assess their exopolysaccharide production. These strains were tentatively identified using standard taxonomic methods.[27]

2.5. *Culture conditions*

Bacterial cultures for EPS production were grown in a basalt salt solution (BSS) containing (g/l); NaCl, 30.0; KCl, 0.75; $MgSO_4$. $7H_2O$, 7.0; NH_4Cl, 1.0; K_2HPO_4, 0.7; KH_2PO_4, 0.3; glucose, 10.0 and 1 ml of traced metal solution.[28] Carbon and nitrogen sources and concentrations, and the concentrations of phosphate were varied as required. pH of the medium was adjusted to 7.5 with 1N NaOH. The medium was sterilized by autoclaving for 20 min at 121°C and was inoculated with 2% (V/V) of an 18-h-old culture grown in the same medium at room temperature on a rotary shaker at 150 rpm.

2.6. *Assessment of EPS production*

Bacterial cultures were grown in the BSS medium wherein glucose was replaced by trisodium citrate (5.0 g/l) for 48 hours. Cells were removed by centrifugation at 6,000 rpm at 4°C for 15 min and discarded. 1 ml supernatant in replicate was used to assess the production of EPS. The EPS was estimated by the phenol–sulphuric acid method as above. Of the cultures examined, the culture SS–21D/8 which was tentatively identified as *Vibrio* sp. produced the highest amount of EPS. This culture was used for further work as described below.

2.7. *Effect of carbon source and concentration on EPS production by Vibrio sp.*

The effect of carbon source on the production of EPS was studied using BSS medium supplemented with 1% of either fructose, galactose, glucose, maltose, sucrose and xylose and 0.5% of citrate as the carbon and energy source. Sucrose concentration in the growth medium was varied from 0.25 to 3% in order to assess the effect of carbon concentrations on EPS production by *Vibrio* sp. After 8 days, cells were removed by centrifugation at 6,000 rpm for 15 min at 4°C. The supernatant was passed through 0.2-μm-pore-size filter. Filtrate was dialysed against distilled water at 4°C to eliminate any low molecular weight sugars and salts using dialysis bags (MW cutoff of 8,000). The dialysed supernatant was again concentrated using rotary vacuum evaporator at 40°C and adjusted to a known volume. A known aliquot was used to estimate EPS by the phenol sulphuric acid method.

2.8. *Effect of nitrogen source and concentration on EPS production*

The BSS growth medium containing 1% sucrose and 0.013% of nitrogen either as ammonium chloride, ammonium sulphate, sodium nitrate or urea was used to assess the effect of nitrogen source on EPS production. The nitrogen (as ammonium sulphate) concentration in the growth medium was varied from 0.001 to 0.1% to assess the effect of nitrogen concentration on the

exopolysaccharide production. The growth medium was inoculated with the 18-h-old *Vibrio* sp. culture grown in the same medium. The culture was grown at room temperature for 8 days on rotary shaker at 150 rpm. Exopolysaccharide was estimated following the method described as above.

2.9. *Effect of phosphate concentration on EPS production*

The BSS medium containing 1% sucrose and 0.013% ammonium sulphate was supplemented with various concentrations of dipotassium hydogen phosphate and potassium dihydrogen phosphate (0.25 to 2.0 μg/ml) to assess the effect of phosphate concentration on the EPS production. The culture was grown and the EPS was estimated as above.

2.10. *Growth and EPS production by Vibrio sp.*

BSS containing 1% sucrose, 0.05% ammonium sulphate (0.006% N) and 1 μg/ml of phosphate was used as a growth medium to monitor the growth of EPS production by *Vibrio* sp. Growth was monitored at room temperature at 150 rpm. At regular intervals, 5-ml aliquots were removed for turbidity measurement (A 540 nm). Samples were centrifuged (6,000 rpm for 15 min) and 1-ml supernatant was removed and dialyzed. A suitable aliquot was used to estimate EPS concentration using phenol sulphuric acid method. The experiment was carried out until the culture reached the stationary growth phase.

2.11. *Isolation of EPS*

Batch culture (2.0 l) was grown in 5-l conical flask using BSS medium containing sucrose (2% W/V), 0.006% nitrogen as ammonium sulphate and 1 μg/ml of phosphate to the stationary phase (72 hours). The cells were removed and the supernatant filtered, concentrated by ultrafilteration using an ultrafilter (MW cutoff of 10,000), dialyzed and concentrated as above. The EPS was precipitated using isopropanol and kept overnight at 4°C. The precipitate was collected by centrifugation at 6,000 rpm for 30 min. The precipitated EPS was dried at 50°C and was used for chemical characterization.

2.12. *Characterization of EPS*

Total carbohydrate was estimated by the phenol sulphuric acid method.[26] Protein was analysed using the method of Smith *et al.*[29] Pyruvate was determined by the method of Slonecker and Orentas.[30] Uronic acids were estimated by the method of Filisetti-Cozzi and Carpita.[31] The FTIR adsorbtion spectra were obtained on Shimadzu spectrophotometer using KBr techniques.

In order to determine the monosaccharide composition of the EPS, a known quantity of the polysaccharide was hydrolyzed with 2N HCl for 2 hours at 100°C in ampoules flushed with nitrogen before sealing. After hydrolysis the solution was evaporated to dryness under reduced pressure at 40°C. The hydrolysate was dissolved in water and passed through a Dowex 50 W-X 8 (H^+ form, 100 mesh, Sigma) using distilled water as an eluant to isolate neutral sugars. Neutral sugars were converted to their alditol acetates and were analyzed by capillary gas chromatography as described earlier with slight modification.[21] A capillary gas chromatograph

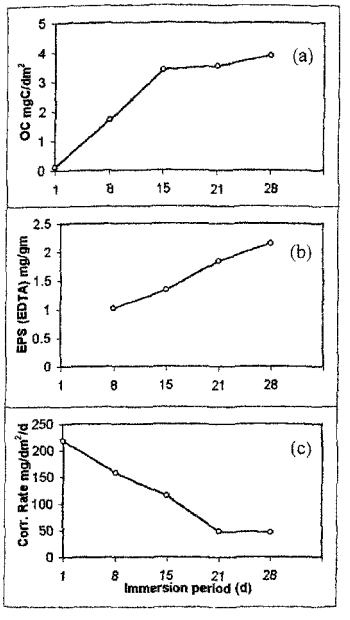

FIG. 1. Changes in the biofilm organic carbon (a) and EPS (b) associated with corrosion products and corrosion rate (c) of mild steel.

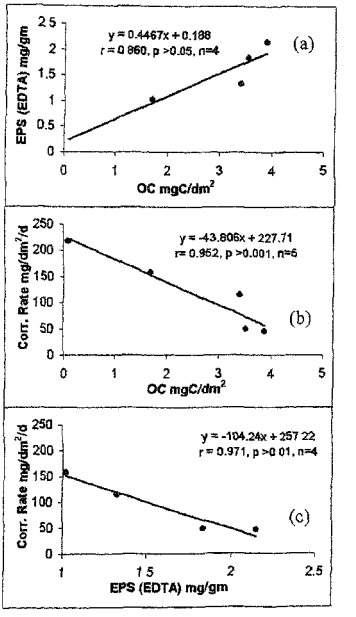

FIG. 2. Linear correlation coeffiient (r) between EPS and organic carbon (a), corrosion rate and organic carbon (b), and corrosion rate and EPS (c).

(Chrompack model CP-9002) equipped with a fused silica capillary column coated with CP Sil–88 (25 m, i.d. = 0.32 mm) and flame ionization detector (FID) was used to separate the alditol acetate mixture. Sample (0.4 μl) was injected using an on-column injector when the initial temperature was 70°C. The oven temperature was then rapidly raised to 150°C and further to 230°C at 3°C/min and maintained at this temperature for 15 min. Quantification of the component was achieved by peak area integration of the GC results using data-handling system installed in the instrument.

3. Results

3.1. *Biofilm biomass, EPS and corrosion of mild steel*

Biofilm biomass (as organic carbon) on the test panels increased over the period of immersion (Fig. 1a). A similar trend was evident for the concentrations of EPS associated with the corro-

sion products of mild steel (Fig. 1b). Corrosion rate of mild steel generally decreased with increasing immersion period (Fig. 1c). Concentration of EPS and organic carbon showed significant positive relationship (Fig. 2a). Interestingly, corrosion rate of mild steel showed significant inverse relationships with both organic carbon (Fig. 2b) and the EPS (Fig. 2c).

3.2. *EPS production and characterization of the culture*

Eight cultures were randomly selected and screened for EPS production (Table I). All the cultures produced EPS; however, production was the highest with culture SS-21D/8. Therefore, this culture was identified and used for further studies. The culture was oxidase-negative, catalase positive, Gram negative, and facultatively anaerobic in nature. It was motile and reduced nitrate to nitrite (Table II). The utilization of maltose, glucose, glycerol, fructose and mannitol was observed. Based on these characteristics the culture was tentatively identified as *Vibrio sp.*

3.3. *Factors influencing the EPS production*

Various factors influencing EPS production by the culture of *Vibrio sp.* were assessed. Of the various carbon sources used sucrose produced the highest amount of EPS (Table III). The production of EPS increased with increase in sucrose concentration (Table IV). Of the various nitrogen sources used, ammonium sulphate, when used at 0.006%, produced the highest quantities of EPS (Table IV). When the growth medium was supplemented with 1 μg/ml of phosphate, the EPS production was the highest (Table IV).

Table I
Screening of biofilm bacteria for EPS production

Bacterial cultures	OD(540 nm)	EPS (mg/l)
MS-15D/10	0.017	5 11
SS-1D/11	0.023	6.53
SS-8D/11	0.056	7.67
SS-8D/13	0 054	6 53
SS-21-D/8	0 588	38.62
SS-28D/12	0.123	5.54
SS-8D/18	0.089	6.96

Table III
Effect of carbon source on EPS production by *Vibrio* sp.

Carbon source	OD(540nm)	EPS (mg/l)
Citrate	2.016	36.82
Glucose	1.291	27.86
Fructose	1.239	32.34
Sucrose	1.285	105.97
Maltose	1.342	48.76
Xylose	1.214	33.83
Galactose	1.096	28.86

Table II
Biochemical characteristics of the culture SS-21D/8

Test	Result
Grams stain	-ve
Motility	motile
Oxidase	–
Catalase	+
Voges Proskauer	–
Methyl red	–
Nitrate reduction	+
Indole test	–
Thornley's	–
Simmon's citrate	+
Hugh Leifson	+
Christensen's urea	–
Ammonia from peptone	+
Starch hydrolysis	+
Gelatin hydrolysis	+
Casein hydrolysis	+
Arginase	–
H_2S production	–
Growth on Na^+-free BSS	–
Growth on TCBS agar	–

Table IV
Effect of sucrose, nitrogen and phophate concentration on EPS production by *Vibrio* sp.

Chemical	OD (540 nm)	EPS mg/l)
Sucrose (%)		
0 25	1.050	050.40
0.50	1.122	136.80
1.00	0.987	231.12
2.00	1.082	338.40
3.00	1.140	432.00
Nitrogen source		
NH_4Cl	1.040	87.07
$(NH_4)_2SO_4$	1.394	774.15
NH_2CONH_2	2.334	262.69
$NaNO_3$	2.334	102.99
Nitrogen (%)		
0.001	0.691	204.12
0 006	1 123	432.18
0 013	1.077	396.27
0.050	1.599	369.18
0.100	1.484	398 79
Phosphate (ug/ml)		
0.25	1.023	321.12
0 50	0 612	320.40
1.00	1.262	447.12
1.43	1.632	383.04
2.00	2.142	259.92

Table V
Chemical composition of EPS produced by *Vibrio* sp.

Component	µg/mg	%
Sugar	510.901	51.09
Uronic acid	148.893	14 88
Pyruvate	85.618	8 56
Protein	17.932	1.79

Table VI
Monosaccharide composition of the EPS produced by *Vibrio* sp.

Monosaccharide	A	B
Rhamnose	0	0
Fucose	1.1	3.7
Ribose	0	0
Arabinose	0	0
Xylose	3.7	6.6
Mannose	3.9	6 9
Galactose	1.8	3.2
Glucose	42.4	75.9

A = µg/mg EPS; B = % of total

3.4. *Growth and EPS production*

To study the production of EPS, growth curve experiments were performed wherein EPS synthesis was measured during the growth in batch cultures (Fig. 3). The culture showed a characteristic sigmoidal growth curve with a lag phase of about 6 h and reached the stationary phase after 29 h. EPS production was observed at all stages of culture growth. It was higher during the stationary phase of growth, and its concentration did not show any decline during 72 h of growth.

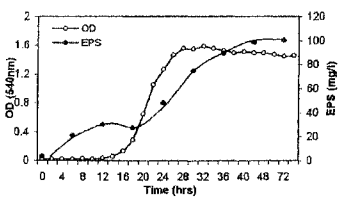

FIG. 3. Growth curve of *Vibrio* sp. and the production of exopolysaccharide (EPS).

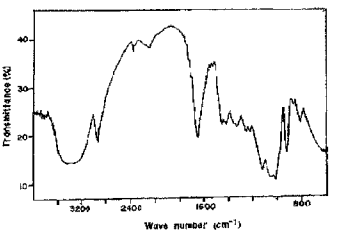

FIG. 4. IR spectrum of exopolysaccharide (EPS).

3.5. *Characterization of EPS*

Carbohydrate, uronic acid, pyruvate and small concentration of protein (1.79%) were the major components of the cold isopropanol-precipitated EPS produced by the culture *Vibrio* sp. (Table V). The polymer was retained by 10,000 Da ultrafilters as well as dialysis bags with MW cut-off of 8,000. The FTIR spectra of the EPS exhibited bands at 2900 cm^{-1}, 1645 cm^{-1} and at 810 cm^{-1} (Fig. 4). Capillary gas chromatography showed that glucose was the major and galactose, mannose, xylose and fucose were the minor components of the total carbohydrates of the EPS (Table VI).

4. Discussion

There was increase in the biofilm biomass measured as organic carbon and decrease in corrosion rates during the period of immersion of mild steel panels in the surface waters of the Dona Paula Bay. With increasing biofilm biomass, the concentrations of EPS also increased over the period of immersion. Biofilm biomass and EPS concentrations showed significant inverse relationships with the corrosion rates of mild steel. While colonizing the surface, microorganisms produce exopolysaccharides which may have direct influence on the corrosion behavior of metals.[32, 33] Biofilm and its exopolysaccharides may provide sites for aeration cells,[34] ion-concentration cells[14] and sites for metal binding.[14, 33] The ability of EPS to bind specific metal ions strongly influences its adhesion to metal surface and its ability to concentrate metal ions from surfaces and bulk media. Binding of metals may be important in both passivation and activation reactions. The observed inverse relationship between EPS and the corrosion rates of mild steel suggests that similar reactions may be occurring in the natural environment leading to the formation of a protective film on the metal surface. Biofilm[33] of a polysaccharide-producing culture *Delya marina* was found to act as a strong corrosion inhibitor with almost complete passivation of mild steel, reducing the corrosion rate by 95%. From this, it is evident that some microorganisms and/or their polysaccharides can act as a strong corrosion inhibitors.

The corrosion of mild steel starts with generation of ferrous ions by anodic oxidation at the surface because of the reaction ($Fe \rightarrow Fe^{+2} + 2\,e^-$) which may undergo further oxidation producing Fe^{+3} species ($Fe^{+2} \rightarrow Fe^{+3} + e^-$). Ferric ions are particularly deleterious for mild steel as they tend to accelerate corrosion by the reaction ($Fe + 2Fe^{3+} \rightarrow 3\,Fe^{2+}$). If ferric ions are immobilized then it may be possible to control the corrosion of mild steel. Some polysaccharides are reported to exhibit strongest stability constant for Fe^{3+} ions.[33] Such a complex may serve as a corrosion inhibitor. The observed inverse relationship between EPS and the corrosion rate of mild steel suggests that such a metal–polysaccharide complex was probably involved in developing a protective film on the metal surface in natural sea water.

Our data suggest that biofilm EPS inhibits the corrosion of mild steel in natural marine waters. All the cultures employed in our study produced EPS although at different levels. Of these cultures, *Vibrio* sp. (isolate SS-21 D/8) produced the highest amount of EPS and therefore was used for the study on production, isolation and chemical characterization of EPS. It is believed that such studies will help in assessing the potential of exopolysaccharides as anticorrosive agents.

When grown in batch culture (2.0 l), using 2% sucrose, 0.006% nitrogen and 1 μg/ml of phosphate, the yield of EPS produced by the culture *Vibrio* sp. was 700 mg. EPS produced by *Vibrio* sp. resembles in many aspects the most common exopolymers of other bacteria. For example, the synthesis of EPS was first detected in early exponential phase and continued at stationary phase as observed in several other bacteria.[5, 35] The production of EPS while growing seems to be an advantage for a bacterium fouling a surface as it may help to increase the strength of adhesive bond and also to build a protective glycocalyx.[5] It was also interesting to note that the level of EPS did not decrease during exponential and stationary phases. This implies that the EPS was not utilized by the organisms as a carbon and nitrogen source and that it served as a structural matrix polymer.

The amount of EPS produced by *Vibrio* sp. was influenced by the source and concentrations of carbon and nitrogen. In contrast, EPS production by some bacteria was unaffected by the source of carbon.[36] The apparent variations were probably due to differences in the uptake and metabolism of the substrates by the organism. Furthermore, some of the metabolic steps involved in EPS synthesis from different carbon sources may also influence the production rates.[37]

The EPS was retained by dialysis bags with MW cutoff 8,000 as well as by the 10,000 Da ultrafilters, indicating its molecular weight being in excess of this value. With the data at hand, it is not known if the EPS was a homogenous sample or a mixture of various components with different lengths or composition or both. Glucose was the most abundant sugar contributing more than 75% of the total carbohydrate of the EPS. Other sugars including mannose, rhamnose, arabinose were also present as minor components. The presence of these sugars suggests that the EPS is a heteropolysaccharide. The occurrence of nonsugar components such as uronic acids and pyruvate suggests the acidic nature of EPS. Acidic heteropolymers have been observed in biofilms[4, 38] and are produced by bacteria isolated from biofilms developed in the natural environments.[5, 10, 24] From these data it appears that acidic heteropolysaccharide are of common occurrence in biofilm developed in various environments.

The physiological factors controlling EPS production have been investigated primarily in marine planktonic bacteria that secrete polysaccharide and in the laboratory cultures. The release of EPS by bacteria is generally low during exponential growth and it accumulates during the stationary phase. For example, secretion of exopolysaccharide by *Pseudomonas putita* and *Pseudomonas fluorescence* increased in stationary cells compared to exponential cells.[5] This agrees well with our findings on the marine fouling bacterium *Vibrio* sp. Environmental conditions, specially availability of nitrogen, affected the carbohydrate and protein content of bacteria.[5] Furthermore, nitrogen starvation may enhance production of carbohydrate. Moreover, the nutrient-deficient cultures may increase their carbohydrate and/or lipid production at the expense of protein. Therefore, higher production of EPS by *Vibrio* sp. during the stationary phase was perhaps associated with the deficiency of nutrients in our growth medium.

Exopolysaccharide produced by the cells of *Vibrio* sp. showed the presence of nonsugar components including uronic acids, pyruvate, sulphate and protein. Although these nonsugar components make up a relatively smaller portion of EPS on a per weight basis, it can be extremely important to the tertiary structure and physical properties of EPS. These components

are most often in the form of residues and side groups on the polysaccharide chains, and contain a variety of carboxyl, amino and sulphate groups. The occurrence of these nonsugar components indicates the acidic nature of the polymer. Acidic polysaccharides have been observed in bacterial biofilm as well.[5] Protein contents of EPS isolated from biofilm organisms vary from 0 to 30%, and the one studied here is relatively low.

The chemical composition of EPS showed the presence of uronic acid which confers an overall negative surface charge and acidic properties to EPS.[39] Such negatively charged surfaces of polysaccharides may play an important role in their metal-complexing capacity. It has been reported that EPS such as alginates which are negatively charged and rich in uronic acids exhibit a high metal-complexing capacity. Further, EPS containing high uronic acid exhibits high copper-binding capacity.[40] If this is so, then the presence of uronic acid in the EPS produced by *Vibrio* sp. may offer some selective ecological advantage to this bacterium as it may be able to grow on surfaces coated with toxic and antifouling compounds such as cuprous oxide. Moreover, selective binding of metal ions by microbial EPS may play an important role in influencing the microbial corrosion of metals. Similarly, sulphated polysaccharides may play an important role in protection, desiccation and cation exchange of the bacterial biofilm.

5. Conclusions

Our data showed that biofilm microorganisms produce EPS, which serve as corrosion inhibitor for mild steel. A number of bacteria isolated from the corrosion products showed potential for EPS production. The isolated polysaccharide appear to be sulphated acidic heteropolysaccharide. Further studies are needed to evaluate the potential of the biofilm exopolysaccharides as anticorrosive agents.

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