Received: 9 June 2022 | Revised: 29 August 2022 | Accepted: 1 October 2022

DOI: 10.1002/jobm.202200352

RESEARCH PAPER

Journal of Basic Microbiology

Characterization of bioemulsifying EPS from *Halobacillus trueperi* MXM-16, a halophilic adhered bacterial isolate from the mangrove ecosystem

Amrita Kharangate-Lad[1] | **Saroj Bhosle**[2]

[1]School of Chemical Sciences, Biochemistry, Goa University, Taleigao-Panaji, Goa, India

[2]Department of Microbiology, Goa University, Taleigao-Panaji, Goa, India

Correspondence
Amrita Kharangate-Lad, School of Chemical Sciences, Biochemistry, Goa University, Taleigao Goa, 403206, India.
Email: amritakharangate@unigoa.ac.in and amritakharangate@gmail.com

Abstract

Exopolymeric substances (EPS) produced by bacterial cells play a crucial role in the interaction of the cells with the surrounding environment. *Halobacillus trueperi* manxer mangrove-16, an adhered bacterial isolate from the mangrove ecosystem was found to produce EPS that was observed by Alcian blue staining and congo red-coomassie blue agar. The EPS of the bacterial isolate exhibited emulsifying properties. Purification of the EPS by dialysis showed an emulsification index of 80% with hexadecane. Qualitative analysis and Fourier's Infrared spectroscopy (FTIR) revealed that the EPS was a glycoprotein in nature. The EPS showed no surface-active properties. Further exploration of the potential of the EPS interaction with metal solutions showed the ability of the bioemulsifier to cause precipitation in the metal solutions and particularly change the color of the Chromium (VI) solution. The scanning electron microscopy-energy-dispersive X-ray spectroscopy (SEM-EDS) of the cells and EPS particularly indicated the interaction of the EPS with the (Fe^0) zerovalent iron nanoparticles and its effect on the cells and EPS of the bacteria. It is therefore concluded that the EPS is a crucial component that anchors the bacteria to particulate matter in the mangrove ecosystem and also plays an important role in interaction with metals and hydrocarbons.

KEYWORDS

bioemulsifier, bioremediation, extracellular polymeric substances (EPS), Fe^0 nanoparticles, glycoprotein, metal-interaction

1 | INTRODUCTION

Mangroves and estuaries are turbid aquatic environments, which are under the constant influence of tides. Since these ecosystems are rich in particulate matter, the bacteria in these ecosystems adhere to it and localize themselves to obtain nutrients and prevent wash-off due to tidal action [1–4]. Attachment to solid surfaces of particulate matter in these aquatic environments occurs using surface appendages and polymers such as pili,

Abbreviations: BSA, bovine serum albumin; DNSA, di-nitro salicylic acid; EPS, extracellular polymeric substances; FTIR, Fourier's Infrared spectroscopy; QODC, qualitative oil drop collapsing test; QODT, qualitative oil displacement test; RP-HPLC, reverse phase high-pressure liquid chromatography; SEM-EDS, scanning electron microscopy-energy-dispersive X-ray spectroscopy; TLC, thin layer chromatography; ZMB, Zobell marine broth.

flagella, capsule, and extracellular polymeric substances (EPS) [5–7]. The interactions between these appendages and the solid surfaces involve chemical and physical bonds such as weak hydrogen bonds, Van der Waals forces of attraction, and ligand–receptor interactions [5, 6]. These interactions also enhance the ability of the cells to acquire metals and compounds present in the surrounding environment and the microorganisms utilize these for their metabolic activities and growth. The importance of EPS in several bacteria from aquatic and terrestrial ecosystems has been well demonstrated and documented. EPS has been studied to be a crucial mechanism involved in cell defense and protection, cell interaction and biofilm formation. Halophilic bacteria known to produce EPS have been studied such as *Halomonas maura*, *Halomonas eurihalina*, *Halomonas ventosa*, *Halomonas anticariensis*, and so on [8, 9]. In bacterial cells, EPS facilitates adhesion to solid surfaces and plays a critical role in the adsorption and immobilization of toxic metals and pollutants in the environment [7]. EPS from many bacteria are found to possess emulsifying properties. EPS from *Pseudomonas* sp. was found to be a biosurfactant that emulsified oil and water by reducing the surface tension between the two phases [10]. Metal chelation by EPS is significant in bioremediation as it involves detoxification of metals such as arsenic, chromium, cadmium, mercury, and copper by bioaccumulation of these metals in their exopolymers. Research studies have demonstrated that the EPS is a complex polymer made up of proteins, carbohydrates, and lipids, DNA and humic acids. Bioemulsifiers are also high molecular weight biopolymers (100,000–230,000 daltons) that are composed of combinations of polysaccharides, proteins, and lipids. Therefore, it is not surprising that the bioemulsifiers form an integral part of the EPS of a microorganism. EPS from a marine bacterium *Pseudoalteromonas* has also been known to exhibit bioemulsifying properties [11]. Though the major component of EPS is polysaccharide, its association with the other macromolecules (proteins and lipids) governs its properties and interactions with the environment [7].

EPS is a fundamental constituent of biofilm. The presence of EPS-producing bacteria in the biofilm community vastly improves the ability of the cells in the biofilm to scavenge for both water and nutrients from the environment when the conditions are limiting [6, 7]. This allows persistent metabolism of the organisms within the biofilm community in atypical conditions.

This study aimed to understand the role of EPS in *Halobacillus trueperi* Manxer mangrove-16 (MXM-16), an adhered halophilic bacteria isolated from the mangrove ecosystem. The bacterial isolate has previously been identified and investigated for its ability to produce pigment, siderophores, and degrade sodium benzoate [12]. Since the bacterial isolate was isolated from particulate matter, it was hypothesized that it would exhibit some adhesion mechanism. The production of EPS by the isolate led us to envisage that the presence of the EPS was crucial to the isolate and aided the isolate in adhesion to the particulate matter in an environment that was dynamic and subject to fluctuations due to tidal influx. Besides adhesion, it was envisaged that the EPS also enhanced the ability of the isolate to interact with the surrounding environment. The EPS was screened for its bioemulsifying properties as emulsification is an important property that is necessary for the acquisition of hydrophobic compounds like hydrocarbons from the environment. The EPS was extracted, purified, analyzed, and characterized qualitatively and quantitatively. This is a novel study that explores the interaction of the EPS from *Halobacillus trueperi* MXM-16 with metals in aqueous solutions. The ability of the EPS to interact with the metal solution also allowed the exploration of the ability of the bacterium to interact with the (Fe^0) zerovalent iron nanoparticles. Since these nanoparticles are the only nanoparticles currently approved for field application it was necessary to understand the effect they could have on the bacteria in the environment [13].

2 | MATERIALS AND METHODS

2.1 | Screening and detection for the production of EPS

The bacterial isolate MXM-16 was identified as *Halobacillus trueperi* MXM-16 and allotted the GenBank accession no KF 379752 [12]. For EPS studies, it was grown on ZMB (Zobell Marine Broth-Himedia) for 24 h at room temperature. The culture was used for Alcian blue staining and Congo red-Coomassie blue agar techniques. For Alcian blue staining, the culture was smeared on clean slides followed by Alcian blue EPS staining protocol [14]. The slides were examined under 40x of light microscope and observed for blue sheath indicating EPS around red bacterial cells. Congo red-coomassie blue agar was used for the detection of EPS. *Halobacillus trueperi* MXM-16 was spot inoculated on congo red-coomassie blue agar [15] and incubated for 24–48 h at room temperature (28°C). The bacterial colonies were observed for pink coloration and smooth glossy appearance indicating the presence of EPS.

2.2 | Determination of bioemulsifying ability of *Halobacillus trueperi* MXM-16

Cell surface hydrophobicity was used to check the bioemulsifying ability of the *Halobacillus trueperi* MXM-16 cells. The isolate was grown overnight on 100 ml of ZMB and pelleted at 8000 rpm/10 min/4°C. The supernatant was used for emulsification index studies. The pellet was resuspended in 50 ml phosphate-buffered saline (PBS) (pH 7.0). The initial absorbance (A0) was measured at 450 nm (Shimadzu UV-2450) for PBS-suspended cells and sterile PBS. For the test, to 2 ml of cell pellet in PBS suspension, 0.5 ml of hexadecane was added. A total of 2 ml PBS and 0.5 ml hexadecane served as control. Both test and control tubes were vortexed (Remi CM101) for 2 min, and allowed to stand undisturbed for 30 min. The final absorbance (A1) was measured in the aqueous layer at the same wavelength. Hydrophobicity was measured as adhesion percentage using the formula $A\% = A0 - A1 \times 100/A0$ [16]. The emulsification index of the supernatant was determined by the E24 method. To 2 ml of the culture supernatant, 2 ml of hexadecane was added. A total of 2 ml of uninoculated sterile ZMB with 2 ml of hexadecane served as control. The tubes were vortexed for 2 min and allowed to stand for 24 h. The emulsification index E24 is given as the percentage of the height of emulsified layer in millimeter divided by the total height of the liquid column [17]. The tests were carried out in triplicates and the graph was plotted with standard deviation.

2.3 | Extraction and partial purification of the EPS

Halobacillus trueperi MXM-16 was grown in 1 L of ZMB for 48 h at 28°C. The cells were pelleted by centrifugation (Eppendorf 5804 R) at 8000 rpm for 20 min at 4°C. The supernatant was transferred to fresh tubes and twice the volume of cold ethanol was added. The mixture was left undisturbed overnight at 4°C. The mixture was centrifuged (Eppendorf 5804 R) at 5000 rpm for 20 min at 4°C and the precipitate was collected. The precipitate was dissolved in deionized distilled water and dialyzed in a dialysis bag with a cut-off value of 12,000 Da against deionized distilled water for 24 h. The dialysate containing the EPS was collected and used for further studies. The emulsification index E24 was measured for the partially purified EPS.

2.4 | Qualitative characterization of the EPS

The partially purified EPS was analyzed for the presence of proteins, sugars, and lipids by the Ninhydrin test, Molisch's test, and Sudan IV test, respectively [14].

2.5 | Characterization of total organic and inorganic content of EPS

A functional EPS is a combination of biological contents and minerals that allows it to function optimally in nature. The ash method was used to determine the total organic and inorganic composition of the EPS. Dry powder of partially purified EPS (1 g) was taken in a previously weighed crucible. The compound was charred in a muffle furnace (Scientech SE-130) at 400°C for 2 h until ash was formed. The weight of the ash formed represented the inorganic component of the compound, while the loss in weight corresponded to the organic content of the EPS.

2.6 | Determination of carbohydrate and protein content

Carbohydrate content was analyzed by phenol sulfuric acid method [18] for total carbohydrates/sugars. For standard 0.1 mg/ml of glucose was used to prepare concentrations ranging from 10 to 100 μg/ml. To estimate the concentration of sugars in an unknown sample, 1 ml of the unknown sample (EPS) was subjected to the phenol-sulfuric acid method. The glucose standard curve was obtained by plotting a graph of Absorbance v/s known concentrations and the concentration of the unknown was deduced from it.

Protein content was analyzed by the Folin–Lowry method [19]. A standard 1 mg/ml solution of bovine serum albumin (BSA) was prepared in distilled water. It was diluted to obtain concentrations of 0–200 μg/ml. To estimate protein in an unknown sample, 1 ml of the unknown sample was subjected to the Folin–Lowry method. The protein standard curve was obtained by plotting a graph of Absorbance v/s known concentrations and the concentration of the unknown was deduced from it.

2.7 | Thin layer chromatography (TLC) of EPS

The EPS was partially hydrolyzed by boiling in 0.5 N NaOH for 30 min followed by neutralization with 0.5 N

HCl. The extract was spotted onto TLC plates (Sd fine-chem aluchrosep silica gel 60/UV 254). The solvent system consisted of n-butanol, distilled water, and acetic acid (12:3:6) for amino acids and sugars. The amino acid composition was visualized by spraying with 0.2% ninhydrin prepared in acetone. Sugars were detected by phenol sulfuric acid reagent.

2.8 | Structural characterization of EPS by Fourier's infrared spectroscopy (FTIR)

The EPS was dried using nitrogen gas until it was completely dry and solid in consistency. Dried EPS was ground with potassium bromide pellets and its infrared spectrum was recorded on an FTIR spectrophotometer (Shimadzu IR Prestige-21) in the 4000–400 cm^{-1} spectral region.

2.9 | Characterization of the sugars by HPLC

Monosaccharides were identified by reversed-phase high-performance liquid chromatography (RP-HPLC) (Agilent Infinity 1260) after precolumn derivation and ultraviolet detection [20]. A total of 200 µl of the EPS was dispensed into a 1.5 ml Eppendorf tube. To this tube, 200 µl each of 0.3 M NaOH and 0.5 M 1-phenyl-3-methyl-5-pyrazolone (PMP) methanol were added while keeping the tube at 70°C for 90 min in a water bath. The solution was neutralized with 200 µl 0.3 M HCl and centrifuged for 5 min. The carbohydrates in the top layer were strained through a 0.45 µm nylon mesh and injected into the C18 column (4.6 mm × 250 mm). The mobile phase used was 0.05 M KH_2PO_4 (pH 6.9) at the flow rate of 1 ml/min, and the column temperature was 30°C. The carbohydrates were identified by comparing them to the reference carbohydrates L-arabinose D-fructose, D-mannose, D-glucose, and D-galactose.

2.10 | Determination of surfactant properties of the EPS

The qualitative oil drop collapsing (QODC) test and qualitative oil displacement test (QODT) were used to determine the surface activity of EPS. In QODC, a drop of oil was placed on a clean grease-free slide. Partially purified EPS was dissolved in deionized distilled water and 10 µl was added as a smaller droplet onto the surface of the larger oil drop. The shape of the oil drop was observed for a minute. A flat drop indicated a positive result for surface activity while a round drop indicated a negative for surface activity [21]. For QODT, a 10 ml mixture of oil-o-red dye in paraffin oil was poured over a petri dish containing 30 ml of distilled water to form a thin red film of oil over water. The EPS was dissolved in deionized distilled water and 10 µl was carefully placed in the center of the film. The appearance of a clear halo within 30 s indicated a positive test for surface activity [22].

2.11 | Interaction of the EPS with metal solutions

A 1 mg/ml solution of the EPS was prepared in deionized distilled water. A total of 0.5 ml of this solution was added to 0.5 ml of 0.2% solutions of cobalt sulfate, cobalt chloride, chromium oxide, ferric chloride, copper sulfate, cadmium chloride, calcium chloride, magnesium sulfate, and manganese sulfate individually and mixed well. The tubes were monitored every 30 min for 2 h for stable precipitation and/or change in color of the metal solution.

2.12 | Interaction of EPS with Fe^0 nanoparticles

Considering the advances in nanotechnology, nanoparticles have found applications in electronics to agriculture and bioremediation. Since Fe^0 nanoparticles are the only nanoparticles approved for field use in water treatment, it was necessary to investigate their impact on bacteria in natural ecosystems. Because mangroves are the ecosystems that connect terrestrial and aquatic ecosystems, nanoparticles are more likely to be washed into this ecosystem by surface runoff from the application site. As a result, the interaction of *Halobacillus trueperi* MXM-16 with Fe^0 nanoparticles piqued our interest. *Halobacillus trueperi* MXM-16 was grown on 100 ml of ZMB with 0.1 g/L of Fe^0 nanoparticles and without Fe^0 nanoparticles under shaking conditions (Remi CIS 24BL) of 150 rpm at 28°C. The cells and the EPS were pelleted and the EPS was precipitated using ethanol and obtained by centrifugation (Eppendorf 5804 R) at 8000 rpm for 20 min. Scanning electron microscopy-energy-dispersive X-ray spectroscopy (SEM-EDS) was performed on the cells and EPS in the Jeol JSM 6360 LV microscope.

3 | RESULTS

3.1 | Screening and detection for the production of EPS

The isolate *Halobacillus trueperi* MXM-16 was positive for the production of EPS Alcian blue staining and

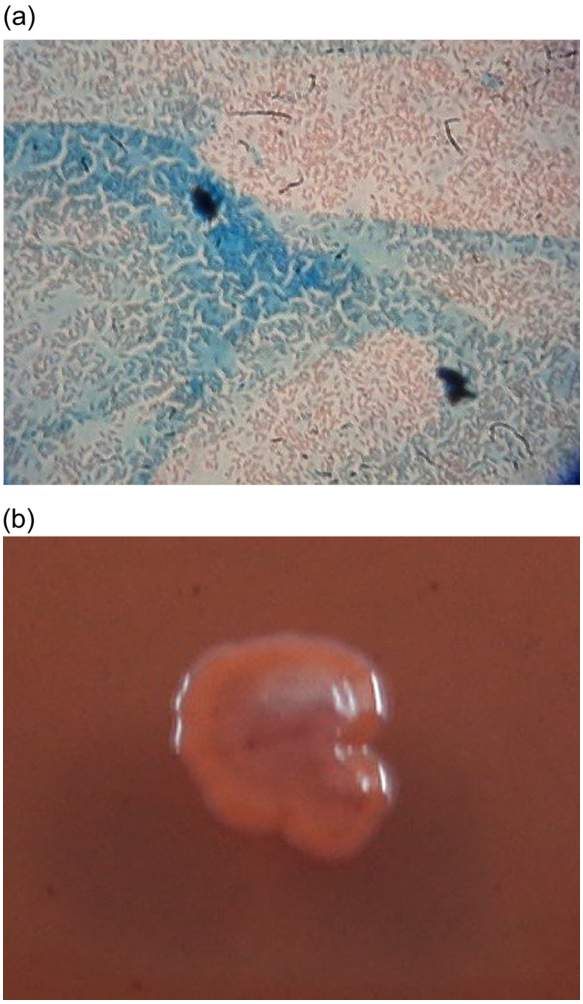

FIGURE 1 (a) Alcian blue staining: *Halobacillus trueperi* manxer mangrove-16 (MXM-16) cells appear red against the blue background of the EPS. (b) Congo red-Coomassie blue agar: Glossy pink colony of *Halobacillus trueperi* MXM-16.

congo-red-coomassie blue agar techniques demonstrated the production of EPS (Figure 1a,b). The Alcian blue staining of the *Halobacillus trueperi* MXM-16 culture suspension showed a blue sheath against red bacterial cells. The Congo red-Coomassie blue agar also showed glossy pink colonies produced by the isolate indicating the presence of EPS.

3.2 | Determination of bioemulsifying ability of *Halobacillus trueperi* MXM-16 cells, supernatant and partially purified EPS

The cell surface hydrophobicity (CSH) of *Halobacillus trueperi* MXM-16 cells was found to be 61% (±0.042) (Figure 2). The emulsification index (E24) of the cell culture supernatant was found to be 57% (±0.025) indicating that the isolate had the capacity to bioemulsify hydrocarbons. The emulsification index E24 of the EPS after dialysis increased to 80% (±0.03).

3.3 | Studies on the bioemulsifier

Like all biological polymers, the EPS is an organic polymer made up of basic elements such as carbon, hydrogen, oxygen, nitrogen, and so on. The EPS from *Halobacillus trueperi* MXM-16 was characterized by qualitative and quantitative analysis. The analysis of organic and inorganic content was carried out by ashing. Ashing is a method of obtaining inorganic residue after the water and organic matter has been removed by heating. The residue is, therefore, the total amount of

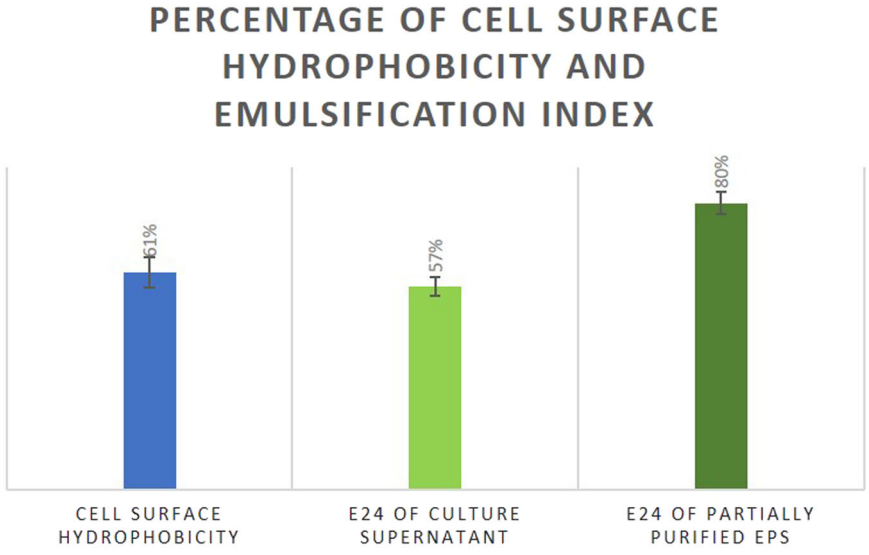

FIGURE 2 Cell surface hydrophobicity of cells and emulsification index of the culture supernatant and partially purified exopolymeric substances (EPS) from *Halobacillus trueperi* manxer mangrove-16 (MXM-16)

minerals in the sample. The ashing of EPS revealed that 86% of the EPS was inorganic and 14% was organic. Thus, 1000 μg/ml (i.e. 1 mg/ml) of the EPS sample contained 860 μg/ml (86%) of inorganic content and 140 μg/ml (14%) of organic content. The inorganic mineral content may include carbon, nitrogen, sulfur, and phosphorous that were part of the glycoprotein EPS, as well as other elements such as iron, magnesium, sodium, potassium, calcium, and other trace metals that play a role in the EPS' functionality. Preliminary qualitative analysis of the EPS showed the ninhydrin test for proteins and the Molisch's test for sugars positive and the Sudan IV test for lipids as negative. Since the qualitative tests for sugar and protein were positive, a quantitative analysis of the two biomolecules was carried out. The total sugar content of the EPS by phenol sulfuric acid method was found to be 40 μg/ml and the total proteins by Folin–Lowry were estimated to be 102 μg/ml. The TLC for amino acids showed spots with Rf 0.72, 0.65, 0.56, 0.27, and 0.12 that corresponded to tryptophan, phenylalanine, tyrosine, serine, and lysine, respectively. The TLC for sugars showed two spots with Rf 0.47 and 0.61 indicating mannose and glucose, respectively. The infrared spectrum (FTIR) (Figure 3) of the EPS showed a symmetric stretch of O–H of polymeric compounds between 3200 and 3320 cm^{-1}. Absorption bands between 1630 and 1660 cm^{-1} were due to the C=O and C–N of amides associated with proteins. Stretching vibrations between 1400 and 1420 cm^{-1} indicated the C=O of carbohydrates. The band between 1060 and 1100 cm^{-1} were characteristic of the O–H fingerprint of polysaccharides and their derivatives. Several bands were visible below 1000 cm^{-1} that indicated the presence of PO_4 and S functional groups in the EPS biomolecule. Based on the qualitative tests and the infrared spectrum it was concluded that the EPS contained proteins and sugars and that it was a glycoprotein in nature. RP-HPLC indicated that the predominant monosaccharide was mannose with a retention time of 6.6 min. Smaller peaks were observed for fructose and glucose with a retention time of 8.42 and 9.30 min, respectively.

3.4 | Determination of surface-active properties of EPS

The EPS of *Halobacillus trueperi* MXM-16 was negative for both the oil displacement and collapsing of the oil drop test. It was observed that upon addition of the bioemulsifier, neither displaced the oil layer nor resulted in the collapse of the oil drop.

3.5 | Interaction with metal solutions

Bioemulsifying EPS from *Bacillus* sp., *Pseudomonas* sp., *Acinetobacter* sp., and *Arthrobacter* sp. have been known to facilitate the degradation of organic pollutants in soil and water [23]. Therefore, it was of interest to see the effect of the EPS on metal solutions. Within 15 min of the addition of the EPS, the metal solutions began to precipitate and the color of the chromium oxide solution

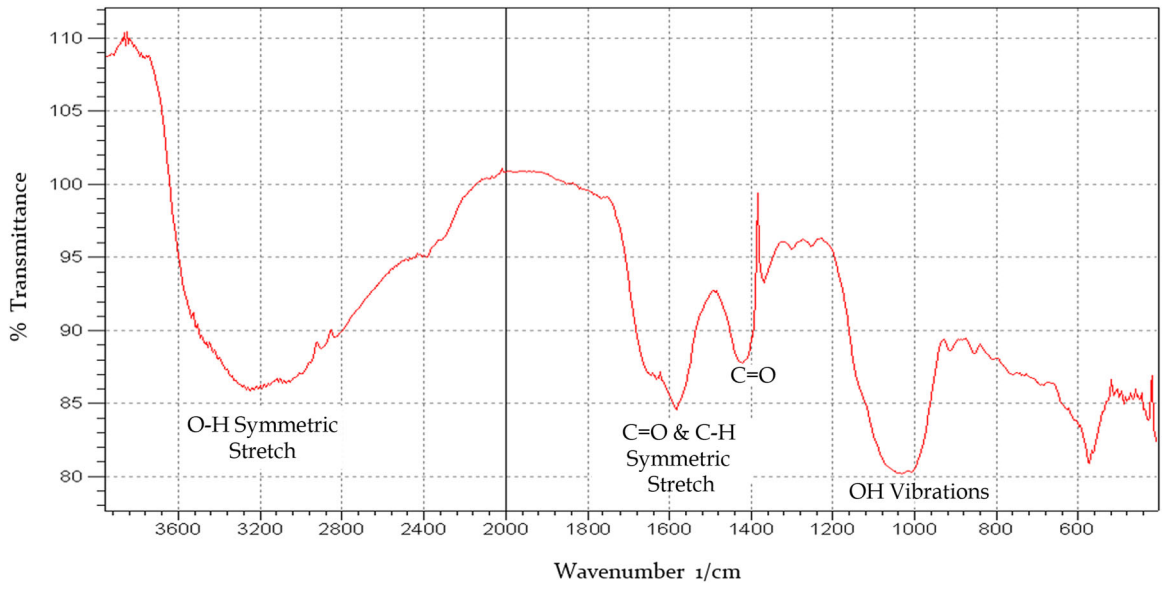

FIGURE 3 Fourier's Infrared spectroscopy (FTIR) spectrum of the bioemulsifier from the adhered bacterial strain *Halobacillus trueperi* manxer mangrove-16 (MXM-16)

changed slightly. The tubes were allowed to stand for 2 h to obtain more stable results on precipitation and color change. All metal solutions showed precipitation at the end of 2 h. The colorless metal solutions showed white precipitate formation. Chromium oxide showed a change in color from orange to brown indicating a reduction of chromium oxide from an oxidation state of +6 (orange color) to +3 (bluish-brown color).

3.6 | Interaction of EPS with Fe^0 nanoparticles

Fe^0 are the only nanoparticles approved for field application. It was interesting to see the interaction of the EPS from *Halobacillus trueperi* MXM-16 with Fe^0 nanoparticles. The SEM showed that the quantity of EPS around the cells was more when the cells were grown in the presence of EPS (Figure 3). The EDS spectra of the EPS showed an increase in the iron content (Figure 4). Interestingly, the bacterial cell surface showed increased carbon content when grown in the presence of Fe0 nanoparticles compared with when the Fe^0 nanoparticles were absent.

4 | DISCUSSION

The mangrove bacterial isolate was found to produce EPS. The pink mucoid colonies indicated the production of EPS. Similar colonies on congo red-coomassie agar have been reported by Narancic and others [15]. It was intriguing to note that the isolate *Halobacillus trueperi* MXM-16 produced EPS in the absence of an inducing agent like a hydrocarbon. This indicated that EPS production was required for the physiological functions of the bacteria, such as adhesion and interaction with the environment for metal acquisition. As a result, the presence of an inducing agent was not a requirement for this isolate to produce an EPS, despite the fact that the EPS is primarily involved in the interaction of the isolate with the hydrocarbon because it extends beyond the cell surface of the isolate.

The production of EPS by *Halobacillus trueperi* MXM-16 was one of the mechanisms that aided the isolate to adhere to particulate matter in the mangrove ecosystem. Other halophilic bacteria such as *Halomonas* sp and *Salipiger mucescens* have been reported to produce EPS which aids the cell in adhesion and interaction with the substrate [8]. Besides adhesion, EPS from microbial cells have also been known to aid in metal tolerance and acquisition of hydrophobic compounds. The cell surface hydrophobicity studies indicated that the bacteria

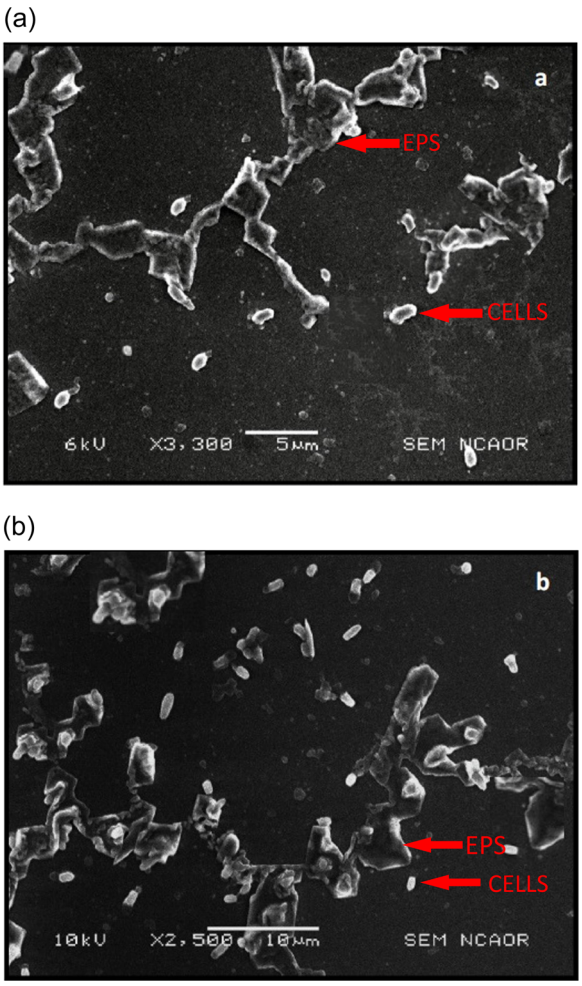

FIGURE 4 Scanning electron microscopy (SEM) images of *Halobacillus trueperi* manxer mangrove-16 (MXM-16) cells and exopolymeric substances (EPS) in the absence (a) and presence (b) of Fe^0 nanoparticles

possessed a hydrophobic cell surface that is beneficial in adhesion, interacting with hydrocarbons and toxic pollutants. Research papers have shown the importance of hydrophobic cell surface in bacteria which aids in the adhesion and interaction of the bacteria with the biotic and abiotic surfaces [24, 25]. The hydrophobic cell surface of the *Halobacillus trueperi* MXM-16 was an important criterion that governed the interactions of the cells in the mangrove ecosystem. The ability of the supernatant and partially purified EPS to emulsify hydrocarbon indicated that the EPS could acquire hydrophobic hydrocarbons from the environment. It was also interesting to see how the bioemulsifying ability of the EPS increased after purification. The standard deviation bars obtained for the supernatant and the purified EPS for the comparison of the emulsification index did not overlap, indicating that the difference in emulsification index for the two samples was significant.

Similar bioemulsifying properties of the EPS from the fungi *Aureobasidium pullulans* RYLF10 have been studied with potential applications in the food industry [26]. Peele [27] reported a marine bacterium *Acinetobacter* sp. which produced an EPS with bioemulsifying and surfactant properties. *Pseudoalteromonas* isolated from the marine environment also produced an EPS that exhibited excellent emulsification of hydrocarbons and food oils [11]. Such emulsifying abilities of bacterial EPS have potential applications in bioremediation and food industries.

The FTIR indicated that the structure contained sugar and protein moieties. Considering that the EPS showed a higher content of protein in comparison to the sugar it was concluded to be a glycoprotein. Similar characterization studies on the EPS from *Enterobacter cancerogenus* revealed that the EPS was composed of sugars and proteins. This EPS also exhibited efficiency in the biosorption of heavy metals such as arsenic, copper, cadmium, and uranium [28]. The identification of sugars by RP-HPLC indicated that mannose was the predominant monosaccharide. However further analysis is required to verify the presence of other monosaccharides and amino acids and their concentration in the EPS. This was consistent with the results obtained for TLC of sugars. Similar observations were reported on EPS of *Bacillus* sp. which revealed that the EPS had a high concentration of mannose [20].

EPS are extracellular polymers composed of various combinations of polysaccharides, lipids, and proteins. The emulsifying properties exhibited by the EPS indicated that the emulsifier was an integral part of the EPS. Bioemulsifiers that are glycoprotein in nature have been reported from microorganisms such as *Salmonella* [29] and *Clavibacter* [30]. Kharangate-Lad and Bhosle [17] reported glycolipid bioemulsifier in *Vibrio* sp. isolated from the saltpans that showed emulsifying and biosurfactant activities with potential applications in oil spill bioremediation. According to the oil displacement and oil drop collapsing tests, EPS did not reduce surface tension between two immiscible phases. Based on the results of the emulsification index, QODC, and QODT tests, it was concluded that the EPS interacted with the hydrocarbon via emulsification and that the EPS lacked the surface-active properties that would have been evident in the QODC and QODT tests, that is, the EPS interacted with the hydrocarbon via emulsification rather than by reducing the surface tension between the two immiscible phases. As a result, it was determined that EPS was a bioemulsifier rather than a biosurfactant. Such surface tension-lowering abilities are critical characteristics of bioemulsifying bacteria like *Pseudomonas* sp., which are being researched for bioremediation of hydrocarbons and oil spills. However, it has been reported that emulsification is more efficient in the remediation of toxic pollutants than surface tension reduction [31, 32].

Interaction with metal solutions showed the ability of the EPS to precipitate metals. EPS being polyanionic have the ability to form complexes with metal cations. This results in the immobilization of the metal in the exopolymer matrix. Similar studies on the precipitation of metals by EPS have been reported by Pohl [33]. Precipitation of metals is a very common technique that is used in the removal of metals during the treatment of wastewater. The EPS, therefore, exhibited an ability to precipitate metals from aqueous solutions, indicating its potential in the treatment of metal-contaminated water. In the case of chromium oxide, it is suggested that the bioemulsifier might serve as a reducing agent capable of donating electrons to chromium oxide for it to be reduced. Bioemulsifiers from *Pseudomonas putida*, *Bacillus subtilis*, *Acinetobacter* sp., and *Actinobacillus* sp. have been studied for their role in the removal of heavy metals such as lead, zinc, and copper from sludge waste from paper industries [34]. Bioremediation using EPS from cyanobacteria and eubacteria has been explored [35, 36].

Earlier studies on *Halobacillus trueperi* MXM-16 have demonstrated that the presence of the Fe^0 nanoparticles influences biomass and EPS (Figure 4) production positively during bacterial cell growth [37]. SEM of the culture suspension showed an increase in the number of cells and the EPS content in the presence of the Fe^0 nanoparticles. Based on this, the SEM-EDS of the EPS and the bacterial cells was carried out that (Figure 5) showed an increase in the content of iron indicating the ability of the bacteria to bioaccumulate the iron using EPS. It is hypothesized that the uptake of iron increased the metabolic activity of the cells which was reflected as an increase in the carbon content of the bacterial cells as observed in EDS of the cells and also led to an increase in biomass of the cells which was noted in the previous study [37]. Similar SEM imaging of cells has been reported in the case of *Pseudomonas aeruginosa* [10] and accumulation of metals by the EPS of bacterial cells has been reported in earlier studies on *Bacillus* sp, *Serratia*, and *Kocuria* [38], *Rhodobium marinum*, and *Rhodobacter sphaeroides* [39]. *Bacillus licheniformis* has exhibited variation in the production of the EPS in the presence of heavy metals such as cadmium, chromium, and mercury. The EPS formation by this bacterium was also associated with a change in the morphology of *Bacillus licheniformis* cells ranging from filamentous to doughnut-shaped cells to resist metal toxicity. Therefore,

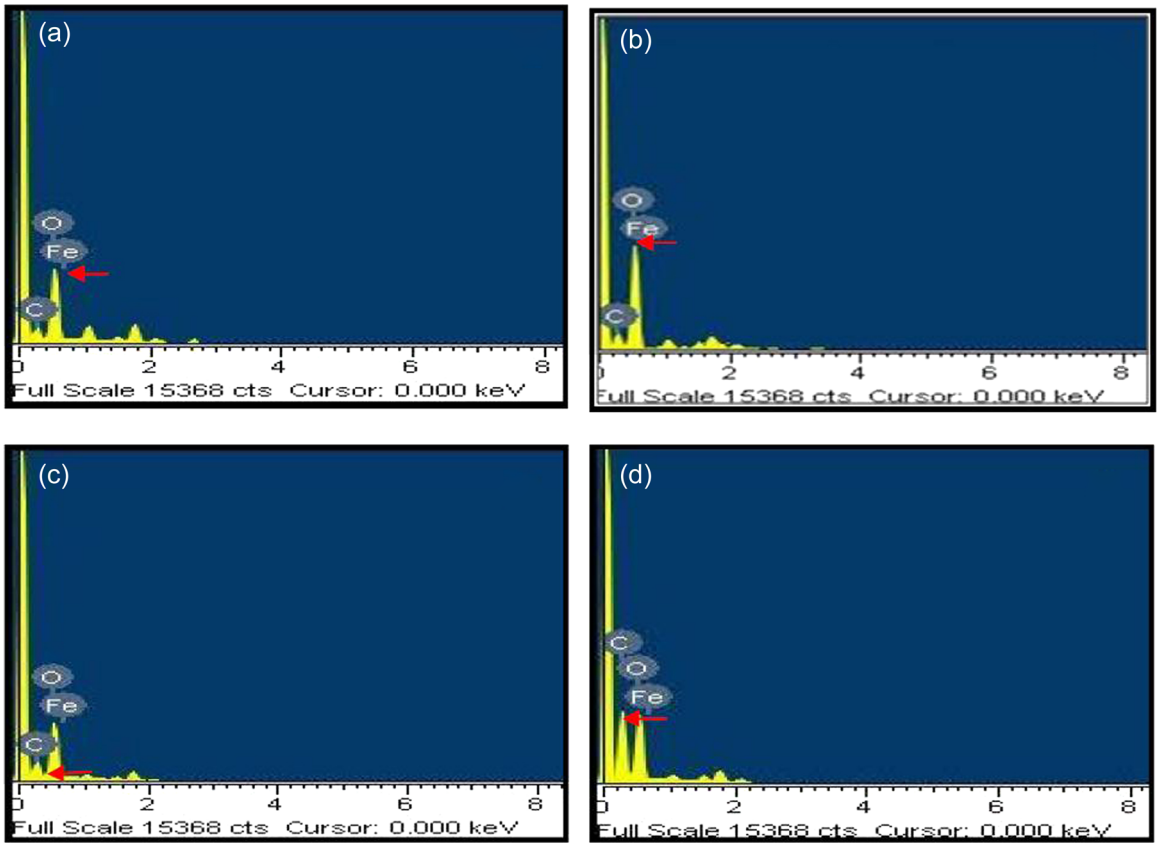

FIGURE 5 Energy-dispersive X-ray spectroscopy (EDS) spectrum of *Halobacillus trueperi* manxer mangrove-16 (MXM-16) EPS without Fe^0 nanoparticles (a) and with Fe^0 nanoparticles (b). EDS spectra of *Halobacillus trueperi* MXM-16 cells without Fe^0 nanoparticles (c) and with Fe^0 nanoparticles (d)

the production of EPS and changes in cell morphology has been identified as important evidence that allows these bacteria to participate in bioremediation [35]. Cyanobacteria like *Gloeothece* sp. have also been reported to resist metal toxicity better when they can form EPS than the mutant non-EPS-producing *Gloeothece* sp. cells [36].

It was interesting to observe that the isolate *Halobacillus trueperi* MXM-16 produced the EPS in the absence of any inducing agent such as hydrocarbon. This indicated that the production of EPS was necessary for the physiological functions of the bacteria such as adhesion and interaction with the environment.

The research work characterized the EPS produced by *Halobacillus trueperi* MXM-16. The characterization of the EPS thus revealed its properties of hydrophobicity, emulsification, and interaction with metals and established the importance of the EPS for the bacteria for survival in the mangrove environment. The increase in the iron and carbon content of the EPS and cells, respectively, in presence of Fe^0 nanoparticles, was evidence of a significant mechanism used by the bacteria in response to the presence of metals in the environment and their bioaccumulation. Apart from bioremediation aspects, the EPS from *Halobacillus trueperi* MXM-16 may also be significant industrially due to its adhesion and bioemulsifying abilities. However, further studies are required to confirm its industrial applicability.

ACKNOWLEDGMENTS

The authors wish to acknowledge Goa University for the studentship provided for Doctoral studies. The authors wish to thank Dr. Indrani Karunasagar, College of Fisheries for the identification studies, Dr. Milind Mohan Naik, School of Biological Sciences and Biotechnology, Goa University and Dr. Rohan Kunkalekar, School of Chemical Sciences, Goa University for the FTIR, Ms. Sahina Gazi, NCPOR Goa for the SEM-EDS studies.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID

Amrita Kharangate-Lad http://orcid.org/0000-0002-6572-4072

REFERENCES

[1] Crump BC, Armbrust EV, Baross JA. Phylogenetic analysis of particle-attached and free-living bacterial communities in the Columbia river, its estuary, and the adjacent coastal ocean. Appl Environ Microbiol. 1999;65:3192–204.

[2] Crump BC, Baross JA. Particle-attached bacteria and heterotrophic plankton associated with the Columbia river estuarine turbidity maxima. Mar Ecol Prog Ser. 1996;1381: 265–73.

[3] Griffith P, Shiah FK, Gloerson K, Ducklow HW, Fletcher M. Activity and distribution of attached bacteria in chesapeake bay. Mar Ecol Prog Ser. 1994;108:1–10.

[4] Gonsalves MJ, Nair S, LokaBharathi PA, Chandramohan D. Abundance and production of particle-associated bacteria and its implication in a mangrove dominated estuary. Aquat Microb Ecol. 2009;57:151–9.

[5] Gottenbos B, Busscher HJ, van der Mei HC, Nieuwenhuis P. Pathogenesis and prevention of biomaterial centered infections. J Mater Sci Mater Med. 2002;13:717–22.

[6] Katsikogianni M, Missirlis YF. Concise review of mechanisms of bacterial adhesion to biomaterials and of techniques used in estimating bacteria-material interactions. Eur Cell Mater. 2004;8:37–57.

[7] Jiao Y, Cody GD, Harding AK, Wilmes PM. Characterization of extracellular polymeric substances from acidophilic microbial biofilms. Appl Environ Microbiol. 2010;76:2916–22.

[8] Martinez-Canovas MJ, Bejar V, Martinez-Checa F, Quesada E. *Halomonas anticariensis* sp. nov.; From Feunte de Piedra, a saline wetland wildfowl reserve in Malaga, Southern Spain. Int J Syst Evol Microbiol. 2004;54:1329–32.

[9] Mata JA, Béjar V, Llamas I, Arias S, Bressollier P, Tallon R, et al. Exopolysaccharides produced by the recently described halophilic bacteria *Halomonas ventosae* and *Halomonas anticariensis*. Res Microbiol. 2006;157:827–35.

[10] de Souza T, Bhosle S. Implications of benzoate induced alterations in cell morphology and physiology in *Pseudomonas aeruginosa* TMR.13 for potential application in bioremediation and monitoring approaches. J Bioremed Biodegrad. 2012;S1(008):2–6.

[11] Dhanya BE, Prabhu A, Rekha PD. Extraction and characterization of an exopolysaccharide from a marine bacterium. Int Microbiol. 2021;25:285–95.

[12] Kharangate-Lad A, Bhosle S. Studies on siderophore and pigment produced by an adhered bacterial strain *Halobacillus trueperi* MXM-16 from the mangrove ecosystem of goa, India. Indian J Microbiol. 2016;56:461–6.

[13] Lo I, Surampalli R, Lai K. Zero-valent iron reactive materials for hazardous waste and inorganics removal. Reston, VA: ASCE Publications; 2006.

[14] Kharangate A. Halophilic and halotolerant bacteria adhered to particulate matter in the mangrove ecosystem. PhD thesis. Goa University. 2015.

[15] Narancic T, Djokic L, Kenny ST, O'connor KE, Radulovic V, Nikodinovic-Runic J, et al. Metabolic versatility of gram-positive microbial isolates from contaminated river sediments. J Hazard Mat. 2012;215:243–51.

[16] Kharangate-Lad A, Bhosle S. Studies on siderophore producing halophilic and halotolerant bacteria adhered to mangrove plant litter. NeBIO. 2014;5:56–60.

[17] Kharangate-Lad A, Bhosle S. Studies on the bioemulsifier production by a bacterial isolate. J Sci Ind Res. 2014;73:674–9.

[18] Dubois M, Gilles KA, Hamilton JK, Rebers PA, Smith F. Colorimetric method for the determination of sugars and related substances. Anal Chem. 1956;28:350–6.

[19] Lowry OH, Nira J, Rosebrough A, Farr L, Randall RJ. Protein measurement with the folin phenol reagent. J Biol Chem. 1951;193:265–75.

[20] Vidhyalakshmi R, Valli N, Narendra K, Sunkar S. *Bacillus circulans* exopolysaccharide: production, characterization and bioactivities. Int J Biol Macromol. 2016;87:405–14.

[21] Youssef NH, Duncan KE, Nagle DP, Savage KN, Knapp RM, McInerney MJ. Comparison of methods to detect biosurfactant production by diverse microorganisms. Appl Environ Microbiol. 2004;70:3152–7.

[22] Morikawa M, Daido H, Takao T, Murata S, Shimonishi Y, Imanaka T. A new lipopeptide biosurfactant produced by *arthrobacter* sp strain MIS38. J Bacteriol. 1993;175:6459–66.

[23] Sinha S, Paul D. Detoxification of heavy metals by biosurfactants. bull environ. Sci Res. 2012;1:1–3.

[24] van Loosdrecht MC, Lyklema J, Norde W, Schraa G, Zehnder AJ. The role of bacterial cell wall hydrophobicity in adhesion. Appl Environ Microbiol. 1987;53:1893–7.

[25] Rozgonyi F, Ljungh Å, Mamo W, Hjertén S, Wadström T. Bacterial cell-surface hydrophobicity. In: Wadström T, Eliasson I, Older I, Ljungh Å Editors. Pathogenesis of wound and biomaterial-associated infections. London: Springer; 1990.

[26] Yadav KL, Rahi DK, Soni SK. Bioemulsifying potential of exopolysaccharide produced by an indigenous species of *Aureobasidium pullulans* RYLF10. PeerJ PrePrints. 2014; 2:e726v1.

[27] Peele KA, Ch VR, Kodali VP. Emulsifying activity of a biosurfactant produced by a marine extracellular bacterium. 3Biotech. 2016;6:177.

[28] Dhanya BE, Athmika, Rekha PD. Characterization of an exopolysaccharide produced by *enterobacter* sp. YU16-RN5 and its potential to alleviate cadmium induced cytotoxicity in vitro. 3Biotech. 2021;11:491.

[29] Gong AS, Bolster CH, Benavides M, Walker SL. Extraction and analysis of extracellular polymeric substances: comparison of methods and polymeric substance levels in *Salmonella pullorum* SA 1685. Environ Eng Sci. 2009;26:1523–33.

[30] van A, Neal K, McMillan BD, Dryden P. The multicomponent extracellular polysaccharide of *Clavibacter michiganenense* subsp. *insidiosum*. Phytopathology. 1987;77:496–502.

[31] Prakash B, Irfan M. *Pseudomonas aeruginosa* is present in crude oil contaminated sites of Barmer Region (India). J Bioremed Biodegrad. 2011;2:129.

[32] Moorthi SP, Deecaraman M, Kalaichelva PT. Bioremediation of automobile oil effluent by *Pseudomonas* sp. Adv Biotechnol. 2008;2:34–7.

[33] Pohl A. Removal of heavy metal ions from water and wastewaters by sulfur-containing precipitation agents. Water Air Soil Pollut. 2020;231:503.

[34] Hidayati N, Surtiningsih T. Ni'matuzahroh. removal of heavy metals pb, zn and cu from sludge waste of paper industries using biosurfactant. J Bioremed Biodegr. 2014;5:255.
[35] Sunilkumar CR, Stephen-Victor E, Naripogu KB, Samanth Kumar J, Nuthan BR, Marulasiddaswamy KM, et al. Differential multi-cellularity is required for the adaptation for *Bacillus licheniformis* to withstand heavy metals toxicity. Indian J Microbiol. 2021;61:524–9.
[36] Pereira S, Micheletti E, Zille A, Santos A, Moradas-Ferreira P, Tamagnini P, et al. Using extracellular polymeric substances (EPS)-producing cyanobacteria for the bioremediation of heavy metals: do cations compete for the EPS functional groups and also accumulate inside the cell? Microbiology. 2011;157:451–8.
[37] Kharangate-Lad A, Pereira F, Fernandes JB, Bhosle S. Studies on the effects of zerovalent iron nanoparticles on bacteria from the mangrove ecosystem. Environ Sci Pollut Res. 2015;23:927–31.
[38] François F, Lombard C, Guigner JM, Soreau P, Brian-Jaisson F, Martino G, et al. Isolation and characterization of environmental bacteria capable of extracellular biosorption of mercury. Appl Environ Microbiol. 2012;78:1097–106.
[39] Panwichian S, Kantachote D, Wittayaweerasak B, Mallavarapu M. Removal of heavy metals by exopolymeric substances produced by resistant purple nonsulfur bacteria isolated from contaminated shrimp ponds. Electron J Biotechnol. 2011;14:2.

How to cite this article: Kharangate-Lad A, Bhosle S. Characterization of bioemulsifying EPS from *Halobacillus trueperi* MXM-16, a halophilic adhered bacterial isolate from the mangrove ecosystem. J Basic Microbiol. 2022;1–11. https://doi.org/10.1002/jobm.202200352