Biomass Conversion and Biorefinery
https://doi.org/10.1007/s13399-023-04808-3

ORIGINAL ARTICLE

Antibiofilm activity of biosurfactant produced by a sponge-associated marine *Cobetia* sp. JCG-23

Govindarajan Ganesan[1] · Chandrasekar Balu[2] · Suganthi Ganesan[3] · Samuel Raj Babu Arulmani[2] · Sabariswaran Kandasamy[4]

Received: 1 July 2023 / Revised: 22 August 2023 / Accepted: 23 August 2023

Abstract
Marine symbiotically associated microbes play a vital role and are an excellent source of natural compounds that exert wide biological activities. In this study, we have reported on the identification, characterization, phylogenetic relationship, and anti-biofilm surface-active compound-producing abilities of marine invertebrate sponge-associated *Cobetia* sp. JCG-23. Among 24 isolates, a total of five strains (JCG2, JCG19, JCG20, JCG22, and JCG23) have active surface molecule producing potential on the emulsification index assay. Interestingly, the potential candidate JCG-23, produces biosurfactants with low surface tension (22 Nm^{-1}) that exert anti-biofilm activity against *Pseudomonas aeruginosa* PAO1. The isolate was identified as genus *Cobetia* sp. JCG-23 with 99.1% sequence similarity to *Cobetia crustatorum* (EU909460) based on 16S rRNA gene sequence analysis. The large-scale production, purification, stability, and characterization of biosurfactant were carried out and its surface activity was determined using the oil drop method. Subsequent spectral analysis such as UV, FT-IR, and GC-MS analysis indicated that the purified biosurfactant was a hydroxyl fatty acid, namely octadecanoic acid ($C_{18}H_{36}O_2$) with a molecular weight of 284 *m/z*. Furthermore, the effect of antibiofilm activity on the viability of *Pseudomonas aeruginosa* PAO1 by static ring tube and light and confocal laser scanning microscopy analysis revealed that the octadecanoic acid from *Cobetia* sp. JCG-23 has strong biofilm dismantle ability against *Pseudomonas aeruginosa* PAO1. Further characterization of the biosurfactant from the isolate *Cobetia* sp. JCG-23 can pave the way for developing novel bioactive agents targeting biofilm-forming pathogens on topical and medical devices.

Keywords Biosurfactant · *Cobetia* sp. · 16S rRNA · CLSM · Antibiofilm

1 Introduction

Marine symbiotic-associated microbe's exhibit specialized metabolic and physiological activities conferring them to synthesize novel biomolecules, notably biosurfactants [1, 2]. The diffusible surface-active chemical entities in marine ecosystems are implicated in ecological niches when they possess antagonistic properties [3]. Biosurfactants are amphipathic molecules exude in microbial cell surfaces which contain both (hydrophobic and hydrophilic) parts that converse the propensity to accumulate between fluid phases, as a result reducing surface and interfacial tension [4, 5]. These biosurfactants are well delineated chemically which categorized into high- and low-molecular-weight molecules [6]. A diverse group of microbes such as bacteria, fungi, and yeasts have been well studied to produces biosurfactants. These microbial procured surfactants have distinctive edge especially, low toxicity, biodegradable capability, and potency at extreme temperatures and pH values over their chemically synthesized surfactants [7–9]. In addition, microbial-derived biosurfactants exert wider biological activities, including antibacterial, antifungal, antibiofilm, anticancer, and antiviral activities, and these

✉ Govindarajan Ganesan
govind2610@gmail.com

[1] Department of Chemistry, Sri Shanmugha College of Engineering and Technology, Salem, Tamil Nadu 637304, India

[2] Department of Molecular Microbiology, School of Biotechnology, Madurai Kamaraj University, Madurai, Tamil Nadu 625021, India

[3] Department of Physics, Government college of Engineering, Dharmapuri, Tamil Nadu 6367041, India

[4] Department of Biotechnology, PSGR Krishnammal College for Women, 641004, Coimbatore, Tamil Nadu, India

Published online: 22 September 2023

properties aid in the development of alternative therapeutics when compared to conventional agents in biomedical practice [2, 8]. For an instant, suppression of uropathogenic biofilms on silicone rubber by biosurfactants (Protein-like) obtained from *Lactobacillus fermentum* RC-14 has been reported [10]. More recently, inhibition of biofilm and bacterial adhesion activities of glycolipid biosurfactant has been assessed from *Lactobacillus rhamnosus* [11]. However, it is well known that biosurfactants with antimicrobial properties being produced in response to the predators, which depicts their ecological significance and reveals the prolific benefit to targets the infectious diseases [12]. Several biosurfactants display antibiofilm potential, which reduces the colonization and adhesion of MDR clinical isolates on medical device surfaces due to the inhibition of pre-formed biofilms [13, 14]. Hence, microbial-derived surface-active molecules were well documented from soil or oil-contaminated environments, whereas these anti-adhesive compounds from marine microorganisms have been poorly studied [15]. Consequently, the microbial-derived biosurfactant can be used as a promising anti-infective alternative molecule to the classical agent to combat infection causing contagious pathogens [16]. In the present study, we report the efficient biosurfactant-producing *Cobetia* sp. JCG-23 strain isolated from marine invertebrates. Additionally, we have identified its molecular phylogenetic position through 16S rRNA gene typing. Furthermore, we have assessed the production, stability, structural characterization, and biofilm susceptibility of the biosurfactant against the biofilm-forming pathogen *Pseudomonas aeruginosa* PAO1.

2 Materials and methods

2.1 Sample collection

The marine sponge was collected at 15m depth in Kayalpattinam of the Bay of Bengal Ocean (8.5667° N, 78.1167° E), Tuticorin district, Tamil Nadu, India. Sponge was collected by using aseptic plastic bags along with sea water and immediately stored at 4°C. Then, collected sponge samples were aseptically transported to the laboratory and stored at 4°C until further use. Fifteen grams of sponge tissue sample was excised from the middle region of *Axinella* sp. by a sterile cutter. The excised sponge was suspended in 10 mL of sterilized distilled water and homogenized tissues using a mortar and pestle. Subsequently, serial dilutions up to 10^{-6} were carried out, and each diluted sample was spread on sterile seawater-containing nutrient agar plates [16]. There were 24 morphologically distinct pure colonies which were isolated and subcultured by using nutrient broth at 30°C for further experimental studies [17].

2.2 Emulsification index

All 24 strains cultured in 200 mL of nutrient broth and kept at 28 °C, 120 rpm for 48 h. The emulsification assay (EI) was determined by mixing of equal volume of cell culture broth and hydrocarbons (1:1 ratio) and vortexing firmly for 1 min. Then, the tubes were kept for 24 h then measured the emulsion layer [18]. Subsequently, different methods were carried out to confirm the efficiency of the strains in terms of biosurfactant production. Methods were (i) hemolytic activity: All the isolates were screened by simple streaking for hemolytic activity on blood agar plates consist of 5% (v/v) human blood and plates were kept at 37 °C for 24 h. Hemolytic activity was observed based on a defined clear zone around colonies [19]. (ii) Oil displacement test: Oil displacement method is used to determine the diameter of the clear zone which can be achieved after exposure of biosurfactant into on oil-water interphase. Twenty milliliters of distilled water were added to a Petri dish (15 cm diameter) and 50 µL of diesel were placed to the surface of water followed by addition of 100 µL supernatant from 24-h culture broth on to the surface of oil (diesel). Occurrence of clear zone was measured after 30 s. The diameter assessment indicates the surface tension reduction potential of biosurfactant. Sterile medium and SDS were used as negative control and positive control respectively [20]. (iii) Hydrocarbon overlay assay: Minimal medium was supplemented with 100 µL of diesel as solitary carbon source separately. Then, the bacterial isolate was spotted on the hydrocarbon amended plate and incubated for 30 °C for 48 h. Colony surrounded by an emulsified halo was considered as positive for biosurfactant producer [21]. Among these, a potential candidate was screened and used for further studies.

2.3 Surface tension measurements

Surface tension was measured for culture broth of isolate JCG-23 by using the standard drop weight method [22, 23]. Surface tension was measured for the culture broth of isolate JCG-23 using the standard drop weight method [22, 23]. To determine surface tension, the cell-free supernatant of *Cobetia* sp. JCG-23 was introduced into a glass column. The lower end of the column was connected to a rubber tube. Subsequently, a pre-weighed clean beaker was placed beneath the column, and the cell-free extract was gently delivered drop by drop into the beaker. A total of 20 drops were collected and their weight was measured. The following equation was employed to calculate the surface tension of the cell-free supernatant [23]

$$M = W2 - W1/\text{total drops}$$

Mass of one drop = W_2 − weight of the sample with beaker-W_1 weight of the empty beaker/total number of drops

Surface tension (T) = $\mathrm{Mg}/\pi \mathrm{r} \times 10^{-3} \times \mathrm{nm}^{-1}$

Where

m	mass of one drop of the liquid
g	acceleration due to gravity
r	radius of the capillary tube
Surface activity	surface tension of the medium alone− surface tension of culture broth.

2.4 CTAB-MB assay

CTAB-MB agar method was subjected to screening the glycolipid-producing abilities. In order to identify the nature of biosurfactant, the JCG-23 strain was cultured in (CTAB-MB) agar plates by supplementing 0.3 g cetyltrimethylammonium bromide (CTAB), 5mg of methylene blue, and 18 g agar in 100mL of mineral medium; then, cultures were incubated for 48 h at 30 °C [24].

2.5 Identification and phylogenetic analysis of the isolate JCG-23

Among 24 isolates, the potential biosurfactant producing isolate JCG-23 was selected for further morphological, biochemical, and physiological parameters, including identifying its molecular phylogeny position. The biochemical features such as starch, cellulose, lipid, casein, catalase, gelatin liquefaction, nitrate reduction, citrate utilization, triple sugar iron, and MR-VP tests were carried out according to Govindarajan et al. and Cappuccino and Sherman [25, 26]. Utilization of carbohydrates was investigated with a basal carbon nutrient medium. The physiological studies such as salinity tolerance (NaCl), pH, and temperature were determined for the isolates JCG-23 as per literature [25, 27]. Furthermore, molecular characterization was done by using 16S rRNA gene sequencing. The 16S rRNA gene of isolate JCG-23 DNA was amplified using primers 27F 5′-AGA GTTTGATCCTGGCTCAG-3′ and 1492R 5′-GGTTACCTT GTTACGACTT-3′ [27]. Then, sequencing was performed with Applied Biosystems 3730XL DNA Analyzer with Big-Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA) [25]. The 16S rRNA gene sequences were analyzed by the BlastN search from the GenBank database. 16S rRNA gene sequences of closely related type strain sequences were retrieved from NCBI BLAST and Ribosomal Database Project (RDP-II) [27, 28]. The phylogenetic tree was constructed by the neighbour-joining method with the help of PHYLIP (version 3.68) software package, and maximum-parsimony methods were adapted to generate bootstrap values over 1000 re-samplings [27, 29]. The 16S rRNA gene sequences were deposited under accession number MH779825 in the GenBank database.

2.6 Biosurfactant stability study

To determine the biosurfactant stabilities, various parameters were checked, such as temperature, pH, and salinity. For temperature stability of biosurfactant, the supernatant was kept at a constant temperature range from 0 to 100 °C for 15 min; subsequently, it is allowed at room temperature for cooling. Likewise, to find out the effect of pH on stability, the pH of the culture broth was adjusted at pH range of 2.5–12.5 using 0.5 N NaOH or 0.5 N HCl. The effect of NaCl (ranges from 1to 10%) that influencing activity of the biosurfactant was also examined [30–32].

2.7 Extraction and purification of biosurfactant

Mother culture was propagated by transferring a pure colony of *Cobetia* sp. JCG-23 into 100 mL of seawater-containing nutrient broth and incubated for 12 h. Ten percent of the prepared seed was subcultured into a 2-L culture flask which consists of 500 ml of fermentation medium (pH 7.0); a total of 10L were incubated at 30°C in 150 rpm for 8 days. Thereafter, the biosurfactant was precipitated by acidifying cell free broth to pH 2.0 with 6N HCl and kept at −20°C for 18 h; then, biosurfactant was recovered by centrifugation at 10,000 rpm for 30 min. Then, it was dissolved in dH_2O to adjusted the pH 7.0 using 1N NaOH. Subsequently, the biosurfactant was extracted with chloroform/methanol/water (2:2:1) ratio and quantified. The product containing phase were separated and evaporated to dryness. Finally, the biosurfactant residue was dissolved in chloroform [33, 34]. Furthermore, biosurfactant was purified using preparative TLC silica gel (mesh size 100–200). The silica gel was prepared and run with mobile phase of chloroform: methanol (6:4v/v), and separation was visualized using an iodine chamber for 30 min. The Rf value was determine as per described methods [35, 36].

2.8 Biosurfactant production kinetics

The kinetics of biosurfactant production was performed in batch cultures for 10 days at optimum conditions by measuring the emulsification assay of supernatant samples obtained after cell separation [37].

2.9 Characterization of biosurfactant

The absorption maxima of the TLC-resolved biosurfactant were checked by UV-Vis (200 nm to 400 nm)

spectrophotometer. FT-IR spectrum of purified biosurfactant was subjected to MCT detector and germanium-coated KBr plate beam splitter for the functional group analysis [30, 34]. GC-MS (gas chromatography-mass spectrometry) (Thermo GC - trace ultra ver: 5.0, thermo MS DSQ II) was used to analyze the purified biosurfactant. The data was processed by DB 35 – MS capillary standard non-polar column with oven temp 70 °C raised to 250 °C for 10 min with flow rate 1mL/min using helium gas. After that, generated peaks were compared with the mass library to determine the fatty acid composition in the biosurfactant [36].

2.10 Antibiofilm assay

The MTP assay were done to study the activity of biosurfactants (100µg/mL) on *Pseudomonas aeruginosa* PAO1 producing biofilm formation. About 1% of 12 h culture of PAO1 (0.5 OD=600 nm) were aseptically transferred into fresh LB medium with 1% glucose in the presence and absence of biosurfactant. The 24-well polystyrene plates were allowed for 16 h at 37°C, planktonic cells along with consumed media were drawn out, and adhering cells were washed with dH_2O and permit to air-dry. After that, biofilms were developed using 0.4% crystal violet (w/v) for 5 min and gently rinsed with dH_2O. 100% ethanol was used to quantify the dye absorbed by the biofilm-forming cells and it was measured at 650nm [38–40]. In addition, a static ring tube test was executed to estimate the outcome of biosurfactant extract on PAO1 biofilms. The PAO1 cells (~OD 0.5= 600 nm) were grown in the presence and absence of biosurfactant ranges from 100 to 500 µL and spawn at 37 °C for 24 h; then, biofilm growth on the surface was estimated [41].

2.11 Anti-biofilm assay by confocal laser scanning microscopy

Twelve-hour culture of PAO1 was exposed to biosurfactant 100µg/mL (w/v) (final concentration) amended with 1.0 mL of LB broth in polystyrene plates (24-well) and cells were grown without biosurfactant as a control. After that, MTP plates were spawn for 24 h at 37 °C, and the planktonic cells and depleted media were removed and adhesive cells (on glass slides) were gently washed with dH_2O and kept for air-dry before being stained [42]. For confocal laser scanning microscopy (CLSM) analysis, 0.1% acridine orange solution (w/v) was used to stain the biofilms, and for light microscopic examination, 0.4% crystal violet was used. Subsequent washing steps were followed as per above said method. The stained biofilms were visualized under CLSM (Model LSM 710, Carl Zeiss, Germany) 480nm and 530nm with 40× magnification and 100× magnification under light microscope [43]. The experiment was performed in triplicates.

2.12 Growth curve

To assess the antimicrobial activity of the biosurfactant against *Pseudomonas aeruginosa* PAO1, a growth curve analysis was conducted both in the presence and absence of the biosurfactant. An overnight culture of PAO1 was adjusted to an optical density (OD) of 0.5 at 600 nm using a fresh sterile medium. Subsequently, 1% of the PAO1 culture was inoculated into 100 mL of LB broth supplemented with 1% glucose. The specified concentration of biosurfactant (10 mg/mL) was then introduced into the culture broth, which was then incubated at 37°C for 24 h. At hourly intervals, the OD was measured at 600 nm. A culture medium without biosurfactant can be used as a negative control [44]. The assay was performed in triplicates.

3 Results and discussion

3.1 Isolation of biosurfactant producers

In our present study, we have successfully isolated *Cobetia* sp. JCG-23, a strain capable of producing a highly effective surface-active compound, from the sponge *Axinella* sp. (Figure S1 in the supporting information). Primarily, the hemolytic assay can be used to screen the non-hemolytic isolates that produce the biosurfactant to control biofilm formation on surfaces and medical devices. Among 24 isolates, only five members exhibited negative hemolysis effects and were selected for biosurfactant assay in the liquid broth. Generally, hemolysis is a rapid method to identify biosurfactant producers [45, 46]. However, the method shows some limitations [47]. Schulz et al. [48] reported that some biosurfactants failed to show any hemolytic effect. Other reports recommended that the hemolytic assay is a primary method for biosurfactant screening, and that can be further confirmed by surface tension measurement [49]. The isolates JCG-23 show the negative hemolytic effect when compared to other screened biosurfactant producers. Bacteria, a symbiotically associated with marine sponges represent a rich source of novel bioactive metabolites. This symbiotic association has sparked specialized biosynthetic pathways, resulting in the production of bioactive metabolites that safeguard soft-bodied invertebrates against pathogenic biofilm-forming pathogens.

3.2 Screening of biosurfactant producers

Assessment of emulsification activity is a versatile screening technique for identifying biosurfactant-producing microbes. The emulsification capability of biosurfactants was evaluated

for non-hemolytic marine isolates with different hydrocarbons such as diesel and kerosene. The emulsification index has been determined after 24 h of incubation. Among these, isolate 23 showed the highest emulsification index with diesel (70%) and kerosene (65%) (Fig. 1a); the emulsification index values are shown in Table 1. The emulsification index assay is the most reliable method to determine the efficiency of biosurfactants and is used to identify the potential bioemulsifier producer [50]. Consequently, this strategy might be considered as an effective assay for screening of surface-active compound producing strains [51]. Thus, isolate JCG-23 had more prominent results than the other biosurfactant producer. All non-hemolytic strains exhibited the highest emulsification index E24 (%) in diesel compared to kerosene. These results suggest that the strain's produced biosurfactant demonstrates a higher emulsification specificity for diesel, whereas its specificity with kerosene is lower. This finding indicates that the emulsification activity primarily relies on an affinity towards the hydrocarbon's nature. Thus, it is evident that the interaction directly with the hydrocarbon, rather than the surface tension of the medium, plays a pivotal role. A previous study also supports these findings [52]. Diesel emerges as the superior substrate, displaying a higher emulsification index of E24 than kerosene. Earlier research by Patel et al. noted the highest emulsification index in n-hexadecane, followed by gasoline and diesel, surpassing kerosene [11].

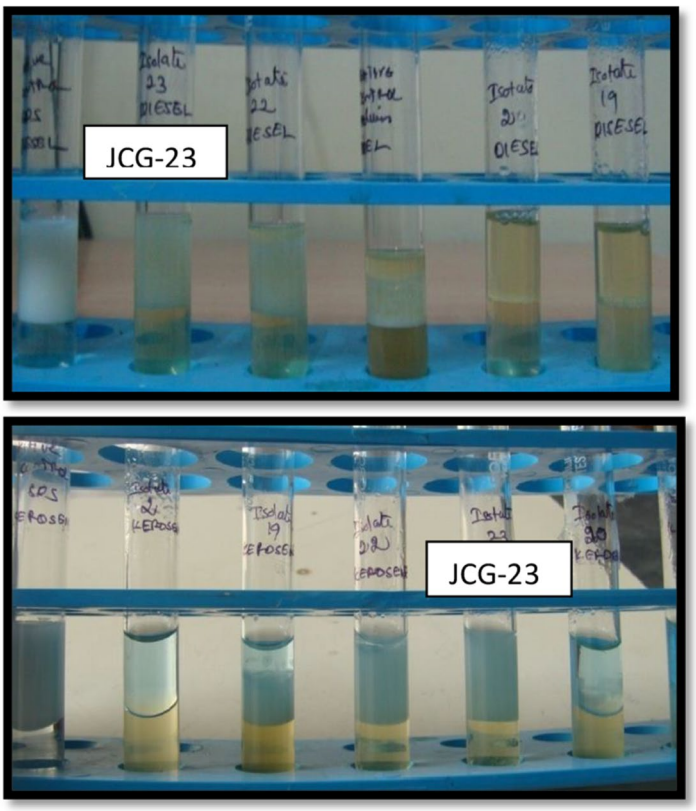
In addition, different biosurfactant screening tests such as oil drop, hydrocarbon overlay, and CTAB-MB agar assays were done for the selected isolates JCG-23. Differences in the ability to displace the oil and spread it in the water were observed. 1% SDS was used as a positive control. Our experimental results show the isolate JCG-23 effectively displaced the oil and made a clear zone of 6.5 cm than the SDS 4.5 cm (Fig. 1b). In general, biosurfactant producing microbes have the ability to displace the oil. Thus, this indirect evaluation of the surface activity of broth culture of isolate against oil represents the reduced surface tension with the promising action of biosurfactant [53, 16]. On the other hand, the hydrocarbon overlay method is another effective screening method to identify the biosurfactant producers. The isolate JCG-23 utilized the carbon source, and the clear zone was observed around the colonies (Fig. 1c). The test confirmed the occurrence of biosurfactants in the cell-free supernatant. Isolate JCG-23 has adequate growth on hydrocarbon-containing solid media due to the biosynthesis of surfactant that facilitates cell adherence on the oil droplets [16, 54]. Similarly, marine *Cobetia* sp. strain MM1IDA2H-1, which influences the formation of micelles or vesicles when grown on dibenzothiophene (DBT) as the unique energy (carbon) source [12]. These lipid-like arrangements are very mandatory for the digestion and metabolism of DBT and also enhance the bioavailability of plausible substrates by stimulating their dissipation and solubilization. In order to identify the nature of biosurfactants, a CTAB-MB agar plate assay was carried out. The strain JCG-23 failed to produce a dark blue zone around the colonies, indicating the absence of rhamnolipid (Fig. 1d). The results obtained from these studies, including hemolytic activity, emulsification index assay, hydrocarbon overlay method, and oil drop method, revealed that the isolate JCG-23 produce a potential surface-active compound. These results were supported by already available reports of biosurfactant screening methods [55–57].

3.3 Surface tension measurement

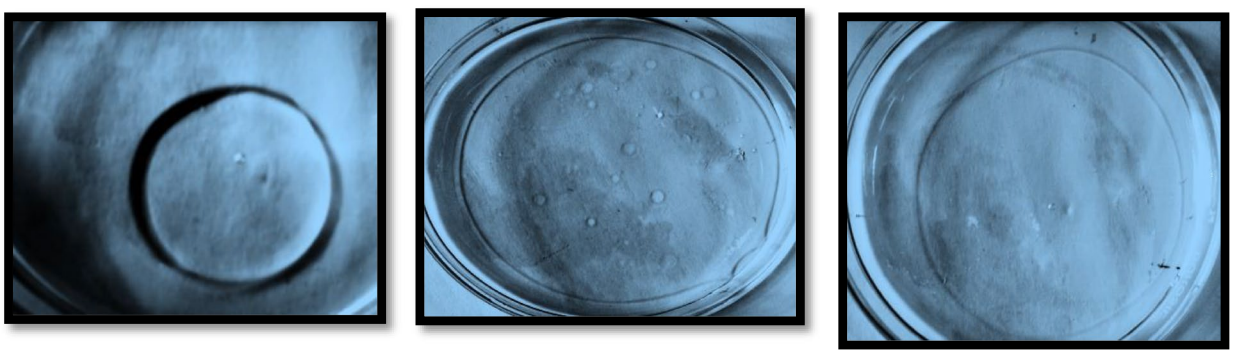
The surface tensions of the culture broth of the isolate JCG-23 were checked, and results show that the *Cobetia* sp. JCG-23 has a low surface tension 22 Nm^{-1} than the surface tension of the pure H_2O (68 Nm^{-1}) and SDS (42 Nm^{-1}) respectively. The surface activity measurement of the cell free culture broth is a simple and highly reliable prefatory screening strategy of biosurfactant inducing microbes. It imparts an impregnable evidence for biosurfactant secretion. Previous reports suggest an effective biosurfactant can reduce the surface tension of water from 72 dynes/cm to 25–30 dynes/cm [58–60]. Based on the literature, *Pseudomonas aeruginosa* widely studied biosurfactant producer, the surfactant molecule produced by the microorganism reduced surface tension to 28 to 27mN/m [61–63]. The decreased surface tension implies the luxury growth of microorganisms [12, 36]. Cooper and Goldenberg [18] suggest that if bacterial strain reduces the surface tension to 40mN/m or less could be consider as good biosurfactant producers. The obtained results suggested that the strain JCG-23 is a potential producer of superior biosurfactant, and the isolate was selected for further physiological, biochemical, and molecular characterization.

3.4 Physiological, biochemical, and molecular phylogenetic analysis of JCG-23

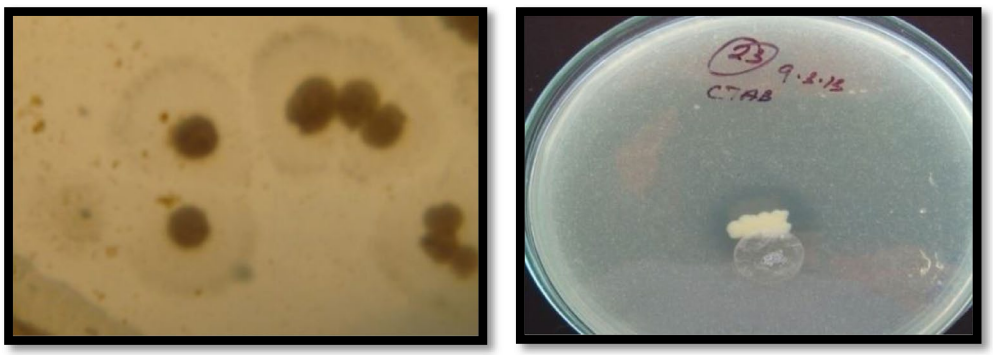
Physiological and biochemical attributes of the isolate JCG-23 were inspected and identified as gram-negative bacteria. The isolate JCG-23 grew effectively in the existence of NaCl (upto-9.0% (w/v), and poor or non-growth was observed in the absence of NaCl. The strains were grown from 25 to 37 °C, and the suitable growth temperature was found at 37 °C. It also suggested that the isolate JCG-23 was a relatively halophilic bacterium native to the marine ecosystem. Ibacache-Quiroga et al. [12] reported moderately halophilic, a hydrocarbon catabolizing marine biosurfactant producing *Cobetia* sp. strain MM1IDA2H-1 which has been isolated from seawater samples. The isolate JCG-23 utilized a wide variety of carbon sources such as cellobiose, sorbitol, adonitol, dextrose, fructose, mannose, inositol, maltose,



a)



b)



c d

◂**Fig. 1** **a** The highest emulsification index of the biosurfactant producing isolate JCG-23. **b** Oil displacement method by culture extract from isolate JCG-23; (i) oil, (ii) oil and culture extract, and (iii) 1% SDS (positive control). **c** and **d** Hydrocarbon overlay and CTAB assay for the isolate JCG-23

rhamnose, melibiose, galactose, and raffinose but not lactose, salicin, dulcitol, mannitol, xylose, and Inulin [28]. Subsequent biochemical characterization revealed that the isolate JCG-23 was positive for citrate and gelatin hydrolysis and negative for TSI, indole production, and methyl red tests (Table S1 in the supporting information).

Based on the primary morphological, physiological and cultural characteristics of the isolate JCG-23 clearly denoted the genus *Cobetia.* The phylogenetic investigation of the 16S rRNA gene showed that the isolate JCG-23 belonged to the genus *Cobetia* and exhibited 99.1% sequence similarity with a type strain, *Cobetia crustatorum* (EU909460) (Fig. 2). The 16S rRNA molecular phylogenetic analysis indicates that the genus exhibit various geographical region with incredible eco-physiological differences [64].

Table 1 Emulsification index E24 (%) of non-hemolytic isolates

Name of the isolate	Diesel	Kerosene
Isolate JCG2	21%	20%
Isolate JCG19	22%	20%
Isolate JCG20	61%	57%
Isolate JCG22	62%	52%
Isolate JCG23	70%	65%
SDS (positive control)	75%	70%

The values are represented as the mean of triplicate experiments

3.5 Determination of biosurfactant stability

3.5.1 Effect of temperature, pH, and NaCl on biosurfactant

To determine the temperature steadiness of the biosurfactant, broth culture of JCG-23 was sustain at a wide temperature ranges from 0 to 100 °C. The effect of different temperature ranges on biosurfactant activity was evaluated by emulsification index assay. The obtained results reveals that the heating the cell free culture broth from 30 to 100 °C caused no significant loss in the biosurfactant performance. The emulsification index (56%) was relatively stable at 100 °C for 24h. This sustained effect of the biosurfactant compound secreted by JCG-23 confirms the thermostability (Fig. 3a). Similarly, biosurfactant stability under various pH ranges were checked by E24 calculation. The obtained results showed that with the expand pH from acidic to alkaline conditions, the emulsification index (E24 =84%) steadily increased results were shown in (Fig. 3b). The more significant effects of biosurfactant are mainly dependent on optimum pH. This worthwhile emulsion under various pH ranges shows that the biosurfactant from isolate JCG-23 was highly stable. The effect of NaCl on biosurfactants was shown in (Fig. 3c). The optimum stability of biosurfactant was observed at 4% NaCl concentration. Minor changes were observed in increased concentrations of NaCl up to 10% (w/v). At higher concentrations of NaCl, the biosurfactant retains 50% of the emulsification activity. The greater usage of biosurfactants is mainly dependent on salinity, temperature, and pH. Based on our study, the biosurfactant produced by *Cobetia* sp. JCG-23 are stable when exposed at different temperature levels up to 100 °C for 24 h. Likewise, biosurfactant activity increased and retained its original properties over pH ranges from acidic to basic (2.5 to 12.5) and withstand salinity up to 10%, confirmed by positive emulsion index assay. Busscher et al. [65] reported *Pseudomonas aeruginosa* P4-derived

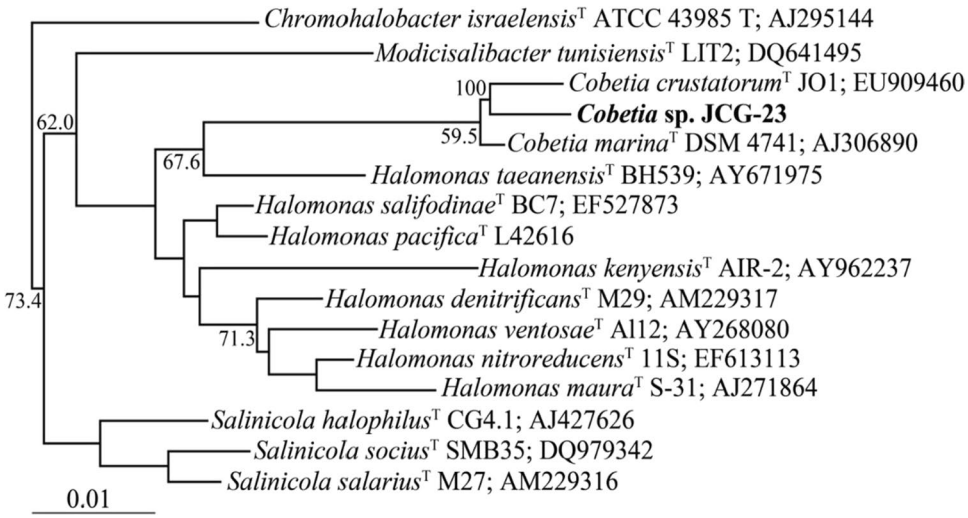
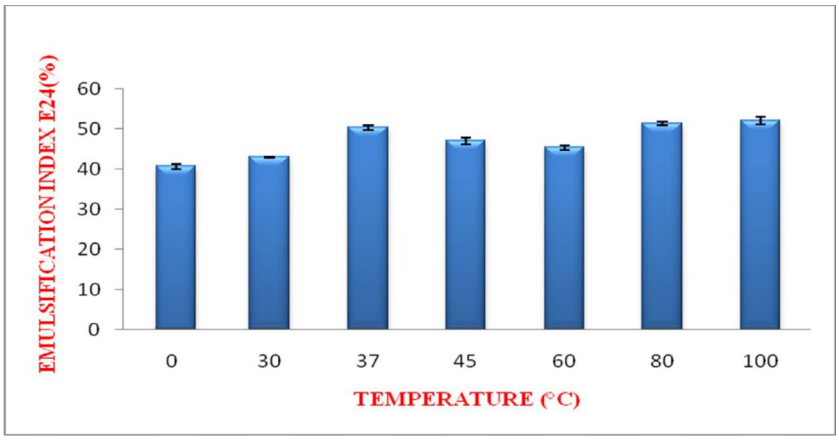
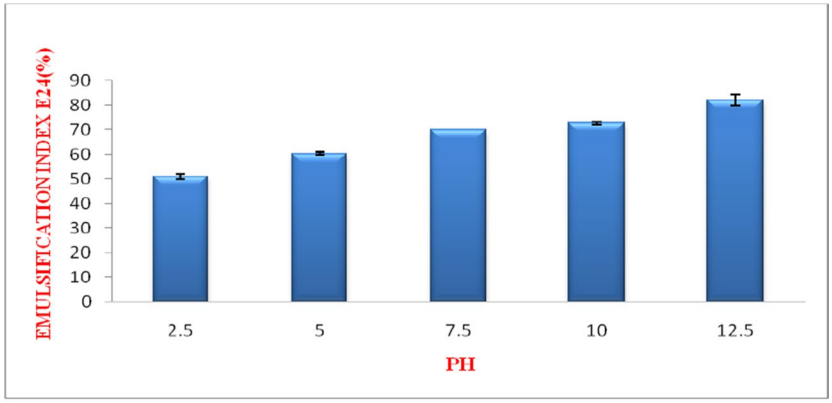


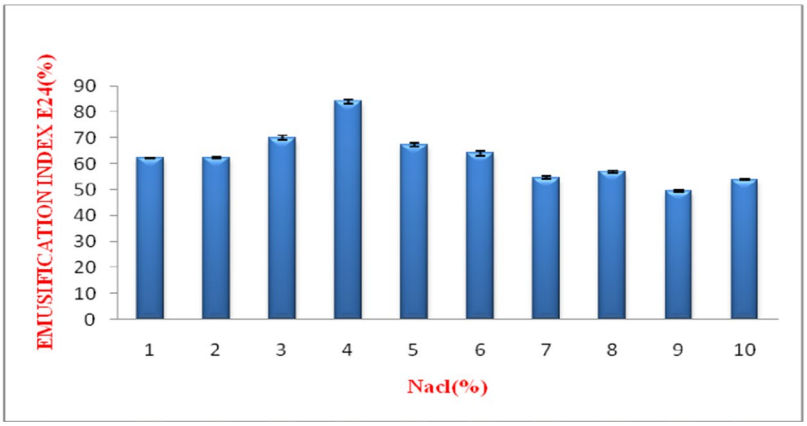
Fig. 2 Phylogenetic tree view of isolate JCG-23, constructed based on neighbor-joining method. The topology of tree was analyzed by bootstrapping with 1000 replicates and 0.01 scale bar indicates the nucleotide substitution level



a



b



c

Fig. 3 **a** Effect of temperature on biosurfactant stability. **b** Effect of pH on biosurfactant stability. **c** Effect of salinity on biosurfactant stability

biosurfactants from petroleum contaminated sites are found to be stable at wide ranges of pH and temperature. In addition, *Candida sphaerica* cell-free extract found as highly stable at pH (1–12), at a temperature of 120 °C and had the ability to withstand a high NaCl concentration of 10% [66]. This study was also supported by other reports [23]. These positive emulsions under various pH ranges, temperatures, and salinity indicated that the biosurfactant from isolate JCG-23 was highly stable and used for wider application.

3.6 Growth characteristics and biosurfactant production of *Cobetia* sp. JCG-23

Biosurfactant production by *Cobetia* sp. JCG-23 was denoted by time course study, and the results were shown in (Fig. 4). It was observed that the organism germination increased moderately until day 6, and that the biosurfactant secretion was observed by emulsification activity assay after the 2nd day of incubation period. After that, their activity was

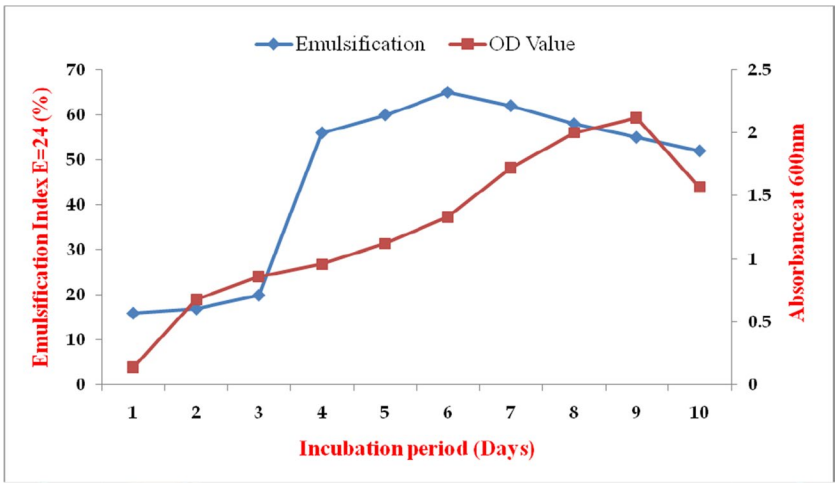


Fig. 4 Growth kinetics and biosurfactant production of *Cobetia* sp. JCG-23

gently increased up to the 9th day of incubation. The results indicate that biosurfactant production mainly depended on the vegetative growth phase. The maximum biosurfactant production can be achieved during log phase by the *Cobetia* sp. JCG-23. The time course study shows that the synthesis of biosurfactants according to the growth rate of the *Cobetia* sp. JCG-23, which begins at the log phase and is sustained until the end of the stationary phase. Similarly, another study reveals that the *L. rhamnosus* biosurfactant synthesis begins at the log phase and abiding until the end of the stationary phase [11]. In addition, *Lactobacillus helveticus* produced biosurfactants are also found to be growth associated, but maximum yield of biosurfactant has been attained at the stationary growth phase [36, 67].

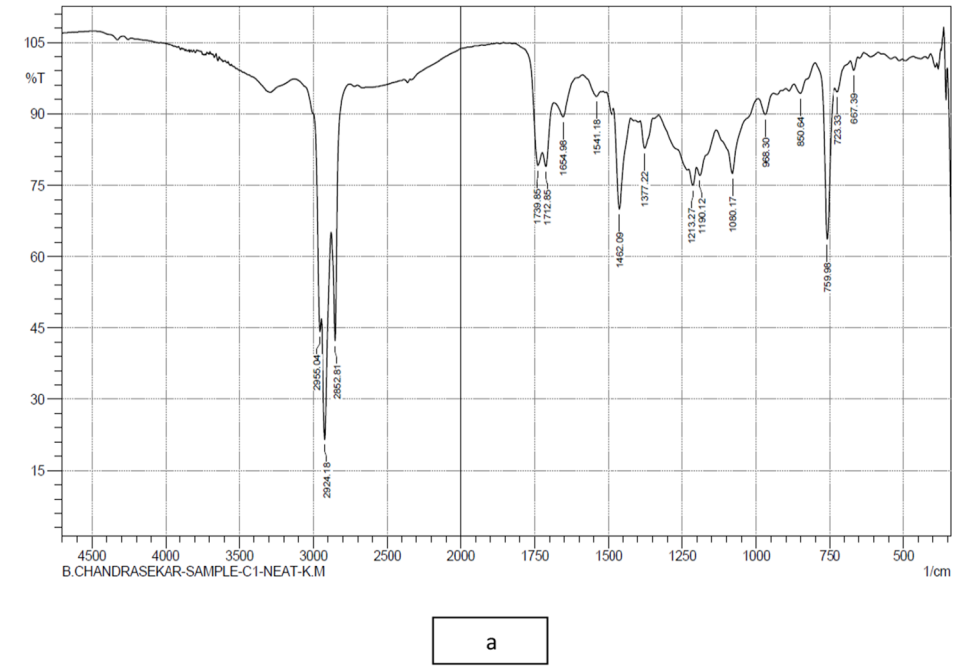
3.7 Purification and characterization of biosurfactant

The fermentation results shows that the isolate JCG-23 might be competent to produce 1.0 g/L of biosurfactant within 8 days of incubation. The obtained TLC results indicated that the purified surface-active molecule has an Rf value of 0.87. Furthermore, UV spectra analysis of the surface-active molecule exhibited maximum absorbance at 280 nm. The functional group of biosurfactant was identified by FT-IR; the strong signals at 2924 cm^{-1}, 1739 cm^{-1}, and 3000 cm^{-1} to 3500 cm^{-1} suggest hydrocarbon, carbonyl, and hydroxyl group, respectively (Fig. 5b). The FT-IR spectrum analysis confirmed the presence of aliphatic chains in the structure of the biosurfactant. Similar function groups were identified from biosurfactants produced by marine *Cobetia* spp. and other genera [12, 36]. GC-MS spectrum of the purified biosurfactant from *Cobetia* sp. JCG-23 suggested that the presence of octadecanoic acid consists of hydroxyl fatty acid (Fig. 5b). Consequently, the UV, FT-IR, and GC-MS spectral data committed the existence of long-chain aliphatic fatty acid octadecanoic acid with a molecular weight of 284 *m/z*. A similar type of structure was also reported by earlier studies [12]. On the other hand, the existence of hydroxyl fatty acids in the cell-free broth were related to outer membrane vesicles (OMVs) [12]. These lipid arrangements are generally synthesis and secreted by Gram-negative bacteria, which plays an important biological role in the nutrient accession or cell-to-cell imparting [68, 12]. The cell wall structure of Gram-negative bacteria has a complex morphological structure consisting of an OM (outer membrane), a narrow shell of peptidoglycan with ample area surrounding the peptidoglycan. The OM contains lipopolysaccharide, which acts as a surface interaction region for various components surrounding the environment and acts as a partial chemical sieve and making them one of the defense systems against toxic compounds. The surfactant molecule can disturb the outer membrane in order to permeabilize the inner membrane [69].

3.8 Antibiofilm activity

The anti-biofilm property of biosurfactant was examined for the biofilm-forming marker strain of *Pseudomonas aeruginosa* PAO1. The static ring tube biofilm experiments result showed the concentration-dependent alleviation in PAO1 biofilm with increasing concentration of biosurfactant, as shown in (Fig. 6). In addition to the static ring tube assay, the light microscopic and CLSM studies on *Pseudomonas aeruginosa* PAO1 biofilm were examined. The images obtained from the light microscopic analysis revealed that the untreated cells illustrate thick biofilm formation of PAO1 compared to PAO1 treated with biosurfactant.

The observed results revealed that reduced biofilm formation compared to the control sample (Figs. 7A and 7B), respectively. To corroborate the findings derived from light microscopy, we conducted confocal laser scanning microscopy (CLSM) analysis to examine further the inherent antibiofilm properties of the biosurfactant against the



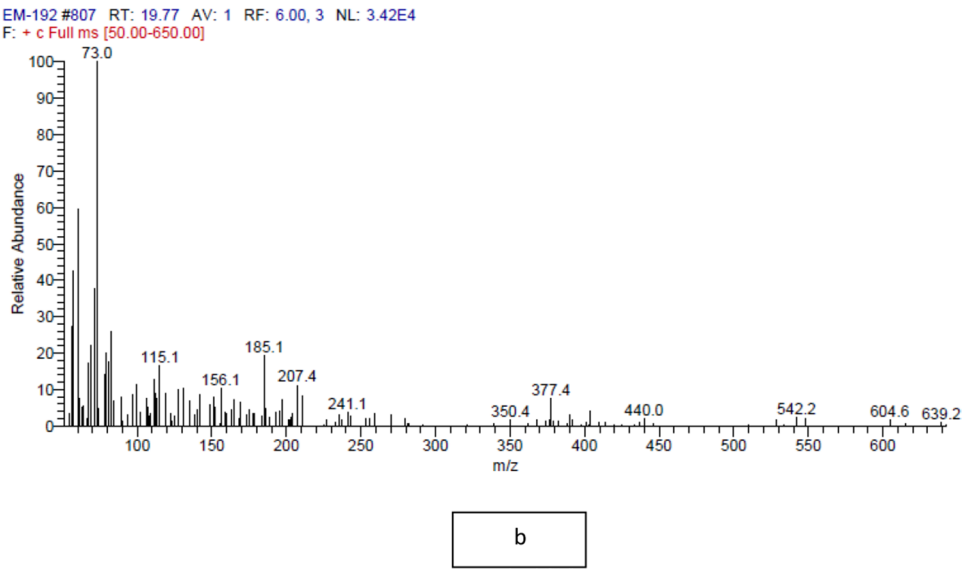


Fig. 5 **a** FT-IR and **b** GC-MS spectrum of biosurfactant from *Cobetia* sp. JCG-23

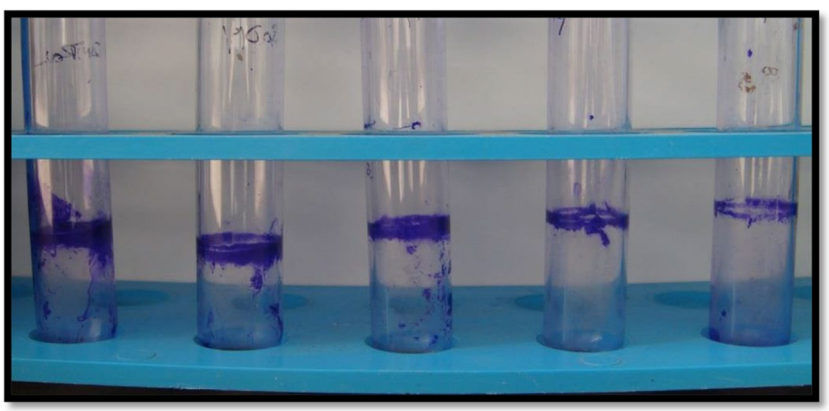


Fig. 6 Static biofilm ring assay of biosurfactant against *Pseudomonas aeruginosa* PAO1

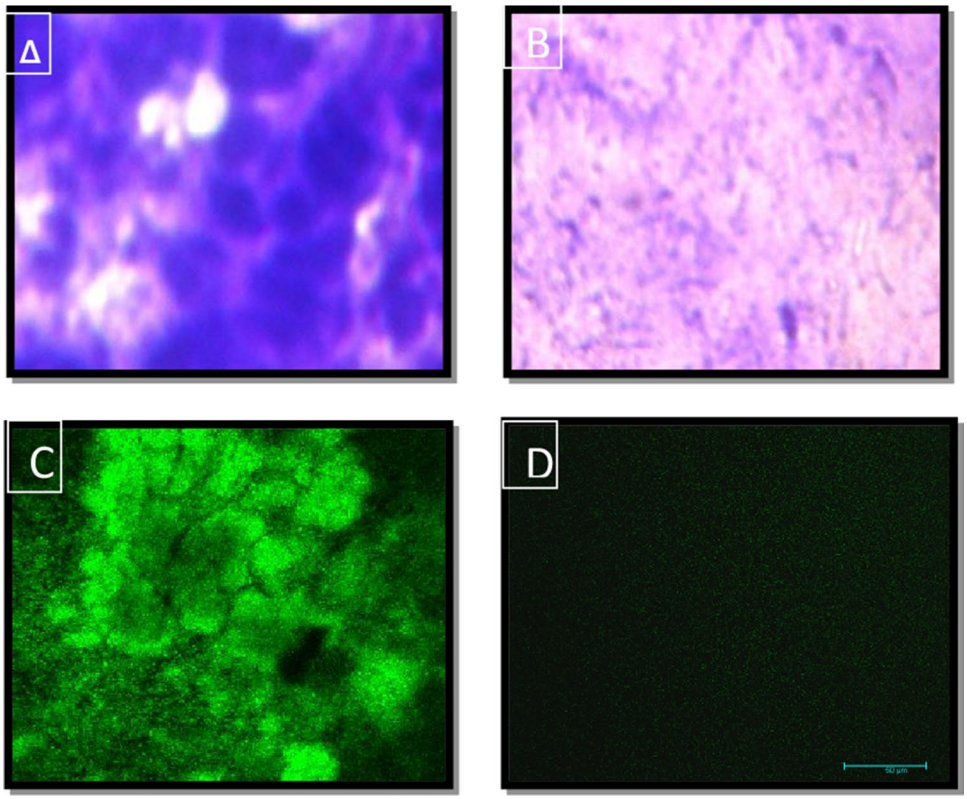


Fig. 7 Light microscopic and CLSM images of *Pseudomonas aeruginosa* PAO1 biofilms grown in the absence and presence of biosurfactant. Light microscopic images of *Pseudomonas aeruginosa* PAO1 in the absence (**A**) and presence (**B**) of biosurfactant. CLSM image of *Pseudomonas aeruginosa* PAO1 biofilms grown in the absence (**C**) and presence (**D**) of biosurfactant

PAO1 strain, as depicted in (Figs. 7C and 7D). The CLSM images unveiled the highly dense biofilm-forming capacity of the reference strain PAO1, attributed to its robust adhesion properties on untreated glass surfaces. In contrast, the biosurfactant-treated samples exhibited a complete disruption in the biofilm architecture of the PAO1 strain. The substantial alteration in the *Pseudomonas aeruginosa* (PAO1) biofilm structure observed through CLSM strongly supports the biosurfactant's potential antibiofilm activity. This showcases its effectiveness in reducing the biofilm formation of the PAO1 strain. The surfactant molecule produced by the *Cobetia* sp. JCG-23 exerted no or very less antimicrobial activity against the Gram-negative *Pseudomonas aeruginosa* PAO1 with huge biofilm dismantling ability. Similarly, a few lactic acid bacteria (LAB) produced biosurfactants that exert anti-biofilm activity against a range of pathogens [70–72]. Recently, antibiofilm effect of *S. haddoni* extract including biofilm inhibition and destruction was reported against different pathogens [40]. In another study, the optimum biofilm inhibition of *P. aeruginosa* (90.86%) has been achieved when cells exposed to the sponge extracts. Furthermore, *Psammocinia* sp. and *Hyattella* sp. derived

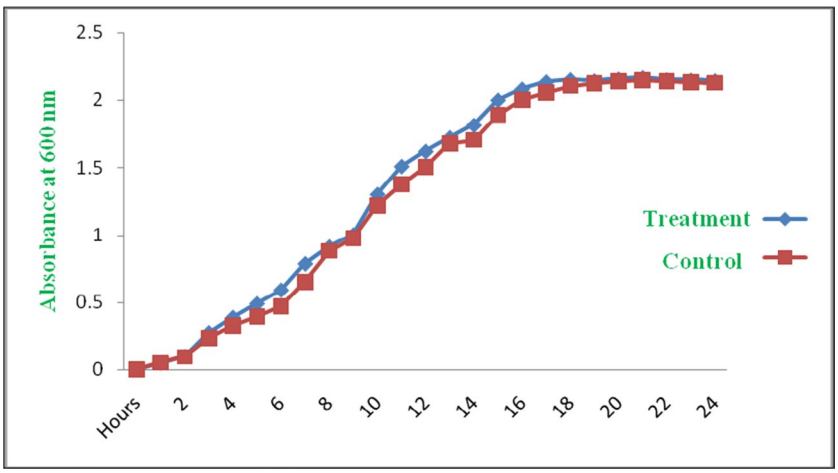


Fig. 8 Growth curve analysis of *Pseudomonas aeruginosa* PAO1 in the presence and absence of biosurfactant

methanolic extracts had exhibited destruction of biofilm architecture against *K.pneumonia* (90.32%) and *E.coli* (94.33%) respectively [39]. The biosurfactants from various microbes are promising applications in many fields. Several studies revealed that many marine bacteria could produce novel anti-biofilm compounds that have not been tapped from terrestrial environments [73]. The biofilm biomass, as determined by the crystal violet adherence and CLSM image analysis, depicted the surfactant produced by the *Cobetia* sp. JCG-23 successfully dismantle the developing bacterial biofilms. The hydroxyl fatty acids from *Cobetia* sp. JCG-23 can serve as disseminative messengers capable of deconstruct the biofilm of PAO1 without any antimicrobial activity. Thus, the reduction of biofilm development by octadecanoic acid of long-chain hydroxyl fatty acid is believed and leads to the development of the novel anti-biofilm surface-active compound. The microbial biofilm dispersing ability exhibited by *Cobetia* sp. JCG-23 can be exploited in healthcare industries to overcome the problems caused by biofilm based community acquired infections.

3.9 Growth curve determination

The growth curve investigations of *Pseudomonas aeruginosa* PAO1 in the existence and non-appearance of biosurfactant was executed to confirm the antibacterial effect, if any. The results show that there is no substantial alteration in terms of bacterial growth in both biosurfactant exposed and un exposed bacterial cultures. This indicates biosurfactants were futile on the growth of PAO1 (Fig. 8). Similarly, two sponge extracts (*Hyatella* sp. and *Psammocinia* sp.) significantly reduce biofilm formation and destruction against WHO declared priority pathogens without exhibiting antibacterial effect [39]. These obtained results provide further supports to demonstrate the effective antibiofilm potential without display an antibacterial effect against tested pathogen PAO1. Further deeper insight into the structure and activity of the biosurfactant from the isolate JCG-23 can pave the way for developing novel bioactive agents that can target the biofilm-forming pathogens.

Cobetia sp. is not previously known for producing antibiofilm biosurfactants derived from symbiotically associated marine sponges. Although *Cobetia* sp. strain MM1DA2H-1 has been documented in hydrocarbon-contaminated environments [12], our study is the first to unveil its potential in antibiofilm biosurfactant production. Notably, the biosurfactant from *Cobetia* sp. JCG-23 displays a markedly lower surface tension of 22 Nm^{-1} compared to SDS at 42 Nm^{-1}, indicating its potential as a biosurfactant producer. Moreover, this strain may enhance the availability of poorly soluble hydrocarbon substrates for effective utilization within hydrocarbon-contaminated sites. Our investigation demonstrates that the biosurfactant secreted by this strain significantly diminishes *Pseudomonas aeruginosa* PAO1 biofilm on glass surfaces without exerting an antibacterial effect. This unique bioactivity holds promise for biomedical applications, offering a means to target biofilm-forming pathogens on surfaces and medical devices. The biosurfactant exhibited stability across wide pH ranges, temperatures, and NaCl concentrations. Stability studies for this biosurfactant derived from *Cobetia* sp. have not discussed in the literature. These physiological attributes of the biosurfactant derived from *Cobetia* sp. have broader implications, including mitigating oceanic oil spills. The findings underscore the potential of *Cobetia* sp. JCG-23 derived biosurfactant is a compelling alternative to antibiotics and chemically synthesized drugs, effectively combating biofilm-forming pathogens.

4 Conclusion

In this study, a potential biosurfactant was isolated from marine *Cobetia* sp. JCG-23, a symbiotic-associated bacteria. The biosurfactant was identified as octadecanoic acid of long-chain hydroxyl fatty acid that exerts strong anti-biofilm activity against *Pseudomonas aeruginosa* PAO1. Consequently, this surface-active compound can be exploited for biopharmaceutical and other applications. However, the mechanism of action of biosurfactants is not elucidated; more studies need to be carried out for deeper insights. Interestingly, this biosurfactant withstands at ranges of pH, temperatures, and NaCl concentration. Perhaps, our study suggested that the biosurfactant produced by *Cobetia* sp. JCG-23 is non-hemolytic potential and is highly stable for wider physiological conditions. Further investigation of the biosurfactant in the *Cobetia* sp. JCG-23 will facilitate the future lead molecule development against biofilm-forming pathogens.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s13399-023-04808-3.

Author contributions Govindarajan Ganesan: supervision, writing—original draft and conceptualization. Chandrasekar Balu performed the experiments. Suganthi Ganesan: technical correction and validation. Samuel Raj Babu Arulmani: writing—review and editing. Sabariswaran Kandasamy: writing—review and editing.

Funding The authors would like to thank the Department of Chemistry, Sri Shanmugha College of Engineering and Technology, Salem 637304, Tamil Nadu, India for their constant support for this work. The authors acknowledge the funding of SBT, Madurai Kamaraj University, 625021, Tamil Nadu and providing equipment support as institutional infrastructure. The authors would like to acknowledge the late Prof. Dr SRD Jebakumar for his valuable guidance.

Data availability All data is available under request.

Declarations

Ethical approval Not applicable.

Competing interests The authors declare no competing interests.

References

1. Carvalho CC, Fernandes P (2010) Production of metabolites as bacterial responses to the marine environment. Mar Drugs 8:705–727
2. Gudina EJ, Teixeira JA, Rodrigues LR (2016) Biosurfactants produced by marine Microorganisms with Therapeutic Applications. Mar Drugs 14:38
3. Mukherjee S, Das P, Sivapathasekaran C, Sen R (2009) Antimicrobial biosurfactants from marine *Bacillus circulans*: extracellular synthesis and purification. Lett Appl Microbiol 48:48281–48288
4. Cunha CD, Rosario M, Rosado AS, Leite SGF (2004) *Serratia* sp. SVGG 16. A promising bio-surfactant producer isolated from tropical soil during growth with ethanol-blended gasoline. Process Biochem 39:2277–2282
5. Mao X, Jiang R, Xiao W, Yu J (2015) Use of surfactants for the remediation of contaminated soils: A review. J Hazard Mater 285:419–435
6. Shekhar S, Sundaramanickam A, Balasubramanian T (2015) Bio surfactant producing microbes and their potential applications: a review. Crit Rev Environ Sci Technol 45:1522–1554
7. Jacobucci DFC, Oriani MRG, Durrant LR (2009) Reducing COD level on oily effluent by utilizing biosurfactant-producing bacteria. Braz Arch BiolTechnol 52:1037–1042
8. Satpute SK, Banat LM, Dhakephalkar PK, Banpirkar AG, Chopade BA (2010) Bio surfactants bio emulsifiers and exopolysaccharides from marine microorganisms. Biotechnol Adv 28:436–450
9. Marchant R, Banat IM (2012) Microbial biosurfactants: challenges and opportunities for future exploitation. Trends Biotechnol 30:558–565
10. Heinemann C, van HylckamaVlieg JE, Janssen DB, Busscher HJ, van der Mei HC, Reid G (2000) Purification and characterization of a surface-binding protein from *Lactobacillus fermentum* RC-14 that inhibits adhesion of *Enterococcus faecalis* 1131. FEMS Microbiol Lett 190:177–180
11. Patel M, Siddiqui AJ, Hamadou WS, Surti M, Awadelkareem AM, Ashraf SA, Alreshidi M, Snoussi M, Rizvi SMD, Bardakci F (2021) Inhibition of bacterial adhesion and antibiofilm activities of a glycolipid biosurfactant from *Lactobacillus rhamnosus* with its physicochemical and functional properties. Antibiotics 10:1546
12. Ibacache-Quiroga C, Ojeda J, Espinoza-Vergara G, Olivero P, Cuellar M, Dinamarca MA (2013) The hydrocarbon-degrading marine bacterium *Cobetia* sp. strain MM1IDA2H-1 produces a biosurfactant that interferes with quorum sensing of fish pathogens by signal hijacking. Braz J Microbiol 48:281–288
13. Rodrigues LR, Banat IM, Teixeira JA, Oliveira R (2006) Biosurfactants: potential applications in medicine. J Antimicrob Chemother 57:609–618
14. Rodrigues LR (2011) Inhibition of bacterial adhesion on medical devices. Adv Exp Med Biol 715:351–367
15. Jackson SA, Borchert E, O'Gara F, Dobson ADW (2015) Metagenomics for the discovery of novel biosurfactants of environmental interest from marine ecosystems. Curr Opin Biotechnol 33:176–182
16. Balakrishnan S, Arunagirinathan N, Rameshkumar MR, Indu P, Vijaykanth N, Almaary KS, Almutairi SM, Chen TW (2002) Molecular characterization of biosurfactant producing marine bacterium isolated from hydrocarbon-contaminated soil using 16S rRNA gene sequencing. J King Saud Univ-Sci 34:101871
17. Janssen PH, Yates PS, Grinton EB, Taylor PM, Sait M (2002) Improved culturability of soil bacteria and isolation in pure culture of novel members of the division Acidobacteria, Actinobacteria, Proteobacteria and *Verrucomicrobia*. Appl Environ Microbiol 68:2391–2396
18. Cooper DG, Goldenberg BG (1987) Surface active agents from two *Bacillus* species. Appl Environ Microbiol 53:224–229
19. Carrillo PG, Mardaraz C, Pitta-Alvarez SI, Giulietti AM (1996) Isolation and selection of biosurfactant-producing bacteria. World J Microbiol Biotechnol 12:82–84
20. Morikawa M, Hirata Y, Imanaka T (2000) A study on the structure-function relationship of lipopeptide biosurfactants. Biochim Biophys Acta 1488:211–218
21. Patil JR, Chopade BA (2001) Studies on bioemulsifier production by *Actinetobacter* strains isolate from human skin. J Appl Microbiol 91:290–298
22. Sabesan R, Dhanalakshmi A, William A, Thangaraj K (2002) Text book of allied Physics, Second edn. Popular Book Depot, pp 65–68
23. Velmurugan M, Baskaran A, Dinesh Kumar S, Sureka I, Jennifer Emelda EA, Sathiyamurthy K (2015) Screening, stability and antibacterial potential of rhamnolipids from *Pseudomonas* sp., isolated from hydrocarbon contaminated soil. J Appl Pharm Sci 5:26–33
24. Sigmund I, Wagner F (1991) New method for detecting rhamnolipids excreted by *Pseudomonas* spp during growth on mineral agar. Biotechnol Tech 5:265–268
25. Govindarajan G, Santhi VS, Jebakumar SRD (2014) Antimicrobial potential of phylogenetically unique actinomycete, *Streptomyces* sp. JRG-04 from marine origin. Biologicals 42:305–311
26. Cappuccino JG, Sherman N (2002) Microbiology. In: A laboratory manual, Sixth edn. Pearson education Inc., San Francisco, California, pp 215–224
27. Govindarajan G, Kamaraj R, Balakrishnan K, SatheejaSanthi V, Jebakumar Solomon RD (2017) *In-vitro* assessment of antimicrobial properties and lymphocytotoxicity assay of Benzoisochromanequinones polyketide from *Streptomyces* sp. JRG-04. Microb Pathog 110:117–127
28. Cole JR, Chai B, Marsh JR, Farris RJ, Wang Q, Kulam SA, Chandra S, McGarrell DM, Schmidt TM, Garrity GM, Tiedje JM (2003) The Ribosomal Database Project (RDP-II): previewing a new autoaligner that allows regular updates and the new prokaryotic taxonomy. Nucl Acids Res 31:442–443
29. Felsenstein J (2008) PHYLIP (Phylogeny Inference Package) version 3.68. Distributed by the author. Seattle: Department of Genome Sciences, University of Washington
30. Ghojavand H, Vahabzadeh F, Roayaei E, Shahraki AK (2008) Production and properties of a bio surfactant obtained from a member of the *Bacillus subtilis* group (PTCC 1696). J Colloid Interface Sci 324:172–176
31. Barakat KM, Hassan SW, Darwesh OM (2017) Biosurfactant production by haloalkaliphilic *Bacillus* strains isolated from Red Sea Egypt. Egypt J Aquat Res 43:205–211
32. Hamedi MM, Abdrabo MAA, Youssif AM (2021) Biosurfactant Production by marine actinomycetes isolates *Streptomyces althioticus* RG3 and *Streptomyces californicus* RG8 as promising sources of antimicrobial and antifouling effects. Microbiol Biotechnol Lett 49:356–366
33. Maneerat S, Phetrong K (2007) Isolation of biosurfactant-producing marine bacteria and characteristics of selected biosurfactant. J SciTechnol 29:781–791

34. Donio MBS, Ronica SFA, ThangaViji V, Velmurugan S, Adlin Jenifer J, Michaelbabu M, Citarasu T (2013) Isolation and characterization of halophilic *Bacillus* sp. BS3 able to produce pharmacologically important bio surfactants. Asian Pac J Trop Med 6:876–883
35. Thavasi R, Jayalakshmi S, Banat IM (2011) Effect of biosurfactant and fertilizer on biodegradation of crude oil by marine isolates of *Bacillus megaterium*, *Corynebacteriumkutscheri* and *Pseudomonas aeruginosa*. Bioresour Technol 102:772–778
36. Sharma D, Saharan BS, Chauhan N, Bansal A, Procha S (2014) Production and structural characterization of *Lactobacillus helveticus* derived bio surfactant. Sci World J:493548
37. Abouseoud M, Maachi R, Amrane A, Boudergua S, Nabi A (2008) Evaluation of different carbon and nitrogen sources in production of biosurfactant by *Pseudomonas fluorescens*. Desalination 223:143–151
38. Adonizio A, Kong KF, Mathee K (2008) Inhibition of quorum sensing-controlled virulence factor production in *Pseudomonas aeruginosa* by south Florida plant extracts. Antimicrob Agents Chemother 52:198–203
39. Hamayeli H, Hassanshahian M, Askari Hesni M (2021) Identification of bioactive compounds and evaluation of the antimicrobial and anti-biofilm effect of *Psammocinia* sp. and *Hyattella* sp. sponges from the Persian Gulf. Thalassas: An Int J Marine Sci 37:357–366
40. Hamayeli H, Hassanshahian M, Askari Hesni M (2019) The antibacterial and antibiofilm activity of sea anemone (*Stichodactyla haddoni*) against antibiotic-resistant bacteria and characterization of bioactive metabolites. Int Aquat Res 11:85–97
41. OToole GA, Kolter R (1998) Initiation of biofilm formation in *Pseudomonas fluorescens* WCS365 proceeds via multiple, convergent signaling pathways: a genetic analysis. Mol Microbiol 28:449–461
42. BabuArulmani SR, Ganamuthu HL, Ashokkumar V, Govindarajan G, Kandasamy S, Zhang H (2020) Biofilm formation and electrochemical metabolic activity of *Ochrobactrum* Sp JSRB-1 and *Cupriavidus* Sp JSRB-2 for energy production. Environ Technol Innov 20:101145
43. You JL, Xue XL, Cao LX, Lu X, Wang J, Zhang LX, Zhou SN (2007) Inhibition of Vibrio biofilm formation by a marine actinomycete strain A66. Appl Microbiol Biotechnol 76:1137–1144
44. Musthafa KS, Saroja V, Karutha Pandian S, Veera Ravi A (2011) Antipathogenic potential of marine Bacillus sp. SS4 on N-acyl-homoserine- lactone-mediated virulence factors production in *Pseudomonas aeruginosa* (PAO1). J Biosci 36:55–67
45. Yonebayashi H, Yoshida S, Ono K, Enomoto H (2000) Screening of microorganisms for microbial enhanced oil recovery process. J Jpn Pet Inst 43:59–69
46. GhayyomiJazeh M, Forghani F, Deog-Hwan OH (2012) Bio surfactant production by *Bacillus* sp. isolated from petroleum contaminated soils of Sirri Island. Am J Appl Sci 9:1–6
47. Jain DK, Collins-Thompson DL, Lee H, Trevors JT (1991) A drop-collapsing test for screening surfactant-producing microorganisms. J Microbiol Methods 13:271–279
48. Schulz D, Passeri A, Schmidt M, Lang S, Wagner F, Wray V, Poremba K, Gunkel W (1991) Marine biosurfactants, I. Screening for biosurfactants among crude oil degrading marine microorganisms from North-Sea. Z Naturforsch C J Biosci 46:197–203
49. Mulligan C, Cooper D, Neufeld R (1984) Selection of microbes producing biosurfactants in media without hydrocarbons. J Fermentation Technol 62:311–314
50. Bonilla M, Olivaro C, Corona M, Vazquez A, Soubes M (2005) Production and characterization of a new bioemulsifier from *Pseudomonas putida* ML2. J Appl Microbiol 98:456–463
51. Satpute SK, Bhawsar BD, Dhakephalkar PK, Chopade BA (2008) Assessment of different screening methods for the selecting biosurfactant producing marine bacteria. Indian J Marine Sci 37:243–250
52. Amiriyan A, Mazarheri Assadi M, Saggadian VV, Noohi A (2004) Bioemulsion production by Iranian oil reservoirs microorganisms. Iranian J Health Sci Eng 1(2):28–35
53. Rosenberg M, Rosenberg E (1981) Role of adherence in growth of *Acinetobacter calcoaceticus* RAG-1 on hexadecane. J Bacteriol 148:51–57
54. Rodrigues LR, Teixeira JA, Van der Mei HC, Oliveira R (2006) Physiochemical and functional characterization of a biosurfactant produced by *Lactococcus lactis* 53. Colloids Surf B Biointerfaces 49:79–86
55. Joshi A, Shekhawat DB (2014) Screening and isolation of biosurfactant producing bacteria from petroleum contaminated soil. Euro J Exp Biol 4:164–169
56. Nwaguma IV, Chikere CB, Okpokwasili GC (2016) Isolation, characterization and application of biosurfactant by Klebsiella pneumonia strain IVN51 isolated from hydrogen-polluted soil in Ogoniland, Nigeria. Bioresour Bioprocess 3:40
57. Garg M, Priyanka CM (2018) Isolation, characterization and antibacterial effects of biosurfactant from *Candida parapsilosis*. Biotechnol Rep 18(e):00251
58. Rosenberg E, Ron EZ (2001) High and low molecular mass microbial surfactants. Appl Microbiol Biotechnol 52:154–162
59. Banat IM, Makkar RS, Cameotra SS (2000) Potential commercial applications of microbial surfactants. Appl Microbiol Biotechnol 53:495–508
60. Silva RCFS, Almeida DG, Luna JM, Rufino RD, Santos VA, Sarubbo LA (2014) Applications of biosurfactants in the petroleum industry and the remediation of oil spills. Int J Mol Sci 15:12523–12542
61. Gautam KK, Tyagi VK (2006) Microbial surfactants: a review. J Oleo Sci 55:155–166
62. Silva SNRL, Farias CBB, Rufino RD, Luna JM, Sarubbo LA (2010) Glycerol as substrate for the production of biosurfactant by *Pseudomonas aeruginosa* UCP0992. Coll Surf B Bioint 79:174–183
63. Shavandi M, Mohebali G, Haddadi A, Shakarami H, Nuhi A (2011) Emulsification potential of a newly isolated biosurfactant-producing bacterium, *Rhodococcus* sp. strain TA6. Coll Surf B Bioint 82:477–482
64. Kim MS, Roh SW, Bae JW (2009) *Cobetia crustatorum sp*. nov., a slightly halophilic bacterium isolated from Korean traditional fermented seafood. Int J Syst Evol Microbiol 60:620–626
65. Busscher HJ, Van Hoogmoed CG, Geertsema-Doornbusch GI, Van der Kuihil-Booij M, van der Mei HC (1997) *Streptococcus thermophiles* and its biosurfactant inhibit adhesion by Candida spp. On silicone rubber. Appl Environ Microbiol 63:3810–3817
66. Luna JM, Rufino RD, Sarubbo LA, Campos-Takaki GM (2013) Characterisation, surface properties and biological activity of a biosurfactant produced from industrial waste by *Candida sphaerica* UCP0995 for application in the petroleum industry. Coll Surf B Bioint 102:202–209
67. Rodrigues L, Van der Mei HC, Teixeira J, Oliveira R (2004) Influence of biosurfactants from probiotic bacteria on formation of biofilms on voice prostheses. Appl Environ Microbiol 70:4408–4410
68. Nevot M, Deroncele V, Messner P, Guinea J, Mercade E (2006) Characterization of outer membrane vesicles released by the psychrotolerant bacterium *Pseudoalteromonasantarctica* NF3. Environ Microbiol 8:1523–1533
69. Nikaido H (2003) Molecular basis of bacterial outer membrane permeability revisited. Microbiol Mol Biol Rev 67:593–656

70. Velraeds MM, van der Mei HC, Reid G, Busscher HJ (1996) Inhibition of initial adhesion of uropathogenic *Enterococcus faecalis* by biosurfactants from *Lactobacillus* isolates. Appl Environ Microbiol 62:1958–1963
71. Rivardo F, Turner RJ, Allegrone G, Ceri H, Martinotti MG (2009) Anti-adhesion activity of two biosurfactants produced by Bacillus spp. prevents biofilm formation of human bacterial pathogens. Appl Microbiol Biotechnol 83:541–553
72. Dusane D, Nancharaiah YV, Zinjarde SS, Venugopalan VP (2010) Rhamnolipid mediated distruption of marine *Bacillus pumilus* biofilms. Coll Surf B Bioint 81:242–248
73. Selvin J, Shanmughapriya S, Gandhimathi R, Kiran G, Ravji RT, Natarajaseenivasan K, Hema TA (2009) Optimization and production of novel antimicrobial agents from sponge associated marine actinomycetes *Nocardiopsis dassonvillei* MAD08. Appl Microbiol Biotechnol. 83:435–445