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RESEARCH PAPER

Effect of biosurfactant derived from *Vibrio natriegens* MK3 against *Vibrio harveyi* biofilm and virulence

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Abstract

Vibrio harveyi is a marine luminous pathogen, which causes biofilm-mediated infections, pressures the search for an innovative alternate approach to strive against vibriosis in aquaculture. This study anticipated to explore the effect of glycolipid biosurfactant as an antipathogenic against *V. harveyi* to control vibriosis. In this study, 27 bacterial strains were isolated from marine soil sediments. Out of these, 11 strains exhibited surfactant activity and the strain MK3 showed high emulsification index. The potent strain was identified as *Vibrio natriegens* and named as *V. natriegens* MK3. The extracted biosurfactant was purified using high-performance liquid chromatography and it was efficient to decrease the surface tension of the growth medium up to 21 mN/m. The functional group and composition of the biosurfactant were determined by Fourier-transform infrared spectroscopy and nuclear magnetic resonance spectroscopy spectral studies and the nature of the biosurfactant was identified as glycolipid. The surfactant was capable of reducing the biofilm formation, bioluminescence, extracellular polysaccharide synthesis, and quorum sensing in marine shrimp pathogen *V. harveyi*. The antagonistic effect of biosurfactant was evaluated against *V.* harveyi-infected brine shrimp *Artemia salina*. This study reveals that biosurfactant can be considered for the management of biofilm-related aquatic infections.

KEYWORDS

biosurfactant, *Vibrio harveyi*, biofilm disruption, brine shrimp, oil dispersant

1 | INTRODUCTION

Trade focus on high-value marine fish species is rising and early-life diseases of marine organisms are a major barrier to the growth of effective production in aquaculture. Diseases with opportunistic pathogens of the Vibrionaceae family, which subsist well in nutrient-elevated levels in dense cultures, seem to be the most prevalent and detrimental [1]. The noticeable fish pathogen in the family of Vibrionaceae is *Vibrio harveyi*, has been extensively documented as a serious infection causing pathogenic agent, which leads to the infections of crustaceans, molluscs, and fish, leading to high death rates and substantial economic losses [2]. Regarding its role as a severe marine animal pathogen, *V. harveyi*'s pathogenicity strategies are still to be completely addressed. Phenotypes that were found to be regulated by the *V. harveyi* quorum-sensing (QS) system in vitro,

comprise of biofilm formation and the production of several virulence factors such as a type III secretion system, extracellular polymeric substances (EPS), which are vital for existence and adaptation, internal and external, of its marine hosts.

Apart from disease-causing, deprived environmental factors, oil pollutants, adverse onsite conditions, which ultimately leads to the disease outbreaks in marine fish farms. Still, no remediation tool has been produced, which can have the capacity to overcome both oil contamination and aquatic disease emergence [3].

Biosurfactants are the surface active and structurally varied group of biological molecules synthesized by microorganisms. Except for the application of biosurfactant in environmental protection management and crude oil recovery, they also have acted as a plausible tool in healthcare and food production trades [4]. They offer a number of benefits above synthetic surfactants, such as negligible toxicity, environmental compatibility, biodegradability due to the modest chemical arrangement, and competence under extreme environment.

Diverse microbial classes such as yeast, filamentous fungi, and bacteria are able to synthesize surfactant through the utilization of constituents such as carbohydrates, hydrocarbons, oils and fats, industrial and agricultural residues or their mixture. Most of the biosurfactants are reported from bacterial species such as *Pseudomonas aeruginosa, Bacillus subtilis*, *Bacillus licheniformis*, *Acinetobacter calcoaceticus*, *Sphingobacterium detergens*, yeast species like *Candida lipolytica*, and marine bacterial species such as *Brevibacterium aureum* [4–7].

But recently, extensive attention has been drawn on biosurfactants of moderately halophilic bacteria and their biotechnological capabilities. Halophilic biosurfactants have been shown to exhibit substantially diverse properties, among which the resistance for elevated salinity with the presence of 1–4 M salt, for its activity and stability is the most critical attribute [8,9]. Although halophiles can possess useful constituents including enzymes and antimicrobial substances many of these have not yet been investigated or applied [10]. Therefore, using the surfactant with both oil dislodging property and antimicrobial potential will become an effective tool to battle against bacterial infections mainly vibriosis and to overcome oil contamination in the aquatic environment. However, reports on marine biosurfactant for oil recovery and aquatic microbial disease control are very few [11].

Therefore, it is important to screen marine microbial systems for their capacity to synthesize biosurfactants. This will give another concoction to surfactants that can be utilized to fight against rising marine bacterial diseases and oil contamination. Henceforth, in the present investigation, we explored the capability of the marine halophilic bacterial biosurfactant against marine bacterial pathogen *V. harveyi*. The efficiency of the biosurfactant as an oil spill dispersant was evaluated by using the shake flask experiment. Further, the toxicity and efficiency of the biosurfactant were evaluated with *in vivo* survival trials in marine microalgae and *Artemia salina*.

2 | MATERIALS AND METHODS

2.1 | Isolation, screening, and identification of marine bacteria

Submarine sandy sediments were collected around Fort Kochi (Arabian sea) at a depth of 10^3–10^6 m and the samples were serially diluted. The diluted samples were inoculated on halophilic alkaline peptone broth (1% bacteriological peptone with 2 M NaCl) with pH 8.5 [12,13]. After incubation at 37°C for 6 to 18 h, 0.1 ml of dilutions were inoculated and spread on Zobell marine agar medium and incubated for 12–48 h. Morphologically different colonies were selected and the strains were further isolated with repeated streaking. After obtaining pure halotolerant strains, drop collapse test and the oil-spreading test were employed to screen for the biosurfactant production. Tributyrin hydrolysis was used to determine the lipase production [14,15]. For molecular level identification of the potent strain, the genomic DNA was extracted by CTAB-NaCl method [16]. PCR amplification was done with the set of primers such as 27 F (5′ AGAGTTTGATCGTGGCTCAG 3′) and 1493 R (5′ CGGTTACCTTGTTACGACTT 3′) [17]. The amplicon obtained was purified and sequenced by dideoxy chain termination sequencing. The nucleotide sequence was correlated with the available 16S sequences in the nucleotide search database with the BLAST program [18]. The correlated sequences were aligned by clustalW and the distance matrices were calculated with high-scored sequences. Mega 6.0.6 software was adopted to construct the phylogenetic tree for the molecular level identification [19].

2.2 | Emulsification activity and surface tension measurements

For the determination of the emulsification index (EI_{24}%), oil hydrocarbon was added to cell-free broth in a ratio of 1:1 and vortexed for 5 min. Subsequently, incubating at room temperature for 24 h, emulsification activity was estimated with the measurement of the

emulsified layer. Emulsification index was calculated using the following formula [20]:

$$\text{Emulsification activity } (EI_{24}\%) = (\text{Height of the emulsified layer} / \text{Total height of the mixture}) \times 100.$$

After calculation of the emulsification index, % emulsion stability was assessed for crude oil, petrol, diesel, motor oil, and kerosene up to 60 h of incubation. The surface tension of the biosurfactant was determined using a stalagmometer. The values represent the average of three independent measurements done at room temperature (25°C). The calculations were made as per the following formula:

$$\gamma_2 = \gamma_1 \, n_1\rho_1/n_2\rho_2,$$

where γ_1 is the surface tension of water (72 dynes/cm), γ_2 is the surface tension of the biosurfactant MK3, n_1 is the number of drops of water, n_2 is the number of drops of biosurfactant MK3, ρ_1 is the density of water, and ρ_2 is the density of biosurfactant.

2.3 | Purification and characterization of the biosurfactant

Vibrio natriegens MK3 was inoculated into 2.5 L of M9 medium and incubated for 24 h (34°C, 160 rpm). The resultant supernatant was purified with acid precipitation followed by the solvent extraction [21]. Cell-free supernatant was acidified with 6 N HCl and refrigerated for 24 h at 4°C. The precipitate was obtained by centrifugation at 13,000 rpm for 20 min and extracted with a solvent mixture of ethyl acetate, diethyl ether, and dichloromethane in the ratio of 3:2:1. The extract was vacuum dried with a rotary vacuum evaporator (Yamato DC 400) and taken as crude biosurfactant. To further refine the surface active molecule, high-performance liquid chromatography (HPLC) was performed with a C_{18} reverse phase column under isocratic mode. The fractions were eluted with solvent combinations of 0.1% trifluoroacetic acid in 80% acetonitrile with the flow rate of 1.0 ml/min. Fractions showing oil-displacement activity were combined, concentrated, and lyophilized. The infrared (IR) spectrum was documented with 4/cm resolution yielding IR traces over the range of 250–4500/cm. For the NMR analysis, the active fraction from HPLC was dissolved in deuterated chloroform and the spectral data was recorded with 5.9 μs pulse duration and 2.6 s acquisition time in a Bruker DRX400 spectrophotometer at 27°C [22].

2.4 | Determination of ionic charge

The agar double-diffusion method was adapted to determine the ionic charge of the biosurfactant [23]. The ζ potential of the biosurfactant was measured to determine the surface charge density. For measurement of ζ potential, 1 mg of biosurfactant was suspended in 1 ml of water and the measurements were taken with the Delsa™ Nano Particle Size and Zeta Potential analyzer Version 2.21/2.03 (Beckman–Coulter Instruments) in the dynamic light-scattering mode.

2.5 | Antagonistic effect of biosurfactant MK3 against *V. harveyi*

2.5.1 | Biofilm disruption assay

The influence of biosurfactant on biofilm formation in *V. harveyi* was evaluated in a high-content screening system (HCS; Operetta; Perkin Elmer). The biofilms were formed in HCS plates (Vision Plate™ 384-wells black sterile plate; Perkin Elmer) with 100 μl of the sterile brain–heart infusion medium for 48 h. After the incubation period, the leftover medium was discarded, washed twice with sterile phosphate buffer (50 mM, pH 7.0) without disturbing the adherent cells and treated with 20 μg of biosurfactant. Finally, the biofilms were stained with 15 μl acridine orange (5 μg/ml; Sigma-Aldrich). The biofilm architecture was evaluated using an HCS System [24]. The inhibition of both biofilm biomass and architecture was evaluated through crystal violet assay in microtitre plates [25]. *V. harveyi* biofilms were developed on presterilized glass surfaces placed in wells of a six-well microtiter plate with 3 mL of the sterile brain–heart infusion medium [26]. One milliliter V. *harveyi* inoculum (3×10^8 cells) was inoculated into each well. The presterilized glass slides (1 × 1 cm) were placed statically in the wells of six-well plates with and without biosurfactant and incubated at 37°C for 48 h. The effect of biosurfactant on the dispersion of preestablished biofilms was monitored by scanning electron micrograph (SEM). Biofilms without surfactant treatment were used as controls.

2.5.2 | Inhibition of luminescence

Inhibition of *V. harveyi* bioluminescence was evaluated by aerobically cultivating the cells in 96-well plates at 30°C [27]. Overnight grown cultures of the *V. harveyi* was diluted with fresh Zobell marine broth with an optical density at 600 nm (OD_{600}) of 0.002 and 10-80 μg of biosurfactant was used as a test. Measurements were carried out for every 1 h up to 24 h. OD_{600} was recorded using a Spectramax Pro 96-well plate reader. The relative

light units (RLU/s) were normalized by dividing the RLU per OD_{600} (RLU/OD_{600}) in Prism 6 (Graph Pad) software [28]. Percentage inhibition was evaluated based on the control and test RLU values.

2.5.3 | Inhibition of EPS production

The EPS was quantified by the total carbohydrate assay [29]. In brief, slide glasses were flooded and grown along with the biosurfactant-treated and -untreated *V. harveyi*. After a 48-h incubation, the glass slides were recovered carefully and eroded with 1% NaCl. An equal volume of 5% equilibrated phenol and five volumes of H_2SO_4 were added with the EPS biofilm suspension. Then, the entire reaction mixture was incubated in the dark for 1 h and the absorbance was measured at 490 nm.

2.5.4 | Inhibition of quorum sensing

As the reticence of a purple-colored pigment violacein, synthesized under the regulation of the QS system by *Chromobacterium violaceum* ATCC 12472, it was adopted to assess the QS inhibition property of biosurfactant [30]. *C. violaceum* ATCC 12472 was cultured in nutrient broth (NB; pH 7 ± 0.2) at 37°C in four-well plates. 0.5 ml of NB medium with and without biosurfactant were added into the wells and inoculated with *C. violaceum* ATCC 12472 and incubated for 3 days at 30°C. The wells were observed for violacein production. One milliliter of culture from all the wells was withdrawn and centrifuged at 12,000 rpm for 20 min. The cell-free supernatant was discarded and the insoluble violacein was pelletized. For the extraction of violacein, the pigment-containing cell pellet was resuspended in 1 ml of dimethyl sulfoxide. After extraction the mixture was vortexed strongly and centrifuged again at 12,000 rpm for 15 min to remove the bacterial cells. The extracted purple pigment violacein was measured spectrophotometrically (U-2800; Hitachi, Japan) at 585 nm.

2.6 | Determination of biosurfactant toxicity

2.6.1 | Toxicity of biosurfactant toward marine microalgae

Marine microalgal sp. *Isochrysis galbana* was obtained from Central Marine Fisheries and Research Institute (CMFRI), Tuticorin, Tamilnadu, India. The stock culture was maintained in Walne medium enriched with natural seawater [31] with the standard laboratory conditions of 23 ± 2°C with the light intensity of $18.75 \pm 2.5\ \mu mol \cdot m^{-2} \cdot s^{-1}$ under 16:8 h light/dark cycle. A seven-day-old culture was taken as mother inoculum at 20% (v/v) for all experiments. The test medium was supplemented with 0 to 1,000 mg/L final concentration of surfactant and the cultures were harvested at the 10th day of growth. The total algal cell concentration was determined using 1-ml Sedgewick–Rafter counting chamber [32,33] under a light microscope (Olympus BX50) and the growth rate was documented with the following equation:

$$\text{Relative cell proliferation (\%)} = (\text{Number of cells in treated} / \text{number of cells in control}) \times 100.$$

2.6.2 | Culturing of *Artemia*

A. salina (Salt Creek Inc., UT) nauplii were emerged out from eggs, subsequently after 48 h hatching and cyst decapsulation. Young *Artemia* nauplii were transported into an augmentation tank of 2 L. During axenic hatching, the pH of the system was sustained by the addition of sodium bicarbonate (0.4 g/L) and the oxygen through aeration systems (5 mg/L). An open-air line pipe was installed at the bottom of the tank to avoid the *Artemia* from settling. After 24 h of hatching-out process, the nauplii were collected and used for further studies.

2.6.3 | Infection testing of *Artemia* with *V. harveyi* and petroleum pollution

Lively nauplii free from eggshells were collected after hatching and used for the assay. Various dilutions of the biosurfactant (from 10 to 200 μg/ml) were added with 10 ml of artificial seawater containing 30 brine shrimp larvae per tube. Percentage of mortality (% M) was calculated after 48 h [34] as:

$$\%M = \%\ \text{of existence in the control} - \%\ \text{of existence in the treatment.}$$

Mortality and survival rate trials were conducted in five different experimental sets using the *A. salina* nauplii, that is, infection positive control (*A. salina* challenged with *V. harveyi* (10^5 CFU/ml), infection test (*A. salina* challenged with *V. harveyi* (10^5 CFU/ml) with 100 μg/ml of biosurfactant), infection and pollution negative control (*A. salina* alone in seawater), pollution positive control (*A. salina* challenged with petroleum covered the surface of the Petri dishes), pollution test (*A. salina* challenged with petroleum covered the surface of the Petri dishes with 100 μg/ml of biosurfactant). Thirty freshly hatched *A. salina* nauplii were kept in each group

in sterile Petri dishes containing 20 ml of autoclaved seawater. The fatality rates of the nauplii were determined up to 48 h by transferring the live *A. salina* into a watch glass and counting manually. The results were analyzed with Prism 6 software and the Kaplan–Meier survival curves were generated [35,36].

2.6.4 | Determination of the bacterial load

After survival trials, the Artemia were collected and dried. The dried nauplii were ground in 1 ml of sterile seawater, serially diluted, and plated in Thiosulfate–citrate–bile salts–sucrose agar (TCBS) for the selective scoring of *Vibrio* sp. Colonies were counted and their respective CFUs were calculated after 48 h of incubation at 37°C.

2.6.5 | Inhibitory activity determination and statistics

Inhibition of activity upon treatment with biosurfactant was calculated by the following formula:

$$\text{Inhibition of virulence (\%)} = [(O.D_C - O.D_T)/O.D_C] \times 100,$$

where $O.D_C$ is the absorbance of the control and $O.D_T$ is the absorbance of treatment.

All the tests were done in a set of biological triplicates, and the results were analyzed by one-way analysis of variance with Prism 6 software.

3 | RESULTS

3.1 | Isolation and screening of microorganisms

In the present study, 27 different strains of halotolerant bacteria that can tolerate 2 M NaCl were isolated from marine soil sediments. The microorganisms isolated were checked for the potential biosurfactant production by surfactant screening assays. Oil-spreading test revealed that 11 isolates were biosurfactant-producing species with varying activity profile (Table S1). All the 11 isolates were lipase positive with variation in the zone of tributyrin hydrolysis.

Emulsification index (EI_{24}%) results showed that the biosurfactant produced by the marine halophilic isolate MK3 has the maximum emulsification capability with the EI_{24}% of 51 with the greater oil dispersant property. The biosurfactant was subjected to emulsify various liquid hydrocarbons and found to form a 75% stable emulsion with crude oil after 60 h of incubation (Figure 1a). The surface morphology of the oil droplet was collapsed within 45–120 sec of incubation compared with the control. In the 16 S rDNA sequence analysis and phylogenetic analysis, strain MK3 was identified and named as *V. natriegens* MK3 (Figure S1). The 16S rDNA sequence of MK3 has been submitted in the GenBank database with accession number KY436581. The growth of MK3, biosurfactant production and emulsification activity were measured simultaneously from 0 to 60 h. After 12–24 h of incubation the biomass increases rapidly and cross the threshold of the log phase of

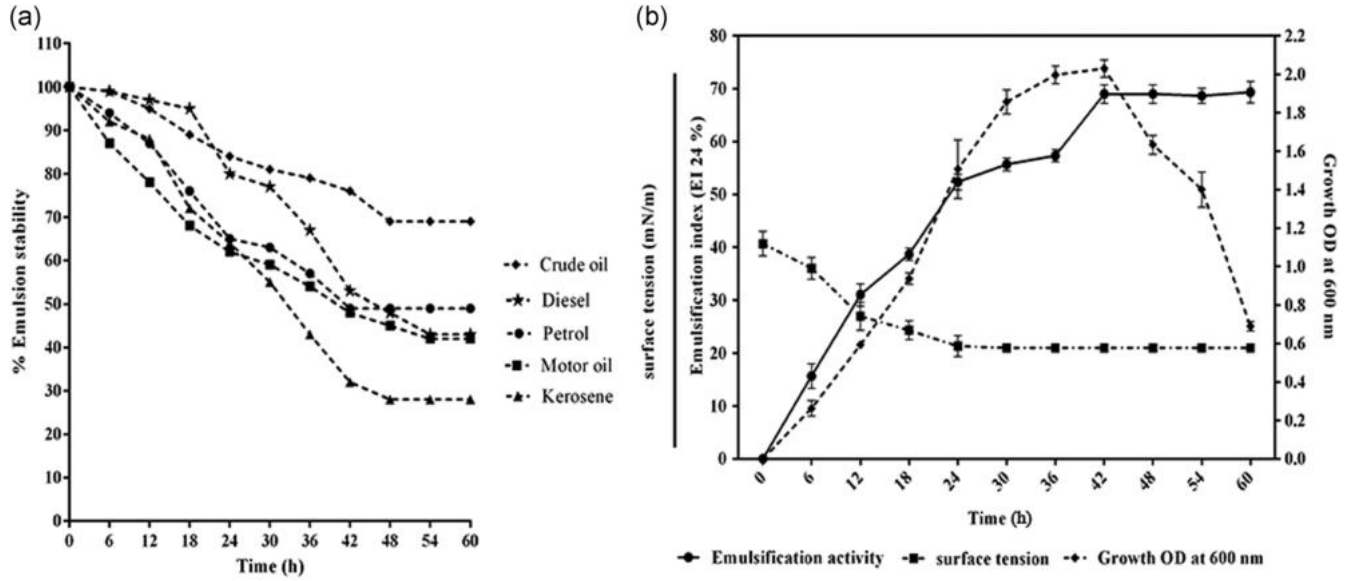

FIGURE 1 (a) Percentage emulsion stability evaluated over a period of 60 h on various oil hydrocarbons by biosurfactant from *Vibrio natriegens* MK3. (b) Biosurfactant production profile with growth OD, surface tension and emulsification activity of MK3 on M9 medium incubated at 34°C, 160 rpm

growth (Figure 1b). After 24 h, the surface tension declined greatly, which confirms that MK3 started to yield surfactant bringing about the critical decrease of surface strain and the surface tension was recorded as 21 mN/m, which is lowermost and retains up in the proceeding with time. At the same time, the highest EI_{24}% of 51 for crude oil was observed at 24 h of incubation with greater emulsion stability. The critical micelle concentration (CMC) of the surfactant was recorded as 0.75 mg/ml and the surface tension was reduced from 45 to 19 mN/m.

3.2 | Production, purification, and characterization of the biosurfactant

MK3 was cultured in 2.5 L M9 medium. The culture-free supernatant was collected and subjected to acid precipitation and solvent extraction. The crude biosurfactant was subjected to HPLC. All the distinct peaks were eluted and screened for their ability to emulsify the hydrocarbons and it was observed that the RP-HPLC (Figure 2a) peak eluted at 3.059 min ($EI_{24}\% = 77$) and at 11.838 min possessed the maximum emulsification index. To identify the functional groups present in the biosurfactant, the Fourier-transform infrared spectrum (Figure 2b) was recorded at 4500–400/cm. The most significant bands were located at 172/cm (for the C═O ester bond), 3408/cm confirming OH stretching and 1400–1460/cm for C═H stretching, which confirmed the presence of glycolipid moieties. The ^{1}H NMR spectrum of the purified biosurfactant confessed that the biosurfactant possesses a typical glycolipid-like structure and characteristic proton chemical shift peaks were also observed (Figure 2c). The two spectra showed two main regions corresponding to resonance of α-carbon protons (3.49–4.95 ppm) and the side chain protons were confirmed with the spectra at 0.07–3.1 ppm. Multiple signals of protons (—CH3) below 1.0 ppm and (—CH2) between 1.0 and 1.25 ppm indicate the presence of a linear alkane-like structure. The presence of intense singlet at 3.687 ppm suggests the presence of one methyl ester group, which describes an increase in its hydrophobicity and therefore increases its surfactant powers and antimicrobial activities.

3.3 | Determination of ionic charge

Agar double-diffusion tests revealed that the precipitation line observed between the biosurfactant produced by MK3 strain and barium chloride (cationic compound) affirms the anionic nature of the biosurfactant, whereas no lines were shaped between the biosurfactant and sodium dodecyl sulfate (anionic compound). The agar double-diffusion results were further supported by ζ potential analysis. The ζ potential value of MK3 biosurfactant was recorded as −5.26 mV with the conductivity of 4.1099 mS cm (Figure S2).

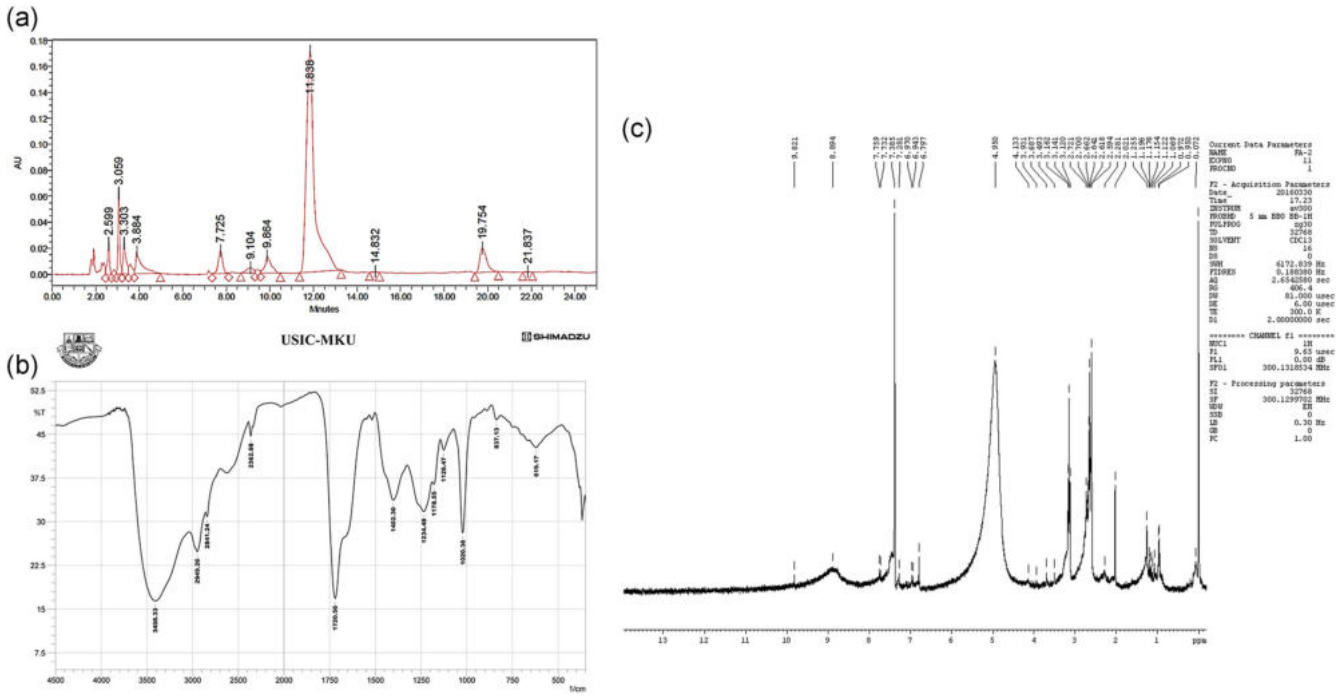

FIGURE 2 Characterization of biosurfactant produced by *Vibrio natriegens* MK3. (a) HPLC analysis of biosurfactant. (b) Fourier-transform infrared spectra of the biosurfactant. (c) ^{1}H nuclear magnetic resonance spectrum of the biosurfactant

3.4 | Biofilm inhibition assay

In view of the outcomes attained from biofilm inhibition assay, the influence of biosurfactant on biofilm formed by *V. harveyi* on 96-well black plates was visualized by light microscopic and HCS imaging system (Figure 3). The biofilm image analysis evidently portrayed the reduction of biofilm formation and disruption of preformed biofilms with the presence of biosurfactant. The preformed biofilms of *V. harveyi* treated with 20 μg/ml biosurfactant and incubated for 10 min efficiently crumbled the biofilm architecture when compared with the untreated control. Compelling results were observed in SEM imaging, which also supported the HCS visualization of antibiofilm activity by biosurfactant. SEM images revealed the formation of profuse biofilm above the exterior of the glass support by *V. harveyi*, whereas the biosurfactant treatment (Figure 4) depicts the antibiofilm activity of the biosurfactant at 20 μg/ml. SEM imaging after the biosurfactant treatment showed noticeable damage in the peripheral cell surface and variations in the cell morphology of *V. harveyi* grown in the presence of biosurfactant. The clear superficial roughness was also recorded in cell surface with the

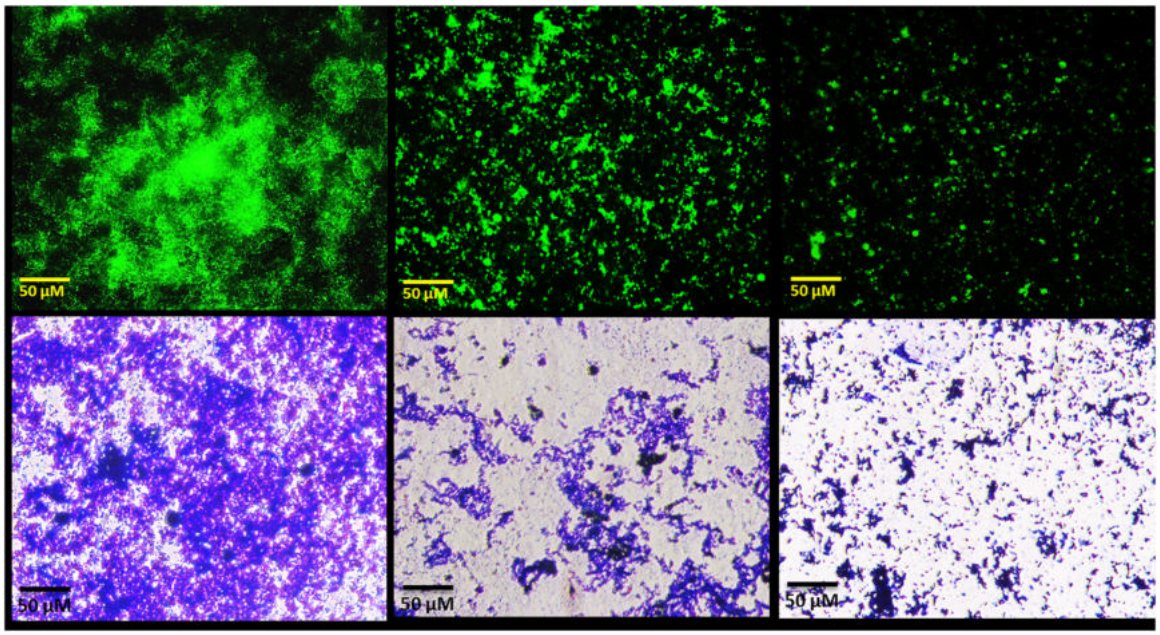

FIGURE 3 The micrographs of biofilms stained with Acridine orange (green film) and crystal violet (violet film) grown in the presence and absence of biosurfactant. (a) Control biofilms of *V. harveyi*; (b) Biofilm after the treatment of biosurfactant (20 μg/ml); (c) Biosurfactant (20 μg/ml) added before the formation of biofilm, which leads to the inhibition of biofilm formation

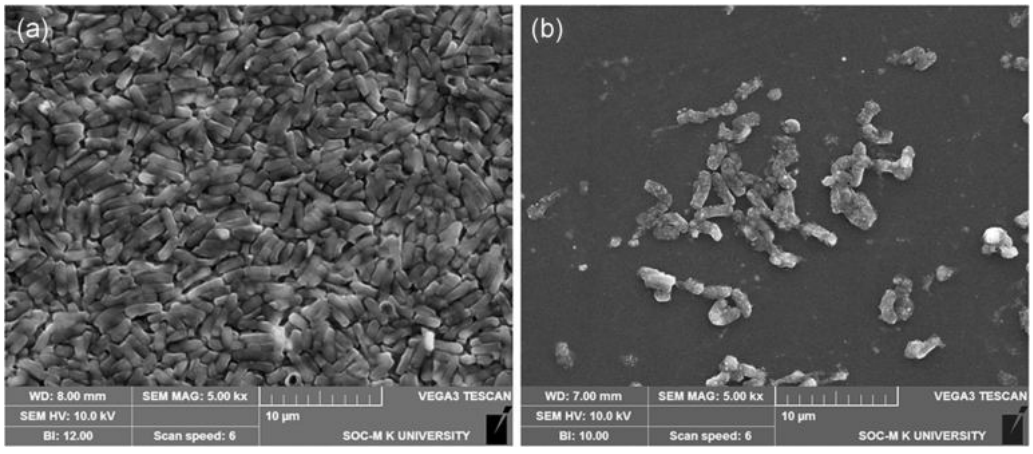

FIGURE 4 Scanning electron micrograph (SEM) of *V. harveyi* biofilm on the glass surface. (a) Control biofilm micrographs illustrating the morphology with the multilayer of cells; High-density attachment and colonization was recorded in the untreated control. (b) Biofilm treated with biosurfactant (20 μg/ml) resulted in the disruption of biofilm with a substantial reduction in bacterial density

effect of biosurfactant. Using SEM it was also noted that the control cells were rod-shaped with an elevated number of attachments in the biofilm state. However, in the case of treated groups, the configuration of the *Vibrio* cells was altered from regularly extended rods to downsize compared with the control. From the biofilm inhibition analysis results (Figure 5), it is clear that biosurfactant potentially impacts both the initiation and also the development of *V. harveyi* biofilm.

3.5 | Inhibition of bioluminescence with biosurfactant treatment

Bioluminescence in *V. harveyi* is one of the fundamental processes in pathogenic phenotype, which is highly governed by QS. Here, we examined the fortuity that biosurfactant may influence bioluminescence in *V. harveyi*. Dose-dependent bioluminescence inhibition up to 97% was attained with the concentration of 60 μg/ml of biosurfactant (Figure 5). Moreover, the maximum rate of bioluminescence arrest with the presence of biosurfactant (10 μg/ml) was recorded between 9 and 13 h of inhibition, whereas, in control, elevated levels of bioluminescence were observed. Bioluminescence exhibited by *V. harveyi* has increased remarkably at 16 h, whereas biosurfactant treatment does not manifest any bioluminescence, which confirms that the biosurfactant inhibits bioluminescence in *V. harveyi*. Further, we examined the impact of this inhibition on the synthesis of several virulence factors in *Vibrio*.

3.6 | Inhibition of EPS production

In addition to inhibition of biofilm formation in *V. harveyi*, the biosurfactant noticeably repressed the EPS production in a dose-dependent manner.

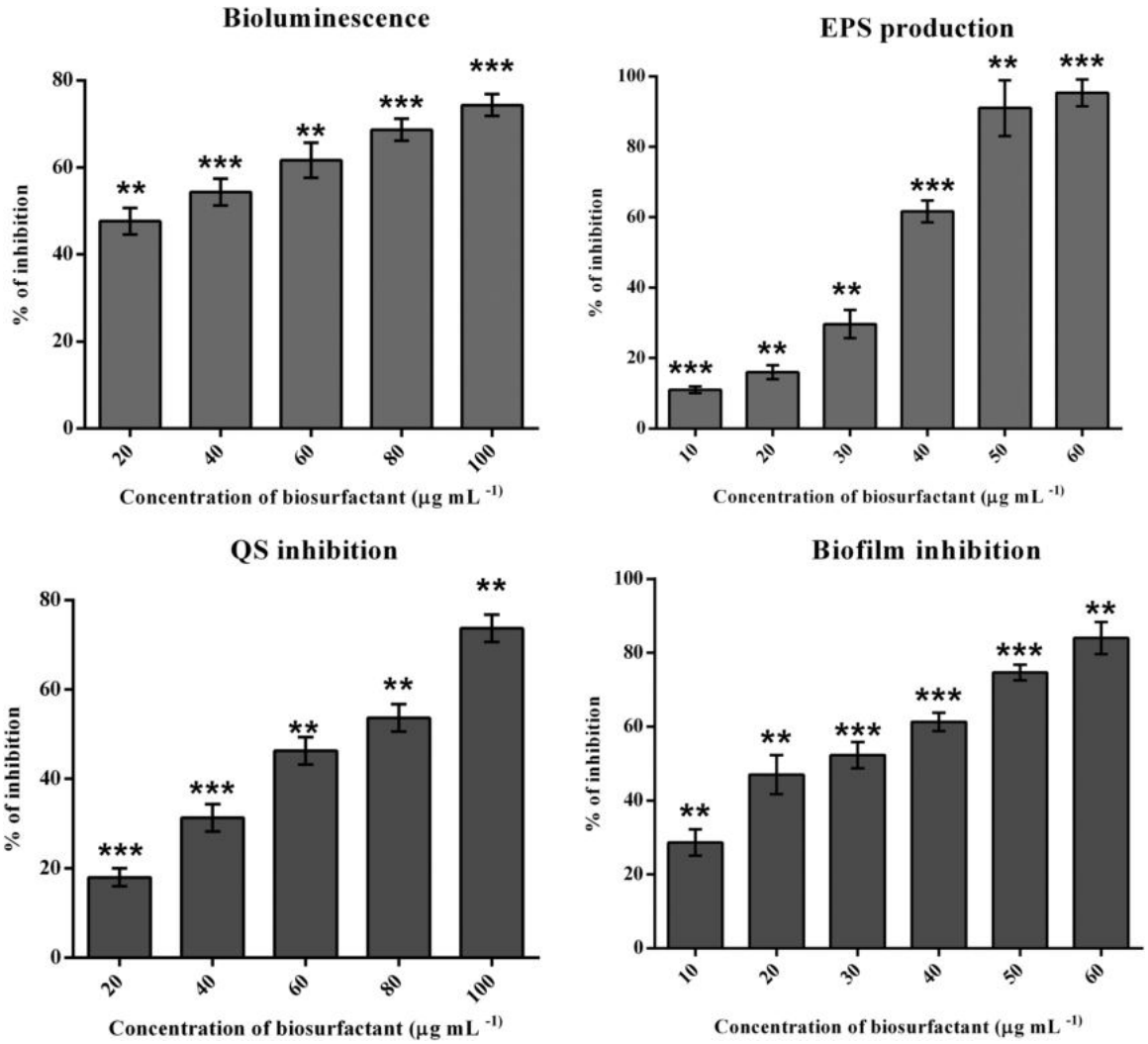

FIGURE 5 Inhibitory effect of biosurfactant on bioluminescence, extracellular polymeric substance (EPS) production, quorum sensing (QS), and biofilm biomass production of *V. harveyi*. Bar graph showing means levels of virulence inhibition in control and biosurfactant-treated samples. Data are shown as mean ± standard deviation (*SD*), $n = 3$, $^{**}p < .05$, and $^{***}p < .01$

Biosurfactant treatment at the range of 20, 40, 60, 80, and 100 μg/ml capably inhibited the EPS production in *V. harveyi* to the range of 20, 32, 47, 53, and 73%, respectively (Figure 5).

3.7 | Inhibition of quorum sensing

QS mechanism inhibition in vibriosis causing bacterial pathogens could eventually result in weakening the virulence and disease severity on aquatic species. In the present investigation, the influence of the MK3 biosurfactant to inhibit QS-dependent violacein production in *C. violaceum* (ATCC 12472) was evaluated. In quantitative analysis, the biosurfactant inhibited violacein production up to 74% (Figure 5).

3.8 | Assessment of biosurfactant toxicity

3.8.1 | Toxicity against microalgae and *A. salina*

The toxicity of MK3 biosurfactant against marine microalgae *I. galbana* was evaluated. Viable count estimates of microalgal populations with a 95% confidence level in the biosurfactant-treated and -untreated flasks were recorded. The growth measured in terms of cell density of algae procured from the medium treated with biosurfactant was more or less similar to that of control, which indicated the absence of toxicity of the biosurfactant. The cell proliferation rate was normal up to 700 mg/L of biosurfactant and it was found to attain a decline phase after 800–850 mg/L. More than 50% of the growth rate was arrested with a concentration of 900 mg/L in the medium. Mortality of the brine shrimp larvae of *A. salina* was measured as a parameter for surfactant toxicity. The elevated concentration of surfactant did not show any significant mortality within 24 h of exposure. No brine shrimp lethality was observed at the concentrations tested (10–200 μg/ml), indicating the nontoxic nature of the biosurfactant. The morphology remains unaffected after the treatment with biosurfactant. The results depict 100% survival of brine shrimp larvae demonstrating the potential of the biosurfactant for use in aquatic environments.

3.8.2 | Infection testing of *A. salina* with *V. harveyi* and crude oil pollution

Brine shrimp challenge assay was performed further to reveal the biosurfactant-mediated control in pathogenicity of *V. harveyi* and the detrimental effect caused by oil pollutants in the axenic condition. Complete tissue dissociation from the anal opening and hindgut region was microscopically recorded with the *V. harveyi*-infected brine shrimps (Figure 6). Hence, more than half of the nauplii population were found to be dead with the infection of *V. harveyi.* Biosurfactant treatment results showed that there is no trace of tissue damage and deformation, the body parts and the gut region appeared clear. The Kaplan–Meier survival plots for *A. salina* affected with either *V. harveyi* or crude oil with their respective biosurfactant treatment and controls are shown in Figure 7. χ^2 statistics (7.476) and the p value for each comparison was obtained and there is a significant difference in survival between shrimps infected, polluted and their treatment and control. On the basis of the survival proportion result, infection positive control and pollution positive control had lower survival rate around 34.20% and 11.88%, respectively. The survival proportion got increased up to 62.06% and 43.11% when treated with MK3 biosurfactant. The natural death rate was recorded in negative control without any treatment and the survival rate observed was 73.34%.

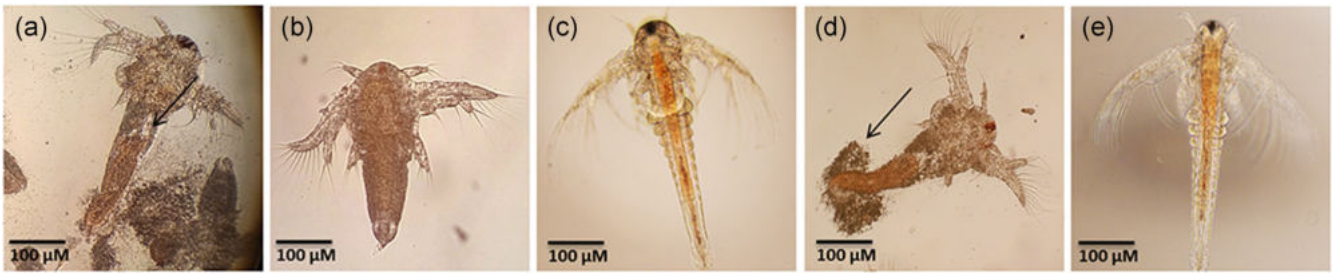

FIGURE 6 Challenge survival and disease experiment with *Artemia salina*. (a) Disease control group—*A. salina* infected with *V. harveyi*; (b) treatment group—infected *A. salina* treated with biosurfactant; (c) negative control without any treatment; (d) pollution control—*A. salina*, grown in the crude oil-contaminated marine environment; (e) pollution treatment—*A. salina,* grown in crude oil-polluted marine environment treated with biosurfactant. Arrow indicates the damage of clear sensorial antennules, antennae, and abdomen due to the infection

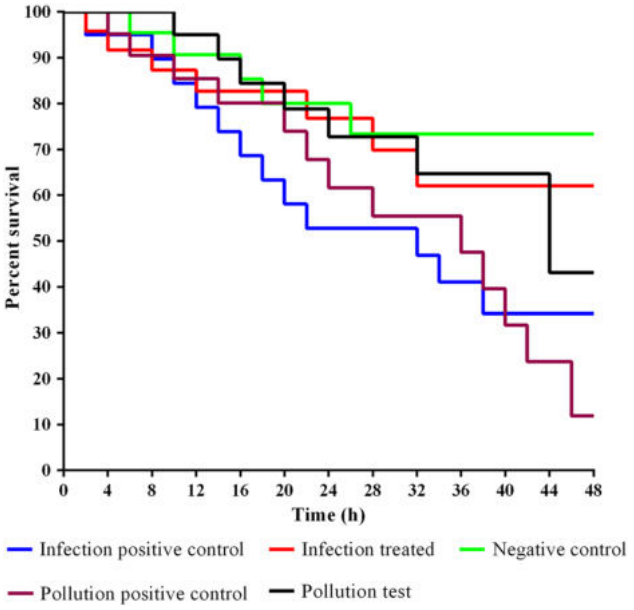

FIGURE 7 Kaplan–Meier survival plans for challenge experiments. The percentage of survival versus time post challenge was plotted

4 | DISCUSSION

The sustainability of marine bacterial communities is directly related to incredible stress triggered by the radical change in surrounding seawater. This indicates that they have developed various structural and metabolic changes that enable them to accommodate for the effects of the adverse condition [37]. Furthermore, metabolites from these marine microorganisms are categorized by renowned features, such as halo tolerance, thermostability, barophilicity, and flexibility to cold, entirely connected to their habitation [38]. Hence, the present study was focused on evaluating the biosurfactant-producing potential of marine bacteria. In the same way, Hentati et al. [39] screened contaminated seawater as a source for biosurfactant producers, as the marine environment, which is considered by its diversity and nutrient availability, represents a rich reservoir of microorganisms. Biosurfactant obtained from the marine environment have been stated to constrain bacterial adhesion and biofilm development. They efficiently express antimicrobial, anti-adhesive, and biofilm disrupting capabilities against pathogenic microorganisms.

In this present study, a total of 27 different strains of halotolerant bacteria were isolated and screened for biosurfactant production [40]. The single-screening method is not suitable to identify biosurfactant producers, therefore, more than one screening methods were included in the primary screening to identify potential biosurfactant producers. Therefore, oil-spreading test, lipase activity, and emulsification activity were used to screen the biosurfactant producer. Among these methods, the oil-spreading test is considered as the consistent and sensitive primary-screening technique, where the surface activity of a tensioactive biomolecule is directly comparative to its concentration in the production medium. Oil-spreading test revealed that isolates were biosurfactant producers with varying activity profile.

Lipase is an enzyme that acts on water-oil surfaces and reduces surface tension. Therefore it was suggested that the lipase production might directly influence the bioemulsifier production [41]. The results suggest that all the 11 isolates were lipase positive with variation in the zone of tributyrin hydrolysis. In addition to the surface tension and interfacial tension disruption, the stability of the emulsion formed between oil and water is commonly used to analyze the surfactant activity [42]. The strain MK3 exhibited higher emulsification index value of 51 against crude oil with the grater emulsion stability confirms its role as a bioemulsifier. An essential attribute of surfactant is the critical micelle concentration (CMC), which is defined as the surfactant concentration capable of forming a micelle. When the surfactant procures CMC, the surface tension continues to be almost constant by virtue of interface saturation between the surfactant and the liquid [43]. Hence, it is important to attain a surfactant with low CMC and as well as the high surface activity, where the surfactant from MK3 exhibited low CMC value at 0.75 mg/ml and reduced the surface tension to a much lower level of 19 mN/m.

The ζ potential (surface charge) of the biosurfactant greatly affects its stability in suspension through the electrostatic repulsion between particles. It further determines the interaction of biosurfactant with the cell membrane of bacteria in vivo. When the biosurfactant is more negatively charged, the chance of the bacterial adhesion will be diminished, and further delays the biofilm formation. The biosurfactant produced by MK3 was found to be negatively charged, which contributes to its efficient biofilm disruption potential. This study, therefore, focusses toward a new way for the development of anionic biosurfactant, which accompanies the antimicrobial and antibiofilm properties and can be used to treat marine infections.

V. harveyi is one of the predominant infectious pathogens of marine reared fish, shellfish, and crustaceans, being responsible for elevated death rates in marketable trades of seafood worldwide. Numerous putative virulence factors have been proposed in *Vibrios* belonging to the species *harveyi*. These include biofilm formation, which allows the pathogens to colonize the host, exopolysaccharides, which enables the bacterial adhesion to the host tissues to form biofilms and protect

them from the host's defense system, and QS mechanisms that allow cell-to-cell communication.

The biofilm image analysis portrayed the reduction in biofilm formation and disruption of preformed biofilms in the presence of biosurfactant. The biosurfactant treatment in the preformed biofilms of *V. harveyi* efficiently crumbled the biofilm architecture when compared with the untreated control as portrayed in *V. cholerae* [44]. The outcome we perceived here is comparable to what has been stated before for *V. harveyi* and *V. parahaemolyticus* with the treatment of ethanolic extract of the red seaweed *Gracilaria fisheri* [45]. The disintegrated biofilm architecture reveals the effectiveness of biosurfactant, which is due to the disturbance in interbacterial adhesion facilitated by the negative charge of the MK3 biosurfactant. The cell surface morphology confers a selective advantage toward bacterial virulence by aiding the process of adhesion toward the host cell and evasion against immune system [44]. In this study, we demonstrated that the biosurfactant could damage the cell wall and were able to destroy the entire cell structure, this would provide a more effective strategy of the biosurfactant to treat against *V. harveyi*. This noticeable damage in the peripheral cell surface potentially impacts the initiation, development, and the survival of *V. harveyi* biofilm. Therefore, the attained outcomes evidently highlight the antibiofilm potential of biosurfactant. The findings of this study also fall in line with the results of Salini et al. [46], wherein, undecanoic acid and auxins significantly repressed the biofilm formation in *Vibrio* sp. by QS inhibition. As the biosurfactant has shown the effective antibiofilm property, it was further evaluated for other antivirulence efficacy.

Bioluminescence is a fundamental pathogenic process governed by QS. Bioluminescence inhibited by MK3 biosurfactant in a dose-dependent manner confirms the influence of biosurfactant on the virulence of *V. harveyi*. The tested combinations showed significant bioluminescence inhibition up to 97% against *V. harveyi*. Hence, any natural compounds that deter the bioluminescence might plausibly decrease the virulence factor production of the bacterial pathogen. The outcomes attained in the bioluminescence study acknowledges with the previous reports of bioluminescence reduction while using phytochemicals against vibrios [47].

EPS secretion may be a defensive reaction of marine pathogens to environmental stresses, which is a crucial feature of biofilm development. EPS offers binding sites for pathogens, and ultimately, the amassed EPS layer entangles pathogenic biomass, making the bacteria tougher to antibacterial substances [48]. Hence, we measured that inhibiting EPS secretion would be a prudent approach to constrain biofilm development, which would make the pathogen become much more sensitive upon treatment. In this study, we validated that the biosurfactant could inhibit EPS secretion by up to 73%.

The effect of the biosurfactant on the cell-to-cell communication of the *V. harveyi* was evaluated by QS inhibition assay. In marine bacterial pathogens such as *V. alginolyticus, V. harveyi,* and *V. vulnificus* the production of virulence factors and also the biofilm formation was highly influenced by AHL-mediated QS machinery [49]. Therefore, the reticence of the QS system in vibriosis instigating pathogens could ultimately effect to diminish the disease progression on aquatic species. It should be noted that several findings already have disclosed the inhibition of AHL-mediated virulence factors and biofilm formation by the extracts from fruits, spices, and medicinal plants in the aquatic bacterial pathogens [47,50]. In the present investigation, we demonstrated the QS inhibition potential of biosurfactant by the restraint of violacein production in the reporter strain *C. violaceum.* The results also support the findings on the inhibition of QS-regulated behaviors in *V. harveyi* by undecanoic acid and auxins [46].

Microalgal biomass seems to be a relatively high-sensitive specification, hence, appears to be an important indicator to evaluate the toxicity, as it is a total measure of microalgal growth with different responses to toxicants. Among various algal species, *I. galbana,* a free-living marine unicellular phytoflagellate plays a tremendous role in aquaculture as a feed for the early larval stages of molluscs, fish, and crustaceans. The cell proliferation rate of microalgal population in the presence of biosurfactant revealed the nontoxic nature of the biosurfactant to the marine environment.

From the brine shrimp toxicity assays, it was found that biosurfactant seems to exhibit antivirulence impacts in vivo and may adversely influence the phenotypic expression of virulence factors essential for the invasion of *V. harveyi* while infecting *A. salina*. The secretion of proteases, phospholipase, hemolysins, and other exotoxins by *V. harveyi* is contributed toward the exerted pathogenicity [51]. These pore-forming cytolysins form stomas in the membranes of the brine shrimps, which eventually lead to the cell lysis and disintegration of tissues to facilitate the subsequent digestion. Oil spills in the marine environment can create long-lasting toxic effects on the aquatic ecosystem. The most common role of biosurfactants is to enhance the dispersal of contaminants in the aqueous phase and increase the bioavailability of the hydrophobic substrate to microorganisms, with subsequent removal of such pollutants through biodegradation. Oil-polluted marine water leads to the structural deformation and lower survival rate in

brine shrimps during growth [52]. Conversely, the shrimps were healthy and the survival rate was high with the treatment of biosurfactant, which collapses and emulsifies the oil pollutants. In conclusion, the outcomes presented in this study are innovative in developing an effective biosurfactant with negligible environmental impact. The biosurfactant produced by the *V. natriegens* MK3 was characterized as glycolipid derivative, which eventually formed a possible source over the chemical surfactants. The "scale-up" production specifies the prospect of large-scale application of the biosurfactant from *V. natriegens* MK3 to overcome the aquatic bacterial pathogens and oil pollutants from the aquatic environments. Besides, distortion of biofilm and motility in the presence of biosurfactants was observed from the SEM and HCS imaging. The halophilic property meets the current need for the use of biosurfactants, which could be applied in high-salinity aquatic environments.

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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