Indian J. Applied & Pure Bio. Vol. 39(1), 632-645 (2024). **ISSN: 0970-2091**
A Web of Science Journal

Isolation and identification of biosurfactant- bacteria from diverse oil samples and their application

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Abstract

Biosurfactants are surface active components produced by various microorganisms as their extracellular or cell wall-associated compounds. And these biosurfactants have future promising properties in various fields because of their biodegradability, low toxicity, good environmental compatibility, high foaming property, high selectivity, and specificity at extreme environmental conditions. The present work aims to isolate and characterize biosurfactant-producing bacteria from oil samples (battery, engine, and crude oil) and evaluate their antimicrobial and biofilm inhibition potential against different pathogens. Samples were collected from different locations in Coimbatore and the bacterial isolates were screened for production of biosurfactants. Biosurfactant activity evaluation was carried out by oil displacement, drop collapse test, oil spreading assay, emulsification assay, and hemolytic assay. 16s rRNA sequence analysis was used for the isolate identification and the isolates were determined as *Metabacillus schmidteae*, *Bacillus* sp. (in: *firmicutes*), and *Pseudomonas aeruginosa*. The same was deposited in the GenBank repository with accession numbers OQ735442.1 (*Metabacillus schmidteae*), OQ745826.1 (*Bacillus* sp. (in: *firmicutes*)) and OQ746451.1 (*Pseudomonas aeruginosa*). The biofilm inhibition assay was determined against *Staphylococcus aureus* and *Klebsiella pneumoniae*, wherein the biosurfactants from all three isolates showed promising potential in comparison with the positive control.

Key words : Biosurfactant - bacteria, Bioflm, *Metabacillus schmidteae, Pseudomonas aeruginosa, Klebsiella pneumeniae.*

Surfactants are surface-active compounds that reduce the system's free energy by replacing bulk molecules of higher energy at an interface. Biosurfactants are those produced by various living surfaces, mostly microorganisms such as Bacteria, Fungi, Yeast, etc which are extracellular or cell wall-associated compounds. These compounds are

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amphiphilic and contain both hydrophilic and hydrophobic ends, allowing them to interact at the interface between aqueous and non-aqueous systems[5]. They also offer several advantages over chemical as well as other synthetic surfactants, such as biodegradability, low toxicity, good environmental compatibility, high foaming property, high selectivity, and specificity at extreme environmental conditions[6,28]. These properties make them ideal for bioremediation, nanotechnology, medicine, pharmaceuticals, food industry, cosmetics, etc[31]. Microbial biosurfactant compounds exhibit emulsifying properties with several known activities, including microbial biofilm solubilization and reducing the surface tension between the compounds. Biosurfactant-producing microorganisms are ubiquitous in nature both in marine and terrestrial regions, especially with a high hydrocarbon content. The surface and interfacial tension-reducing properties of biosurfactants produce excellent detergency and emulsifying, foaming, and dispersing traits making them one of the most versatile products[35].

Petroleum hydrocarbons such as crude oil and other diverse oil samples are considered the major and hazardous contaminants worldwide. They are contagious in both aquatic and terrestrial environments in this developing world, because of their drunken usage and mishandling during their production, transfer, and management[40]. Commonly, petroleum products like crude oils are described and assumed as hard settings for the growth of microorganisms, However, microbes can thrive on crude petroleum that contains sole hydrocarbon sources, especially Bacteria and Fungi. Already many studies and research reveal mounting evidence for the wide distribution of microbial diversity, including *Pseudomonas, Bacillus, Streptomyces,* and *Stenotrophomonas* species, were associated with oil wells (Yoshida *et al.,* 2005; Cai *et al.,* 2015)[1]. The microorganisms that can grow in crude petroleum environments have the ability to degrade the hydrocarbon as their energy source and encapsulate the heavy metals, these properties make them ideal for a wide range of applications, especially bioremediation[32]. In this study, we examine the three oil samples including crude oil, engine oil, and battery oil, because they are rich in hydrocarbon sources, which the microorganisms utilize as their carbon source and produce biosurfactants.

Biosurfactants have various applications in many industries and fields, among these thousands of applications one of the milestone implementations is in the Biomedical industry. They act as inhibitors of microbial adhesion and biofilm formation[36]. The biofilm is a growth of microorganisms as a thick layer, which forms a colony. The formation of the biofilm on living and non-living materials is an aggregation of microorganisms, that attaches to a surface with an extracellular polymeric substance matrix which aids in protecting these microorganisms[4]. Within the biofilm, bacteria show many characteristics which make them hard to eliminate. Because of these particular characteristics, biofilms are a protected type of growth that is highly hard conditions that are not suitable for the usual phases[15]. Moreover, the bulge presence of the microbial cell densities within the biofilm eventually intensifies the chance of horizontal gene transfer between the cells, which enhances the chances of the appearance of highly resistant

or mutated strains[29]. The resistance of biofilm toward the antibiotics and immune systems of the hosts depends on many aspects such as changes in physiology, steady growth rate, neutralization of the antimicrobial agents, changes in expression of genes, synthesis of extracellular polymers, the age of the biofilm, appearance of small colony variants and dysfunction of the local neutrophils, etc.[23]. Biofilms of bacteria are typically pathogenic and responsible for nosocomial infections. About 60 to 80% of chronic infections are due to the formation of biofilm[16,20,23,27]. Presently, biofilm is a serious problem around the globe, which causes a severe impact and ultimately huge losses to the food, dairy, oceanic, aquaculture, beverage, environment, and biomedical industries[20]. Therefore, biofilm removal is a global challenge that necessitates developing novel natural bioactive compounds to control biofilms, as an alternative to antibiotics or chemically synthesized agents[29].

Biosurfactants can play a critical role in the inhibition of the adherence ability of numerous pathogens to the surfaces, an essential step for the formation and proliferation of biofilms. The adsorption of biosurfactants to solid surfaces might constitute its strategic role to reduce microbial adhesion and combat the colonization of contaminants or pathogenic microorganisms. Thus, biological compounds with antimicrobial properties and the capability to inhibit the adhesion potential of pathogens on different types of surfaces can be developed as a potent antibiofilm agent and it finds application as an anti-adhesive agent in the biomedical field[7]. In this context, different biosurfactants have been demonstrated to reduce the adhesion of pathogenic microbes on different matrices such as glass[8], silicone rubber[9], surgical implants[4], and voice prostheses[12]. Biosurfactants possess bacteriostatic, bactericidal, and biofilm disruption ability, which makes them an ideal antimicrobial agent[14]. Numerous reports are available that show the effectiveness of biosurfactants against different pathogens. For example, Foschi and others reported antimicrobial effects against *Neisseria gonorrhoeae*[17]. Similarly, Morais and others observed against *Candida albicans*[30], and Dusane and others reported biofilm degradative behavior of rhamnolipid surfactant against *Bacillus pumilus*[9]. However, biosurfactants produced by microbial strains act differentially during pathogen inhibition. For instance, Rhamnolipids possess activity through The amphipathic nature of rhamnolipids binding with the charges of the bacterial cell membrane and changing their hydrophobicity. This prevents biofilm formation and makes the pathogen highly susceptible to antimicrobial agents[22,26]. Several studies have suggested that rhamnolipids may act more effectively against Gram-positive bacteria than Gram-negative bacteria due to the absence of an outer membrane. The presence of the outer layer may exclude biosurfactant molecules[22,37]. However, the lipopolysaccharides biosurfactants attribute antimicrobial properties via penetrating or damaging the lipid. The charge imbalance led to pore formation in the cell membrane lipids, which ultimately caused damage to or death of the pathogens, especially Gram-negative bacteria[22,39].

This study aims to isolate the biosurfactant-producing microorganisms from crude oil, battery oil, and engine oil respectively, and to identify them. Objectives of the study

include[1] Isolation of biosurfactant-producing bacteria from the respective oil samples[2] Characterization and identification of biosurfactant-producing bacteria[3] Evaluation of biosurfactant activity from isolates and [4] Application of the biosurfactant in biofilm inhibition.

Isolation and enrichment of biosurfactant-producing bacteria :

Bacteria were isolated from the collected diverse oil samples after incubation for 24 hours on a nutrient agar plate at 28°C (Fig. 1). Isolated bacteria were enriched by incubating isolated bacterial culture in carbon-amended Bushnell Hass broth for 7 days at 30°C in the rotary shaker. Then cell-free supernatant was collected by centrifugation at 6500 RPM for 20 minutes at 4°C.

Molecular identification :

16s rRNA sequence analysis was used for the isolate identification and the isolates were determined as *Metabacillus schmidteae*, *Bacillus* sp. (in: *firmicutes*), and *Pseudomonas aeruginosa*. The same was deposited in the GenBank repository with accession numbers OQ735442.1 (*Metabacillus schmidteae*), OQ745826.1 (*Bacillus* sp. (in: *firmicutes*)) and OQ746451.1 (*Pseudomonas aeruginosa*).

Assay for biosurfactant production :

Drop collapse assay :

In this assay the engine oil shows positive results that is when we place a drop of culture supernatant on the crude oil-coated surface, it gives a flattened drop. While crude oil and battery oil gives round-shaped drops, it indicates negative results or contains less biosurfactant concentration.

This assay relies on the destabilization of liquid droplets by surfactants. Therefore, drops of a culture supernatant are placed on an oil-coated, solid surface. If the liquid does not contain surfactants, the polar water molecules are repelled from the hydrophobic surface and the drops remain stable. If the liquid contains surfactants, the drops spread or even collapse because the force or interfacial tension between the liquid drop and the hydrophobic surface is reduced. Drops' stability depends on surfactant concentration and correlates with surface and interfacial tension.

Oil spreading assay :

Here Engine oil and Battery oil show a positive result, that is there is a clear zone formed when the culture supernatant is placed on the crude oil layer over the distilled water. The crude oil sample shows negative results *i.e.* no clear zone was formed.

If biosurfactant is present in the supernatant, the oil is displaced and a clearing zone is formed. The diameter of this clearing zone on the oil surface correlates to surfactant activity. So it is also called displacement activity.

Emulsification assay :

The optical density of the emulsion of crude oil with each sample is compared with the optical density of positive control Triton X 100. The optical density of the crude oil and positive control are almost the same and the

battery oil has little variation. The engine oil has an optical density larger than the positive control.

Hemolytic assay :

It indicates that every three samples produce the biosurfactant, but the crude oil and Engine oil show moderate hemolysis(++) and battery oil shows partial hemolysis, So produces less amount of biosurfactant than the other two samples (Fig. 2).

Biosurfactants can cause lysis of erythrocytes. This principle is used for the hemolysis assay. Positive strains will cause lysis of the blood cells and exhibit a colorless, transparent ring around the colonies.

CTAB agar assay :

Engine oil and Battery oil show blue halos around the biosurfactant-filled wells in the CTAB agar plate, while crude oil doesn't show the halos around the well (Fig. 3). The

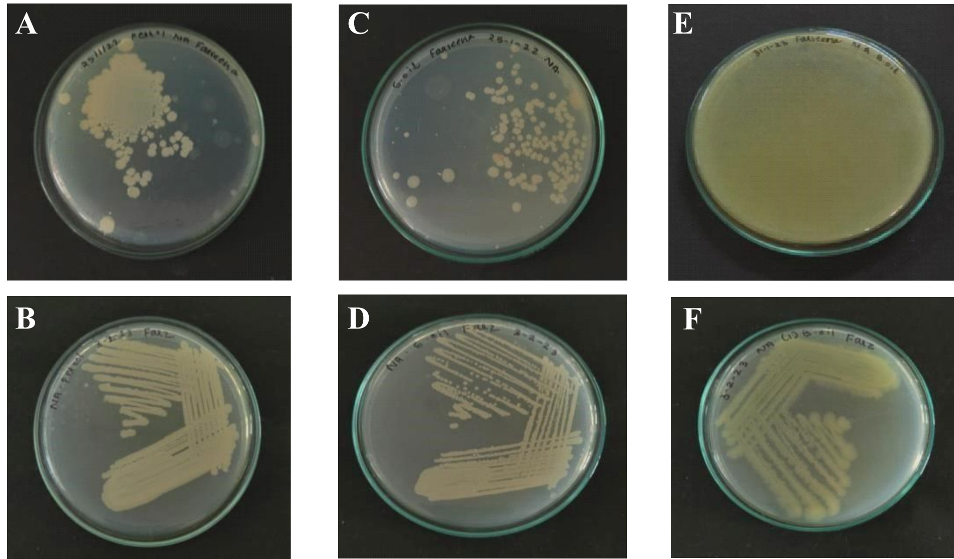

Fig. 1 Isolation of bacteria from diverse oil-contaminated soil samples (A, B) bacteria isolated from Crude oil, (C, D) bacteria isolated from Engine oil, (E, F) bacteria isolated from Battery oil

Fig. 2. Bacterial strains isolated from different oil samples (Blood Agar). A. Crude oil, B. Engine oil C. Battery oil.

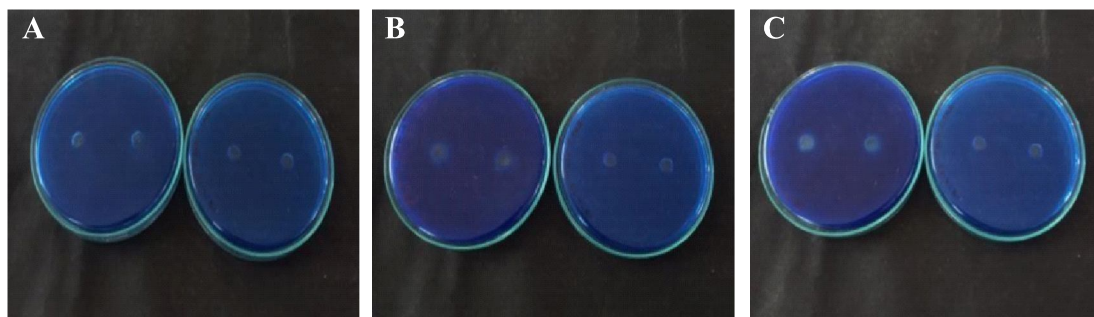

Fig. 3. Bacterial strains isolated from different oil samples (CTAB Agar) A. Crude oil, B. Engine oil C. Battery oil

results indicate that the engine oil and battery oil might produce a type of anionic surfactant or glycolipids, while sample crude oil did not produce these respective types of microbial surfactant.

The CTAB agar plate method is a semi-quantitative assay for the detection of extracellular glycolipids or other anionic surfactants. If anionic surfactants are secreted by the microbes growing on the plate, they form a dark blue, insoluble ion pair with cetyltrimethylammonium bromide and methylene blue. Thus, productive colonies are surrounded by dark blue halos.

Application – biofilm inhibition assay :

Here biofilm inhibition assay of biosurfactant derived from every three samples is done against two biofilm-producing microorganisms, *Staphylococcus aureus,* and *Klebsiella pneumoniae.* The percentage of biofilm inhibition is calculated by the equation, % Microbial inhibition of adhesion = [(1 - (Ac/Ao)]×100 where Ac represents the absorbance of the well with known biosurfactant concentration c and Ao represents the absorbance of the control well.

Biofilm inhibition against Staphylococcus aureus :

Biosurfactant produced by *Metabacillus schmidteae,* which was isolated from a crude oil sample shows 26.5%, 63.4%, 66.6%, and 79.1% of biofilm inhibition against *Staphylococcus aureus* in the concentrations of 50µl, 100µl, 150µl, 200µl respectively.

Biosurfactant produced by*, Pseudomonas aeruginosa* which was isolated from an Engine oil sample shows 0.34%, 14.3%, 57%, and 57.9% of biofilm inhibition against *Staphylococcus aureus* in the concentrations of 50µl, 100µl, 150µl, 200µl respectively And the biosurfactant produced by*, Bacillus firmicutes* which were isolated from a battery oil sample shows 3.8%, 11%, 32.9%, and 56.8% of biofilm inhibition against *Staphylococcus aureus* in the concentrations of 50µl, 100µl, 150µl, 200µl respectively (Fig. 4).

Biofilm inhibition against Klebsiella pneumoniae :

Biosurfactant produced by *Metabacillus schmidteae,* which was isolated from a crude oil sample shows 1.5%, 8.2%, 23.4%, and

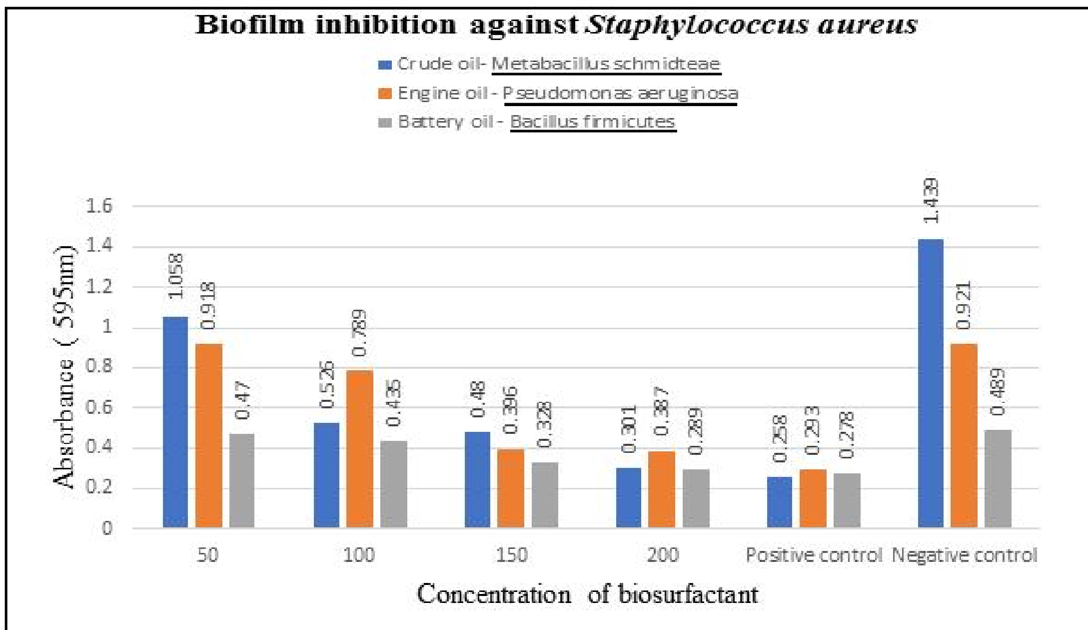

Fig. 4. Biofilm inhibition assay. Inhibition of *Staphylococcus aureus* at different biosurfactant concertation

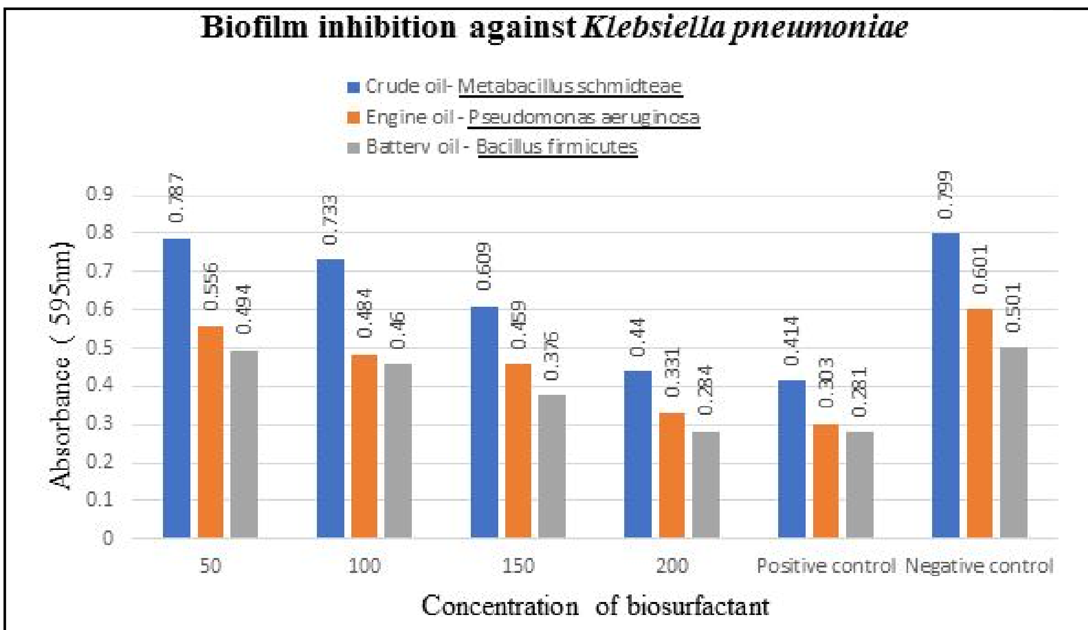

Fig. 5. Biofilm inhibition assay. Inhibition of *Klebsiella pneumoniae* at different biosurfactant concertation.

44.7% of biofilm inhibition against *Klebsiella pneumoniae* in the concentrations of 50µl, 100µl, 150µl, 200µl respectively Biosurfactant produced by, *Pseudomonas aeruginosa* which was isolated from an Engine oil sample shows 7.4%, 19.4%, 23.6%, and 44.9% of biofilm inhibition against *Klebsiella pneumoniae* in the concentrations of 50µl, 100µl, 150µl, 200µl respectively.

And the biosurfactant produced by, *Bacillus firmicutes* which were isolated from battery oil sample shows 1.3%, 8.1%, 24.9%, and 43.3% of biofilm inhibition against *Klebsiella pneumoniae* in the concentrations of 50µl, 100µl, 150µl, 200µl respectively (Fig 5).

Summary :

Biosurfactant-producing microorganisms were isolated from oil samples, *i.e,* engine oil, battery oil, and crude oil, the isolated strains were given for the 16srRNA sequencing for the species identification, and isolated the biosurfactant-producing *Pseudomonas aeruginosa, Bacillus firmicutes, Metabacillus schmidteae* respectively. In the oil spreading technique, drop collapse assay, hemolytic assay, CTAB agar assay, and emulsification activity were performed for the screening of the biosurfactant-producing bacteria, and it confirm the production of biosurfactant. The biosurfactant has high value and various applications in the biomedical industry and other fields. Here we expose the ability of biosurfactants to inhibit biofilm production as a biomedical application. In the pre-biofilm inhibition assay with the biosurfactant cultures, they exhibited inhibition activity against the test organisms *Staphylococcus aureus* and *Klebsiella pneumoniae*. Despite many laboratory-based successes in biosurfactant production and its immense commercial applications, the production of biosurfactants at a plant scale remains a challenging issue as the composition of the final product is affected by nutrient, micronutrient, and environmental factors. Guidelines and regulations should be formulated for the use of biosurfactants in different sectors. It is expected that in the future, we could apply this biosurfactant to inhibit biofilm formation by pathogenic organisms in hospitals, laboratories, and biomedical sectors.

Supplementary materials :

Chemicals :

$MgSO_4{\cdot}7H_2O$, $CaCl_2$, KH_2PO_4, K_2HPO_4, NH_4NO_3, $FeCl_3$, Glucose, Fructose, Glycerol, Mannitol, Olive oil, Triton X-100, Cetyltrimethylammonium bromide, Methylene blue, Agar, 50 mM Tris buffer (pH 8.0), phosphate-buffered saline (PBS), Methanol (99% purity), 2% crystal violet solution, 33% glacial acetic acid.

Sample :

Diverse oil samples were collected from different areas of Coimbatore. Crude oil was collected from the petrol bunk, Peelamedu, Coimbatore. Engine oil and battery oil were collected from an automobile workshop, at Nava India, and Sullur, Coimbatore respectively.

Culture media :

Nutrient broth (NB) was composed of, in g/L de-ionized water: peptone 10, beef

extract 5, and NaCl 5. For the preparation of nutrient agar plates or slants, 15.0 g/L agar (strength 1300) was added.

Luria Bertani broth was prepared by composing 950 ml distilled water: 10 g Tryptone, 10 g Sodium Chloride (NaCl), and 5 g Yeast Extract. Then make the final volume into 1000 ml.

Bushnell and Haas (BHM) broth, composed of Magnesium sulfate, Calcium chloride potassium phosphate, Dipotassium phosphate, Ammonium nitrate, and Ferric chloride supplemented with 2% of each glucose, fructose, glycerol, mannitol, and olive oil (w/v or v/v) as sole sources of carbon.

CTAB agar plates were prepared by adding 0.15 g of cetyltrimethylammonium bromide, 0.005 g Methylene blue (Sigma-Aldrich), and 12 g of agar to 1 L of distilled water.

Blood agar plates were prepared by adding 5% (v/v) of sheep blood (Fisher Scientific) to a sterilized mixture of NaCl (10 g), yeast (5 g), tryptone (10 g), and agar (15 g) in 1 L of distilled water[37].

The pH of the media was adjusted to 7 using 1 N NaOH and autoclaved at 121°C for 20 min.

Isolation and enrichment of biosurfactant-producing bacteria :

The samples were prepared under sterile conditions at a Laminar air flow hood to avoid any contamination. 10 ml of each sample was mixed with an equal volume of distilled water and the emulsion was continuously agitated for 2 hours on a rotary shaker. Then separate the aqueous phase from the emulsion. 100 µl of each prepared sample were plated on nutrient agar media and incubated at 28°C for 24 hours. After incubation, the grown colonies were streaked on a nutrient agar plate for three rounds to obtain pure form.

For efficient degradation of complex hydrocarbon oil and the production of biosurfactants, Bushnell and Haas (BHM), composed of Magnesium sulfate, Calcium chloride potassium phosphate, Dipotassium phosphate, Ammonium nitrate, Ferric chloride[1] supplemented with 2% of each glucose, fructose, glycerol, mannitol, and olive oil(w/v or v/v) as sole sources of carbon[11], adjusted to pH 7.0 and sterilized at 21 psi for 20 min was used. A 1 mL volume of bacterial cultures (grown at 22oC for 18–24 h with agitation in LB broth) with an OD600 between 0.6 and 1.0 was transferred to 100 mL of the carbon-amended BHM media. Inoculated media was incubated with continuous agitation at 30oC for 7 days and then the cell-free supernatant was collected by centrifugation (6500 × g at 4oC for 20 min). Kept at 4oC until further use.

Molecular identification :

The polymerase chain reaction (PCR) technique was utilized to identify and confirm the bacteria. Bacterial samples were initially processed to extract DNA by the instruction provided by Helini pure fast DNA bacterial genomic DNA mini spin prep kit (Helini Biomolecules, Chennai, India). Finally, sequencing of the PCR results was transmitted to the applied biosystem, Chennai.

Assay for biosurfactant production :

Bacterial isolates originating from

crude oil samples were screened for biosurfactant production by applying the most commonly used assays in the literature; the oil spreading test and drop collapse assay[18,24]. Unless otherwise stated, Triton X-100 was used as the positive control in all assays. All tests were done for each bacterial strain. Based on the above-mentioned criteria, the top biosurfactant producers were further screened using the CTAB agar method, emulsification assay, and hemolytic assay.

Drop Collapse Assay :

This assay relies on the destabilization of liquid droplets by surfactants. Therefore, cell suspension or culture supernatant drops are placed on an oil-coated, solid surface. If the liquid does not contain surfactants, the polar water molecules are repelled from the hydrophobic surface and the drops remain stable. If the liquid contains surfactants, the drops spread or even collapse because the force or interfacial tension between the liquid drop and the hydrophobic surface is reduced. Drops' stability depends on surfactant concentration and correlates with surface and interfacial tension.

The wells of a polystyrene 96 well microplate were coated with 2 µL of crude oil and left to dry for 24 h at 22°C. Filtered cell-free supernatant (5 µL) was transferred to the center of the oil-coated well. The results were recorded after 1–2 min and considered positive for biosurfactant production when the oil drop was flat. Those that gave rounded drops were scored negative, an indication of the absence of biosurfactant production[33,38].

Oil Spreading Assay :

The oil spreading method is rapid and easy to carry out, requires no specialized equipment, and just a small volume of sample. It can be applied when the activity and quantity of biosurfactants are low. If biosurfactant is present in the supernatant, the oil is displaced and a clearing zone is formed. The diameter of this clearing zone on the oil surface correlates to surfactant activity, also called oil displacement activity.

The oil-spreading assay was performed in polystyrene petri dishes containing 20 µL of crude oil that was carefully layered over 20 mL of distilled water. A drop (~10 µL) of filtered supernatant was carefully pipetted onto the center of the oil layer[13].

CTAB Agar Assay :

The CTAB agar plate method is a semi-quantitative assay for the detection of extracellular glycolipids or other anionic surfactants. The microbes of interest are cultivated on a light blue mineral salt agar plate containing the cationic surfactant cetyltrimethylammonium bromide and the basic dye methylene blue. If anionic surfactants are secreted by the microbes growing on the plate, they form a dark blue, insoluble ion pair with cetyltrimethylammonium bromide and methylene blue. Thus, productive colonies are surrounded by dark blue halos.

CTAB agar plates were prepared by adding 0.15 g of cetyltrimethylammonium bromide, 0.005 g Methylene blue (Sigma-Aldrich), and 12 g of agar to 1 L of distilled water, adjusted to pH 7 and sterilized. Two

holes were made in the CTAB plates, and approximately 150 µL of filtered cell-free supernatant was loaded inside each hole. Plates were incubated at 37°C for 48 h. Cell-free supernatant containing anionic surfactant produced blue halos around the wells in which they were placed. The diameter of the halo was measured and compared with positive and negative controls[33,34].

Emulsification Assay :

Evaluating the emulsification capacity is a simple screening method suitable for the first screening of biosurfactant-producing microbe. A volume of 1 mL of the cell-free supernatant was added to 5 mL of 50 mM Tris buffer (pH 8.0) in a 30 mL screw-capped test tube. Crude oil was tested for emulsification activity. Crude oil (5 mg) was added to both layers and vortexed for 1 min and then the emulsion mixture was allowed to settle for 20 min. The optical density of the emulsified mixture was measured at 610 nm. A negative control consisted of only a buffer solution and crude oil with Triton X100 was used as the positive control[34].

Hemolytic Activity :

Biosurfactants can cause lysis of erythrocytes. This principle is used for the hemolysis assay. The blood agar method is often used for the preliminary screening of microorganisms for the ability to produce biosurfactants on hydrophilic media. Blood agar is a rich growth medium for many organisms. But the method has some limitations. First, the method is not specific, as lytic enzymes can also lead to clearing zones. Second, this assay cannot include hydrophobic substrates as the sole carbon source. Third, diffusion restriction of the surfactant can inhibit the formation of clearing zones. In addition, some biosurfactants do not show any hemolytic activity at all. It can give a lot of false negative and false positive results.

Blood agar plates were prepared by adding 5% (v/v) of sheep blood (Fisher Scientific) to a sterilized mixture of NaCl (10 g), yeast (5 g), tryptone (10 g), and agar (15 g) in 1 L of distilled water. Approximately 150 µL of filtered cell-free supernatant of each bacterial isolate was loaded into each well made by a cork borer in the blood agar plates and incubated at 30°C for 24–48 hr. Biosurfactant biosynthesis was confirmed by hemolysis activity as indicated by the presence of clearing zones around the wells[1,21,38].

Application – Biofilm inhibition assay :

The anti-biofilm activity of the isolated biosurfactant against the biofilm-forming bacterial strains was quantified according to a pre-adhesion inhibition assay[19] and all the bacterial strains used in this study were predetermined to possess strong adhesion-forming ability. The predetermination was based on bacterial adhesion to the polystyrene microtitre plate which was performed using the same applied conditions and crystal violet staining was used for the pre-adhesion inhibition assay as described below with a single modification. This procedure devoid of the pre-incubation of biosurfactant at different concentrations to the well plates and adhesion potential of all the bacterial strains were classified as strongly adherent based on the scheme of Stepanović and coworkers[36] which

is as follows: Non-adherent: OD ≤ ODc; weakly adherent: ODc < OD ≤ 2 × ODc; moderately adherent: 2 × ODc < OD ≤ 4 × ODc; strongly adherent: 4 × ODc < OD. This classification is based upon the cutoff of the optical density (ODc) value defined as three standard deviation values above the mean OD of the negative control[39].

Biofilm inhibition assay against Staphylococcus aureus :

The required number of wells of the 96-well flat-bottom polystyrene microtitre plate with lid (Tarsons, India) were filled with 200 µL of biosurfactant solution prepared in phosphate-buffered saline (PBS) at different concentrations ranging from 2 mg/mL to 3.9 µg/mL. After incubation at 4°C for 18 h, the wells were washed twice using PBS and the control wells contained PBS buffer only. The bacterial strains to be tested, that is *Staphylococcus aureus* were grown at 37°C overnight under aerobic conditions. An aliquot of 200 µL of the bacterial strain was added and incubated in the wells at 4°C for 4 h. After incubation, the unattached microorganisms were removed by washing the wells three times with PBS and the adherent microorganisms were fixed using 200 µL of methanol (99% purity) per well. After 15 min, the methanol in the wells was poured out and then allowed to air dry. Later, the wells were stained for 5 min using 200 µL of 2% crystal violet solution. Excess stain was rinsed off by running tap water and allowed to air dry. Subsequently, the dye bound to the adherent microorganisms was resolubilized with 200 µL of 33% (v/v) glacial acetic acid per well, and the absorbance of each well was measured at 595 nm using a microplate reader (Biotek Elx808, WI, USA). The microbial inhibition percentages at different biosurfactant concentrations for each microorganism were calculated as follows: % Microbial inhibition of adhesion = [(1 - (Ac/Ao)] ×100 where Ac represents the absorbance of the well with known biosurfactant concentration c and Ao represents the absorbance of the control well. The anti-adhesion properties were determined based on the microtitre-plate anti-adhesion assay which determines the percentage of microbial adhesion reduction in the control wells, which were set at 0% to indicate the absence of biosurfactant[39].

Biofilm inhibition assay against Klebsiella pneumoniae :

The required number of wells of the 96-well flat-bottom polystyrene microtitre plate with lid (Tarsons, India) were filled with 200 µL of biosurfactant solution prepared in phosphate-buffered saline (PBS) at different concentrations ranging from 2 mg/mL to 3.9 µg/mL. After incubation at 4°C for 18 hr, the wells were washed twice using PBS and the control wells contained PBS buffer only. The bacterial strains to be tested, that is *Klebsiella pneumoniae* were grown at 37°C overnight under aerobic conditions. An aliquot of 200 µL of the bacterial strain was added and incubated in the wells at 4°C for 4 h. After incubation, the unattached microorganisms were removed by washing the wells three times with PBS and the adherent microorganisms were fixed using 200 µL of methanol (99% purity) per well. After 15 min, the methanol in the wells was poured out and then allowed to air dry. Later, the wells were stained for 5 min using 200 µL of 2% crystal violet solution. Excess stain was rinsed off by running tap water and allowed to air dry. Subsequently, the dye bound

to the adherent microorganisms was resolubilized with 200 µL of 33% (v/v) glacial acetic acid per well, and the absorbance of each well was measured at 595 nm using a microplate reader (Biotek Elx808, WI, USA). The microbial inhibition percentages at different biosurfactant concentrations for each microorganism were calculated as follows: % Microbial inhibition of adhesion = [(1 - (Ac/Ao)] ×100 where Ac represents the absorbance of the well with known biosurfactant concentration c and Ao represents the absorbance of the control well. The anti-adhesion properties were determined based on the microtitre-plate anti-adhesion assay which determines the percentage of microbial adhesion reduction in the control wells, which were set at 0% to indicate the absence of biosurfactant[39].

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