Antibiofilm Activity of Biosurfactant Producing Coral Associated Bacteria Isolated from Gulf of Mannar

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Abstract Coral Associated Bacteria (CAB) ($N = 22$) isolated from the mucus of the coral *Acropora digitifera* were screened for biosurfactants using classical screening methods; hemolysis test, lipase production, oil displacement, drop collapse test and emulsifying activity. Six CAB (U7, U9, U10, U13, U14, and U16) were found to produce biosurfactants and were identified by 16S ribosomal RNA gene sequencing as *Providencia rettgeri*, *Psychrobacter* sp., *Bacillus flexus*, *Bacillus anthracis*, *Psychrobacter* sp., and *Bacillus pumilus* respectively. Their cell surface hydrophobicity was determined by Microbial adhesion to hydrocarbon assay and the biosurfactants produced were extracted and characterized by Fourier Transform Infrared spectroscopy. Since the biosurfactants are known for their surface modifying capabilities, antibiofilm activity of positive isolates was evaluated against biofilm forming *Pseudomonas aeruginosa* ATCC10145. Stability of the active principle exhibiting antibiofilm activity was tested through various temperature treatments ranging from 60 to 100 °C and Proteinase K treatment. CAB isolates U7 and U9 exhibited stable antibiofilm activity even after exposure to higher temperatures which is promising for the development of novel antifouling agents for diverse industrial applications. Further, this is the first report on biosurfactant production by a coral symbiont.

Keywords Coral associated bacteria · Biosurfactant · Antibiofilm · Lipopeptide

Introduction

Microbial surfactants or biosurfactants are low molecular weight, amphipathic, surface active compounds produced by microorganisms. Biosurfactants are characteristic of reducing the surface and interfacial tensions between individual molecules at the surface and interface respectively in both aqueous solutions and hydrocarbon mixtures. Most biosurfactants are diverse, complex molecules, comprising of different structures that include glycolipids, lipopeptides, lipoproteins, fatty acids, phospholipids, neutral lipids, polymeric and particulate biosurfactants. Biosurfactants have received enormous attention because of their versatile applications in petroleum industry, environmental remediation, food processing, agro industry, cosmetics and pharmaceuticals [1]. They are highly preferred over their synthetic counterparts due to their reduced toxicity, higher biodegradability, ecological acceptability and specific activity in wide range of temperature, pH and salinity [2].

Besides their regular applications in bioremediation and hydrocarbon degradation, they are employed in formation of ion channels in lipid membranes and inhibition of fibrin clot formation. They are also explored for their antitumor, antiviral and anti-mycoplasmic activities [3, 4]. In recent years, biosurfactants are considered as potential drug candidates and looked up for their explicit biomedical applications such as antibacterial, antifungal, antiviral and anti-adhesive agents against several pathogens [4–8]. Biosurfactants have also been proved as potential immunomodulators and enzyme inhibitors [9]. They have shown

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pronounced antimicrobial and antibiofilm activity against several drug resistant pathogens [7, 8].

Biofilms are complex aggregation of bacteria enfolded in an extracellular polymeric substance (EPS) which forms a protective barrier for the indwelling microbes from the hostile environment. The bacterial pathogens that adapt biofilm mode of growth are several fold resistant to bactericidal agents than their planktonic counterparts. There are reports which exemplify that antibiotic resistance is attributed to the complex nature of biofilms formed by the microbes in vivo [10, 11]. Antibiotics were believed to be the remedy for all ailments caused by bacterial pathogens, until the incidence of drug resistance emerged. Continuous and indiscriminate usage of antibiotics have paved way for the development of antibiotic resistant bacteria and few which were resistant to multiple antibiotics. Antibiotics have become ineffective in controlling the virulence of bacterial pathogens [11–15]. Hence, novel antimicrobial agents which selectively reduce the virulence of pathogens rather than inducing antimicrobial resistance are the need of the hour [16]. Antibiofilm agents are one such a kind which selectively eradicate the persistent biofilms thereby facilitate the diffusion of antibiotics into the complex biofilm ambience [17, 18]. Identification and characterization of new biosurfactants and antibiofilm agents with profuse biological activities will aid mankind to combat recurrent infections caused by drug resistant biofilm forming pathogens [19].

Though hydrocarbon contaminated sites are the most promising source for the isolation of biosurfactant producers, there are few reports which elucidated the isolation of biosurfactant producers from diverse environments [20]. It has been reported that microorganisms from marine origin have shown to produce novel biosurfactants with excellent bioactivity [21]. Marine environment is an excellent source of novel biomolecules due to their species richness and the chemical uniqueness of the metabolites produced by marine organisms and associated microorganisms. Coral reefs are the most inimitable and species-rich ecosystem existing in the marine environment which harbour protected marine mammals, fishes and other endangered marine biota. With this milieu, coral ecosystem, a hitherto under explored reserve for novel bacteria, was screened for biosurfactant producers and evaluated for their antibiofilm activity with the hope that biomolecules from such novel bacteria will be of new-fangled type.

Materials and Methods

Bacterial Strains and Media Used

CAB used in this study were isolated earlier by our group from the mucus of the coral *Acropora digitifera* collected

from Gulf of Mannar (9°12'N, 79°5'E) and maintained in glycerol stocks at –86 °C. The isolates were revived and grown aerobically in Zobell marine broth (ZMB) (HiMedia Laboratories, Mumbai) at 30 °C and 160 rpm for 24 h on a rotary shaker. The cultures were centrifuged at 12,000 rpm for 10 min at 4 °C and the supernatants were filtered through 0.22 µm cellulose acetate filter to obtain cell free culture supernatants (CFCS). This CFCS was used for preliminary screening and the assays wherever mentioned. *Pseudomonas aeruginosa* ATCC10145 used for antibiofilm assays was maintained in Luria–Bertani broth (HiMedia Laboratories, Mumbai) at 37 °C.

Screening of Biosurfactant Producers

Hemolytic Test

Biosurfactants, due to their amphiphilic nature induce hemolysis of red blood cells. Hemolytic assay was carried out in blood agar plates containing 5 % (v/v) sheep blood. Blood agar plates patched with CAB isolates were incubated at room temperature for 24 h and the hemolytic activity was determined by the formation of hemolytic zones around the colonies [7].

Screening for Lipase Production

Lipases which play specific role in the modification and emulsification of biosurfactants were employed as a primary screening method. CAB isolates were patched on tributyrin agar (TBA) plates containing 1 % (v/v) tributyrin and incubated at room temperature for 48 h. The plates were inspected for zone of clearance around the colonies [7].

Oil Displacement Test

The activity of biosurfactants to form oil displaced circles was employed as a preliminary screening method [7]. For this screening, 20 µL of olive oil was overlaid on the surface of 20 mL of distilled water in a petri dish followed by the addition of 10 µL of CAB CFCS onto the centre of the pre-formed oil layer. The oil displaced circles formed by CFCS were visualized under visible light. Uninoculated ZMB served as a negative control in all the assays performed with CFCS.

Drop Collapse Test

Drop collapse test was carried out in the polystyrene lid of a sterile 24 well tissue culture plate [7]. The lid was pre-coated with olive oil and equilibrated at 37 °C for 1 h followed by the addition of 20 µL of CAB CFCS to the

surface of the oil and observed for the change in the shape of the drop on oil. Biosurfactant containing drops were collapsed and construed as positive and those that remain as bead shaped drops were scored as negative.

Emulsifying Activity

Emulsification potential of the biosurfactants was tested as described previously [7]. The screw capped tubes containing 2 mL of CAB CFCS with equal volume of petrol were vortexed vigorously for 2 min and the tubes were incubated at room temperature for 24 h. The emulsification index was calculated after incubation using the given formula:

Emulsification index (E_{24}) = (Height of emulsified layer/height of total liquid column) $\times$ 100.

Molecular Identification of the Biosurfactant Producers by 16S Ribosomal RNA Gene Sequencing

Genomic DNA was isolated from biosurfactant producers. Eubacterial primers (Forward primer p63F 5'-CAG-GCCTAACACATGCAAGTC-3' and reverse primer R1378R 5'-CGGTGTGTACAAGGCCCGGGAACG-3') were used to amplify 16S ribosomal RNA gene and the amplified products were sequenced (Macrogen Inc., S. Korea). The sequences were aligned using the freeware Contig Assembly Program V. 3 (<http://doua.prabi.fr/software/cap3>) and the assembled sequences were searched for similar sequences in public database, NCBI using Nucleotide BLAST. The sequences were further submitted in GenBank for public availability and their accession numbers are given in results section.

Standardization of Biosurfactant Production Time

One percent of overnight grown biosurfactant producers were diluted (1:100) in ZMB and incubated on a rotary shaker (160 rpm) at 30 °C. Triplicate samples were taken at different time intervals (0, 6, 12, 18, 24, 30, 42, 48, 66, 72 and 80 h) and the emulsification index was calculated to standardize the biosurfactant production time.

Surface Tension Measurement

Reduction in surface tension of culture broth was measured with a Du-Nouy ring tensiometer (Kruss, Germany). All measurements were carried out at room temperature using the CAB CFCS obtained by centrifugation of 12, 18, 24 and 48 h old cultures at 12,000 rpm for 25 min at 4 °C.

Microbial Adhesion to Hydrocarbon (MATH) Assay

Cell surface hydrophobicity of CAB was measured by MATH assay as described below. One mL of overnight culture of 0.8 OD was taken and added with equal volume of toluene. The mixture was vortexed vigorously for 2 min and then allowed for phase separation at room temperature. Absorbance of the aqueous phase was measured at 600 nm and the percentage of hydrophobicity was calculated using the formula:

Percentage hydrophobicity = $[1 - (\text{OD}_{600} \text{ of lower aqueous phase after vortexing} / \text{OD}_{600} \text{ of culture before vortexing}) \times 100]$.

Extraction of Biosurfactant

CAB isolates were cultured aerobically in ZMB at 30 °C for 24 h at 160 rpm. The CFCS obtained were acidified to pH 2.0 with concentrated hydrochloric acid and incubated overnight at 4 °C. The acidified CFCS was spun at 10,000 rpm for 30 min at 4 °C. The white pellet obtained after centrifugation was washed with absolute ethanol and dissolved in sterile Milli-Q water, adjusted to pH 7.0 using 1 N NaOH and lyophilized (Alpha 2-4 CD plus, Christ, Germany) and the crude biosurfactants obtained were stored at –26 °C until further analysis.

Characterization of Biosurfactants by FTIR Analysis

The chemical components (functional groups) of biosurfactants were determined by Fourier transform infrared spectroscopy. The lyophilized biosurfactants were ground with potassium bromide (KBr) and scanned in the range of 400–4,000 cm^{-1} with a spectral resolution of 4 cm^{-1} . The spectra were recorded on the Nexus-670 FT-IR Spectrometer (Thermo Electron Corporation, Yokohama, Japan). KBr pellet alone was used as a background reference.

Antibiofilm Activity

Antibiofilm activity of CAB isolates was tested against biofilm forming *P. aeruginosa* ATCC10145 in 24 well polystyrene plates [22]. One mL of LB broth was supplemented with 1 % overnight grown *P. aeruginosa* ATCC10145 and 10 % of 24 h grown CAB CFCS and the plates were incubated at 37 °C for 18 h. *P. aeruginosa* ATCC10145 alone in LB broth served as control. After incubation, the absorbance of planktonic cells was read at 600 nm using multi label plate reader (Spectramax M3, Molecular Devices, USA). The wells were washed with sterile distilled water and stained with 0.4 % crystal violet followed by destaining with 70 % ethanol for 30 min and

measured at 570 nm. Biofilm inhibition was calculated by the following formula

$$\text{Biofilm inhibition (\%)} = \left[\frac{(\text{Control OD}_{570\text{nm}} - \text{treated OD}_{570\text{nm}})}{\text{Control OD}_{570\text{nm}}} \right] \times 100$$

Microscopic Observation of Antibiofilm Activity

Antibiofilm activity of CAB isolates against *P. aeruginosa* ATCC10145 was confirmed by CLSM analysis [22]. The biofilms were grown on glass pieces (1 × 1 cm) placed in 24-well polystyrene plates supplemented with and without the biosurfactants. Each biosurfactant with a concentration of 100 µg mL⁻¹ was supplemented in LB broth containing the test pathogen (10⁶ CFU mL⁻¹) separately. The plates were incubated at 37 °C for 18 h. After incubation, the glass slides were washed with sterile phosphate buffered saline, stained with 0.1 % acridine orange and visualized under confocal laser scanning microscope (CLSM) (Zeiss LSM 710, Carl Zeiss, Germany). The thickness of the biofilms was measured by Z-stack mode in the Zen 2009 CLSM control software (Carl Zeiss, Germany).

Thermal Stability and Proteolytic Stability of Antibiofilm Nature of CAB Isolates

The CFCS of CAB isolates were individually subjected to different temperature (60, 70, 80, 90 and 100 °C) treatment and Proteinase K (Sigma-Aldrich) treatment (1 mg mL⁻¹) at 55 °C for 1 h and tested for antibiofilm activity against *P. aeruginosa* ATCC10145 as described above.

Determination Biofilm Inhibitory Concentration (BIC) of Crude Biosurfactants

The biofilm inhibitory concentration was determined as the minimal concentration of CAB biosurfactants required for maximum biofilm inhibition. Different concentrations of lyophilized biosurfactants (20, 40, 60, 80, 100, 200, 500 and 1 mg) were tested to determine the BICs.

Statistical Analysis

All the assays were performed in triplicates and the values expressed are mean ± SD. Statistical analysis was done by student *t* test.

Results and Discussion

In the present study, 22 CAB strains (U1–U22) isolated from the mucus of the coral *Acropora digitifera* were screened for the production of biosurfactants employing five classical screening methods (hemolysis, lipase production, oil

Table 1 Emulsification index, surface tension and hydrophobicity (%) values of CAB isolates

CAB isolate	Emulsification index (%)	Surface tension (mN m ⁻¹)	% Hydrophobicity
<i>P. rettgeri</i> (U7)	60	60	83.25
<i>Psychrobacter</i> sp. (U9)	52	58	71.98
<i>B. flexus</i> (U10)	61.2	58	74.38
<i>B. anthracis</i> (U13)	52.5	58	74.75
<i>Psychrobacter</i> sp. (U14)	56.25	58	76.73
<i>B. pumilus</i> (U16)	68.75	58	91.59
Uninoculated ZMB	0	66	0

displacement, drop collapse and emulsifying activity). Of the 22 isolates screened, 6 (U7, U9, U10, U13, U14 and U16) were confirmed as biosurfactant producers by comparing the results of all five methods. Emulsifying property of CAB supernatants was also examined with petrol as a source of hydrocarbon. Emulsification index (E₂₄) values tabulated in Table 1, revealed the stability of the emulsions formed by CAB isolates. The strains which could able to maintain 50 % of emulsions after 24 h are considered as biosurfactant producers [23]. Six of the isolates (U7, U9, U10, U13, U14, and U16) formed stable emulsions and the reduction in their surface tension was measured with Du-Nuoy Tensiometer (Table 1). The minimal surface tension values were recorded at 24 h of incubation which coincide with the biosurfactant production time. Further incubation led to an increase in the surface tension. A minimal reduction of 6–8 mN m⁻¹ was observed in CFCS of CAB isolates with that of uninoculated ZMB. The small reduction observed in the surface tension implies that these strains produced low level of extracellular biosurfactants which goes well with previous report [5].

The biosurfactant producers were identified to genus and species level by 16S ribosomal RNA gene sequencing. Sequencing and further sequence analysis revealed U7, U9, U10, U13, U14, and U16 as *Providencia rettgeri* (GenBank Accession Number JN315773), *Psychrobacter* sp. (GenBank Accession Number JN315776), *Bacillus flexus* (GenBank Accession Number GU297611), *Bacillus anthracis* (GenBank Accession Number GU297610), *Psychrobacter* sp. (GenBank Accession Number JN315774) and *Bacillus pumilus* (GenBank Accession Number JN315777) respectively. MATH assay result revealed the higher hydrophobicity of biosurfactant producers towards hydrocarbons which implies the ability to adhere hydrophobic substrates in order to facilitate the emulsification which

Fig. 1 CLSM 2-dimensional images of biofilms formed by *P. aeruginosa* ATCC10145 in the absence (a) and presence of 100 µg mL⁻¹ of biosurfactant from U7 (b), U9 (c), U10 (d), U13 (e), U14 (f), U16 (g)

further paves way for the degradation and utilization of hydrocarbons as carbon source (Table 1).

FTIR spectrum of biosurfactant (Supplementary Fig. 1a) produced by *B. flexus* (U10) revealed peaks at 3305, 1662, 1550, 1115 cm⁻¹ corresponding to NH stretching, CO–N stretching, C=O and C–O–C bonds respectively. The absorption bands located at 3054, 2961, 1446 cm⁻¹ revealed the presence of aliphatic chains in the compound [24]. FTIR spectra (Supplementary Fig. 1b) of *B. pumilus* (U16) showed prominent peaks at 3408, 1652, 1544, 1405 and 1138 cm⁻¹ corresponding to N–H stretching, CO–N, peptide bond, and C–O–C stretching of esters respectively. The absorption bands located at 2,879 and 2,825 cm⁻¹ revealed the presence of alkyl side chains in the compound [25]. Hence we presume that the biosurfactants produced by *B. flexus*, *B. pumilus* could be lipopeptide biosurfactants. FTIR spectrum of *P. rettgeri* (Supplementary Fig. 1c), *Psychrobacter* sp. (Supplementary Fig. 1d, e) and *B. anthracis* (Supplementary Fig. 1f) (U7, U9, U14 and U13) showed various peaks in the finger print region (1,640–990 cm⁻¹) and exhibited stretching vibrations in the range of 3,300–3,500 cm⁻¹ along with a peak at 2,070–2,090 cm⁻¹ region. FTIR spectra of U7, U9, U13 and U14 have no resemblance with any of the spectra of known biosurfactants. Thus, the biosurfactants produced by *P. rettgeri*, *Psychrobacter* sp. and *B. anthracis* could be novel and further detailed structural analyses are warranted to conclude their chemical nature.

Antibiofilm activity of biosurfactant producing CAB isolates provides a new avenue besides their regular applications. Quantification of *P. aeruginosa* ATCC10145

biofilms formed in the presence and absence of CAB CFCS' epitomised the prominent biofilm inhibiting activity of CAB isolates (Supplementary Fig. 2). CAB isolates, U7, U9, U10, U13, U14 and U16 significantly inhibited the biofilm formation in *P. aeruginosa* ATCC10145 about 89, 85, 79, 88, 86 and 79 % respectively. Biofilm mediated resistance and persistent infections caused by drug resistant biofilm forming bacterial pathogens wreak havoc on the life of human beings, animals and aquaculture organisms [26, 27]. They also contribute to environmental menace by creating biofouling related issues. Previous investigations unveiled that bacteria present in biofilms are up to 1,000-fold resistant towards antibiotics than their planktonic counterparts [10]. Antibiofilm agents promise a better future due to their ability to inhibit the initial adherence and preventing subsequent attachment of biofilm forming pathogens which are crucial prerequisite for the development of a persistent biofilm [28–32]. Some of the antibiofilm agents reported so far even possess disruption potential of pre-formed mature biofilms by disposing the extracellular polymeric substances. CAB treatment did not alter the planktonic growth of *P. aeruginosa* ATCC10145 (Supplementary Fig. 2), which further supports the statement that the CAB biosurfactants are acting as anti-virulence agents but not the growth inhibiting bactericidal agents which gain resistance in due course. Supplementary Fig. 2 explains the influential role of CAB isolates in controlling the biofilm formation without affecting the bacterial survival. Selective antibiofilm activity offers a positive hope for the development of new antimicrobial agents which primarily inhibit the virulence of pathogens

rather than their growth which in turn completely reduces the risk of resistance development [27, 28, 33].

The quest for novel bioactive agents which are efficient and stable under extreme conditions is incessant [35, 36]. Against this backdrop, the solidity of antibiofilm activity displayed by CAB isolates was tested at high temperature treatment. CFCS of isolates U7 and U9 were able to maintain their antibiofilm activity up to 76 and 77 % respectively even after the temperature treatment of about 100 °C (Supplementary Fig. 3). Proteinase K treatment of CFCS of CAB isolates revealed that the antibiofilm activity is not attributed to any enzymatic secretions (Supplementary Fig. 3). CFCS of isolates U10, U13, U14 and U16 were found to be susceptible to temperature treatment which depreciates their utility in diverse industrial setup. Until now, very few heat stable antibiofilm agents were reported which gives space for the search of novel heat stable antibiofilm agents [34]. The results are statistically significant ($p < 0.05$) when compared to their untreated control.

CLSM analysis of control and treated biofilms revealed significant antibiofilm activity of biosurfactants even at lower concentration ($100 \mu\text{g mL}^{-1}$). The biofilm thickness was quantified by CLSM. *P. aeruginosa* ATCC10145 control biofilm accounted for 26.2 μm thickness, whereas it was reduced up to 9.22, 7.73, 14.61, 12.61, 16.32 and 14.77 μm upon treatment with U7, U9, U10, U13, U14 and U16 biosurfactants respectively. Visible reductions in biofilm formation are shown two dimensionally in Fig. 1. BIC determination revealed that U7 and U9 biosurfactants were significantly ($p < 0.05$) effective in controlling the maximum biofilm formation at 80 and $100 \mu\text{g mL}^{-1}$ respectively (Supplementary Fig. 4). Though there are few studies available to date which demonstrate the biofilm disruption potential of biosurfactants, studies pertaining to the inhibition of *P. aeruginosa* biofilms by the same are scarce [7, 37]. To the best of our knowledge, the study by Luna et al. [8], is the only one that reports the biofilm inhibitory potential of lunasan biosurfactant against *P. aeruginosa*. Even then, the concentration of the lunasan biosurfactant isolated from *Candida sphaerica* UCP 0995 required to inhibit the biofilms of *P. aeruginosa* is very high (10 mg mL^{-1}) than the one reported in this study. Considering the lower BIC values obtained in the present study, further studies with these isolates could reveal additional novel biosurfactants with potential industrial and biomedical applications. Non-protein and thermo stable nature of the biosurfactants holds the promise for the development of antibiofilm agents for the application in extreme environmental conditions.

In conclusion, the present study unveils the potentials of coral associated bacteria as a promising source for biosurfactants. To the best of our knowledge this is the first report of biosurfactants from coral symbiont bacteria and

biosurfactant production from *Bacillus anthracis* and *Psychrobacter* sp. of marine origin. Outcome of the MATH assay has proved the higher affinity of CAB cultures to hydrocarbon, a typical characteristic feature of a biosurfactant producer which could be exploited for the bioremediation of hydrocarbon contaminated environments. Further characterization of the biosurfactants from *P. rettgeri* (U7), *Psychrobacter* sp. (U9 and U14) and *B. anthracis* (U13) is expected to reveal novel biosurfactants with feasible biological activities. Antibiofilm potency of biosurfactants at minimal concentrations will give an affluent lead for the development of novel anti-adhesive agents from marine microorganisms.

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Conflict of interest The authors declare that they have no conflict of interest.

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