Pankaj Kumar
Ramesh Chandra Dubey *Editors*

Multifunctional Microbial Biosurfactants

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Editors
Pankaj Kumar
Department of Botany and Microbiology
H.N.B. Garhwal University
(A Central University)
Srinagar Garhwal, Uttarakhand, India

Ramesh Chandra Dubey
Department of Botany and Microbiology
Gurukul Kangri Vishawavidyalaya
Haridwar, Uttarakhand, India

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Mātṛ Devo Bhav.
Pitṛ Devo Bhav.
Ācārya Devo Bhav.

(Tattirīya Upaniṣad 1.11.2)
(See God in Mother, Father and Teachers)

Preface

The book entitled 'Multifunctional Microbial Biosurfactants' encompasses 22 chapters on various types of microbial biosurfactants and their applications in different area. The chapters also provide an overview of the different production process of biosurfactants and its future aspect. Therefore, this book will be beneficial for post-graduate students, research scholars and scientists working in various areas of biosurfactants.

Biosurfactants are attractive amphiphilic surface-active molecules derived from microorganisms (bacteria, fungi, actinomycetes and cyanobacteria) and differ in their structural and physico-chemical properties depending on the organism. Different techniques are used to isolate these surface-active agents. Biosurfactants are environment-friendly and an alternative of synthetic surfactants, which are highly selective, biodegradable and impart limited toxicity.

Biosurfactants are usually applied as emulsifiers and reducers of surface tension in different fields, mainly in the oil and detergent industries. As compared to chemical surfactants, biological surfactants have better surfactant properties and are essential for the remediation of soil and sea water. In recent times, the bio-pesticides have gained attention in the management of fungi, pest and insects and have long been boosted as potential alternative of chemical pesticide. Lipopeptide and rhamnolipid biosurfactants are low in toxicity to the ecosystem and highly biodegradable in nature and promising surface-active compound that could be used as bio-pesticides. The biosurfactant producing PGPRs are important to raise the disease-free crop and help in counteracting the problem of food security.

Besides, the biosurfactants are the best alternative for the biological control of mosquito. The two vital features promoting the applications in markets are economic growth and cost-effectiveness. Biosurfactants are safer for the environment, less poisonous and easily decomposable than the chemical surfactants. It has numerous applications in the food industry, healthcare and cosmetic industries. Biosurfactants are used for oil clean-up, soil remediation, pesticide manufacturing, plant growth promoters, drug delivery, medicine, agriculture and environmental safety. Cosmeceuticals are cosmetic products having some specific therapeutic effects.

The role of biosurfactants in cosmetic and detergents industry and their applications have also been covered in this book.

Biosurfactants also exhibit antioxidant, antimicrobial, anti-ageing, cytotoxicity and anti-inflammatory antiviral activities. It kills herpesvirus, retrovirus and coronavirus by interacting with viral membrane and carbon atoms in surfactin's acyl chain. It is predicted that biosurfactants might be the potential inhibitors of SARS-CoV-2. Biosurfactants-mediated nanoparticles have exhibited multipurpose roles in biomedicine, particularly as antibacterial, antifungal, antibiofilm, anticancer, wound healing, anti-inflammatory, mosquitocidal and dermal drug delivery agents without showing toxicity to the normal cells.

We extend our heartiest thanks to all contributors for providing an insight into these important areas of research and development. We also thank Dr. Sofia Costa and the entire team of SPRINGER-NATURE for publishing this book. We are indebted to our teachers, parents and family members because this tedious journey could not be completed without their blessing and support.

Srinagar Garhwal, Uttarakhand, India — Pankaj Kumar
Haridwar, Uttarakhand, India — Ramesh Chandra Dubey

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About the Editors

Pankaj Kumar completed his master's and doctorate degrees at the Gurukula Kangri Vishwavidyalaya, Haridwar, Uttarakhand (India). He has more than ten years of teaching and research experience. His research interest and expertise are rhizosphere microbiology, biosurfactants, and biofertilizers etc. He has published several research papers in national and international reputed journals. He has edited two books *Rhizosphere Engineering* (2022), *Macrophomina Phaseolina: Ecobiology, Pathology and Management* (2023) published by Academic Press (Elsevier), along with Prof R.C. Dubey. He is also a coauthor of An Objective Compendium on Food Science (Brillion publishing, India, 2022). He is life member of the Association of Microbiology of India (AMI), New Delhi and Indian Science Congress, Kolkata (West Bengal) and serving as reviewer and editorial board member of several national and international reputed journals. Currently, Dr. Pankaj is associated with the Department of Botany and Microbiology, H.N.B. Garhwal University (A Central University), Srinagar Garhwal, Uttarakhand, India.

Ramesh Chandra Dubey is a Professor, the Director (Research and Development Cell), Dean-Research, Dean-Faculty of Medical Science and Health, and the former Head of the Department of Botany and Microbiology, Gurukula Kangri Vishwavidyalaya, Haridwar, Uttarakhand (India). He obtained his M.Sc. and Ph.D. degrees from the internationally renowned Banaras Hindu University, Varanasi (India). He has more than 35 years of teaching and research experience in the field of Agricultural Microbiology and Biotechnology. He has published more than 208 research papers in the national and international journals of repute. His Google citation index is above 5138 till date. He has authored 7 books and co-edited 10 books under different subjects such as agriculture, microbiology and biotechnology. In 2022, Dr. Dubey published a book, *Vedic Microbiology* with Motilal Banarsidass International, New Delhi (India).

Dr. Dubey is a Life Member and Fellow of the Indian Botanical Society, Indian Phytopathological Society and the International Society for Conservation of Natural Resources. He also previously served as the Chairman of the Institutional Animal Ethical Committee (IAEC) for 6 years (2015–2021) and the Councillor of the Indian Botanical Society for 3 years (2011–2013).

Screening Methods for Biosurfactant-Producing Microorganisms

Sumeyra Gurkok and Murat Ozdal

Abbreviations

$(NH_4)_2SO_4$	Ammonium sulfate
BATH	Bacterial adhesion to hydrocarbon
BTB	Bromothymol blue
CMC	Critical Micelle concentration
CTAB	Cetyltrimethylammonium bromide
EU	Emulsification activity
FC	Foaming capacity
FTIR	Fourier transform infrared spectroscopy
GC-MS	Gas chromatography-mass spectrometry
H_2SO_4	Sulfuric acid
HCl	Hydrochloric acid
HIC	Hydrophobic interaction chromatography
HPLC	High-performance liquid chromatography
LC-MS	Liquid chromatography-mass spectrometry
MATH	Microbial adhesion to hydrocarbon
NaCl	Sodium chloride
NaOH	Sodium hydroxide
nm	Nanometer
NMR	Nuclear magnetic resonance spectroscopy
OD	Optical density
SDS	Sodium dodecyl sulfate
TBA	Tributyrin agar
TLC	Thin layer chromatography
ZMA	Zobell marine agar

S. Gurkok (✉) · M. Ozdal
Department of Biology, Science Faculty, Ataturk University, Erzurum, Turkey

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1 Introduction

Surfactants are amphiphilic chemical compounds containing hydrophilic and hydrophobic moieties that partition at physical interfaces and reduce the surface and/or interfacial tension between different phases (Santos et al. 2016). Biosurfactants, on the other hand, are attractive surface-active molecules derived from mostly microorganisms. Biosurfactants have many advantages over synthetic surfactants in terms of structural diversity, lower toxicity, lower critical micelle concentration (CMC), stability, and biodegradability (Edwards et al. 2003; Jahan et al. 2020). Therefore, they are in great demand in different industries with emulsification, foam formation, detergent, and oil dispersion activities.

The biggest obstacle that limits the use of biosurfactants in different industrial sectors instead of their synthetic counterparts is that they are produced in low quantities and at high cost. Majority of the chemical surfactants are obtained from petrochemical industry and can therefore be produced with high efficiency and low cost. However, this mode of production is widely considered as unsustainable and contrary to sustainable green economy strategies, and also damages the ecosystem due to toxicity and bio-incompatibility issues. They are also not preferred because of consumers' tendency toward natural products and sustainable production systems. On the other hand, biosurfactants avoid the concerns related to the use of petrochemical sources. Cost-effective production is extremely important in increasing the application areas of biosurfactants instead of synthetic surfactants. Using cheap substrates and waste materials in production and optimizing the production process are frequently applied strategies (Ozdal et al. 2017; Rastogi and Kumar 2021). However, the isolation of strains that efficiently produce novel biosurfactants with diverse properties is the critical step to overcome the economic constraints of biosurfactant production. The production of biosurfactant has gained importance recently, and in parallel with this, the discovery of novel biosurfactant-producing organisms is kept in the foreground. The process for microbial biosurfactant production begins with sampling, and areas contaminated with hydrocarbons are among the most suitable environments for sampling. Following sampling, various screening methods are used for the detection and isolation of promising microbial strains able to produce high yields of biosurfactants. While majority of the screening methods rely directly on the surface and/or interface activity of the cell culture supernatant. Others are dependent on the microbial cell surface hydrophobicity, which is an indication of biosurfactant synthesis. An ideal screening assay should (1) be able to detect the promising microorganisms, (2) even if it is a qualitative method, it should provide an idea about the production yield and ensure the selection of the most effective microorganisms, (3) be economical and easy to implement, (4) allow screening of a large number of candidates simultaneously, and (5) give fast results and save time.

Microbial biosurfactants have a great structural diversity and can be classified according to different criteria, such as their microbial origin, molecular weight, biochemical structure, and mechanism of action. According to their molecular

weights, they are generally divided into two large groups as high-molecular-weight and low-molecular-weight biosurfactants. Protein, lipoprotein, lipopolysaccharide, polysaccharide, and biopolymers are classified as high-molecular-weight biosurfactants. Lipopeptide, glycolipid, phospholipid, and fatty acids have been grouped as low-molecular-weight biosurfactants (Rosenberg and Ron 1999). Biosurfactants are also classified according to their biochemical structure and the microorganisms that produce them. The five main biosurfactant classes are: (1) glycolipids, (2) phospholipids and fatty acids, (3) lipopeptide/lipoproteins, (4) polymeric surfactants, and (5) particulate surfactants (Parra et al. 1989). Since it is not possible to screen all types of biosurfactants with a single method, it is necessary to use various screening methods in combination for effective, reliable, and accurate screening and isolation (Ariech and Guechi 2015; Gurkok 2022). Based on this, the current chapter provides commonly used methods involved in screening for biosurfactant-producing microorganisms.

2 Sampling for the Isolation of Biosurfactant-Producing Microorganisms

Biosurfactants can be obtained by sampling from a wide variety of environments as seen in Table 1. Although the isolation of biosurfactant-producing microorganisms can be performed by sampling from various undisturbed and contaminated areas (Bodour et al. 2003; Gurkok 2022), hydrocarbon contaminated sites are mostly preferred. Hydrocarbon contamination often results from leakage of crude oil from storage facilities or tanks, spills during transportation of petroleum products, and deliberate discharge of petroleum derivatives and by-products into soil or water. Environments contaminated with hydrocarbons for such various reasons have proven to be good sources for biosurfactant-producing microorganisms in many studies (Shoeb et al. 2015; Joy et al. 2017; Patowary et al. 2017; Astuti et al. 2019; Balakrishnan et al. 2022).

Biosurfactants produced by extremophiles are also of interest for different biotechnological purposes, and therefore, extreme habitats are also preferred as sampling areas (Cameotra and Makkar 1998; Schultz and Rosado 2020). Numerous studies have shown that hot environments, such as deserts, volcanoes, and hot springs (Zarinviarsagh et al. 2017) and cold environments, such as alpines, glaciers, permafrost, ice caves, deep and polar oceans (Perfumo et al. 2018), extreme pH (Arulazhagan et al. 2017), and saline (Sarafin et al. 2014) environments can also be used for sampling.

Table 1 Biosurfactant-producing strains, sampling areas, and screening methods

Strain	Sampling area	Screening method	References
Pseudomonas mendocina ADY2b	Hydrocarbon-contaminated soil of Chennai Harbor	Drop collapse, E_{24}, hemolysis, oil spreading	Balakrishnan et al. (2022)
Bacillus sp., *Streptomyces* sp., *Microbacterium* sp., *Micrococcus* sp., *Rhodococcus., Pseudomonas, Arthrobacter* sp., *Staphylococcus* sp.	Oil batteries, Chauvin, Alberta	CTAB, drop collapse, oil spreading, emulsification, hemolysis	Rani et al. (2020)
B. subtilis, P. aeruginosa, B. tequilensis, B. safensis	Potwar oil fields, Pakistan	Drop collapse, E_{24}, emulsification assay, hemolysis, tilted glass slide, oil spreading	Sohail and Jamil (2020)
Serratia sp., *Paenibacillus* sp., *Citrobacter* sp.	Soil, Amapaense Amazon	Drop collapse, E_{24}, oil spreading, surface tension	Oliveira et al. (2021)
B. subtilis	Brackish water of Chilika Lake, Odisha, India	Bath, drop collapse, E_{24}, oil spreading, surface tension	Nayarisseri et al. (2018)
Pseudoxanthomonas sp.	Petroleum reservoir, South Sumatra	E_{24}, hemolysis, interfacial tension, oil spreading	Astuti et al. (2019)
Geotrichum candidum, Galactomyces pseudocandidum, Candida tropicalis	Rhizosphere soil, Egypt	CTAB, E_{24}, hydrocarbon overlay agar plate, hemolysis, oil spreading, Parafilm-M, phenol sulfuric acid, phenol red test, surface tension	Eldin et al. (2019)
Franconibacter sp.	Soil from Lakwa oil field, Assam	CTAB, drop-collapse, E_{24}, oil spreading, Parafilm M	Sharma et al. (2022)
C. parapsilosis	Contaminated dairy products, India	Drop collapse, E_{24}, hemolysis, oil spreading	Garg and Chatterjee (2018)
Aspergillus terrus, A. fumigatus	Crude oil sludge, Malaysia	Drop collapse, E_{24}, oil spreading, parafilm test, surface tension	Othman et al. (2022)
Brevibacterium casei	Textile wastewater	E_{24}, oil spreading, surface tension	Carolin et al. (2021)
Halomonas elongata	Khewra slat mines, Pakistan	CTAB, drop collapse, E_{24}, hemolysis	Fariq and Yasmin (2020)
B. halotolerance	Oil fields, CNPC, China	Oil spreading	Wang et al. (2022)
Pseudomonas sp.	Motor oil-contaminated soil, Tunisia	E_{24}, oil spreading, surface tension	Chebbi et al. (2017)
S. marcescens	Hydrocarbon-contaminated site in Melaka, Malaysia	E_{24}, surface tension	Almansoory et al. (2019)

(continued)

Table 1 (continued)

Strain	Sampling area	Screening method	References
S. quinivorans, Psychrobacter arcticus	Pony Lake, Ross Island, Antarctica	E_{24}, oil spreading	Trudgeon et al. (2020)
Janthinobacterium svalbardensis	Cotton glacier, Transantarctic Mountains, Antarctica	E_{24}, oil spreading	Trudgeon et al. (2020)
Ochrobactrum intermedium	GheynarjeNir hot spring Ardebil, Iran	Drop collapse, E_{24}, hemolysis, oil spreading	Zarinviarsagh et al. (2017)
Kocuria marina	Condenser pond of Kovalam, India	Drop collapse, E_{24}, hemolysis, oil spreading	Sarafin et al. (2014)

3 Methods for Screening Biosurfactant-Producing Microorganisms

Biosurfactants are structurally heterogeneous amphiphilic molecules derived from mostly microorganisms. As a result of the heterogeneity, diverse approaches for screening prospective biosurfactant-producing microorganisms have been well-established, devised, and implemented as shown in Table 1.

Some of the screening methods including, bacterial adherence to hydrocarbon, hydrophobic interaction chromatography, salt aggregation test, and replica plate tests are based on measuring cell surface hydrophobicity, which is directly related to biosurfactant production (Pruthi and Cameotra 1997). In most screening methods, crude oil, hexane, n-hexadecane, xylene, and sunflower oil are used as hydrocarbons. In the following screening methods, controls are not individually specified for each method, but typically, 1% (w/v) SDS or Triton X-100 is used as the positive control, and distilled water or a buffer is applied as the negative control.

3.1 Bacterial Adhesion to Hydrocarbons Assay

The test known as Bacterial Adhesion to Hydrocarbon (BATH) or Microbial Adhesion to Hydrocarbon (MATH) was first used for measuring cell-surface hydrophobicity by Rosenberg et al. (1980). According to this method, biosurfactant producer microorganisms attach to hydrocarbons due to their hydrophobic cell surfaces. By measuring the hydrophobicity of the cell, this method offers a simple and rapid spectrophotometric test for prescreening for biosurfactant-producing microorganisms. Detection of biosurfactant-producing bacterial strains of *Acinetobacter calcoaceticus*, *Bacillus pumilus*, *B. laterosporus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Serratia marcescens,* and *Staphylococcus aceticus* was achieved rapidly

by testing cell surface hydrophobicity, which has a direct correlation with biosurfactant production (Pruthi and Cameotra 1997). Using this method, numerous microorganisms producing various types of biosurfactants including lipopeptides, phospholipids, glycolipids, fatty acids, and polymeric biosurfactants have been identified (Nayarisseri et al. 2018).

In this assay, after 18 to 24 h of incubation in liquid medium, cells are harvested by centrifugation. Cell pellets are washed several times with phosphate buffer and suspended in the same buffer to reach an optical density of about 0.5 at 600 nm. In a test tube, 2 mL of cell suspension and 100 μL of a hydrocarbon, such as octane, hexane, xylene, hexadecane, or crude oil, are mixed and vortexed briefly for 2 to 3 min. The mixture is left for approximately 1 h to allow separation of aqueous phase and hydrocarbon phase. The OD of the aqueous solution is then determined at 600 nm to estimate the reduction in turbidity. The formula below is used to calculate the percentage of cell adhesion to the hydrocarbon.

$$\text{Cell adhesion } (\%) = [1-(\text{OD}_{600\text{ aqueous solution}}/\text{OD}_{600\text{ starting cell solution}})] \times 100.$$

3.2 *Bromothymol Blue (BTB) Assay*

BTB assay is a quantitative colorimetric assay used to screen the strains producing lipopeptide-containing biosurfactants by mixing a solution of BTB (0.2 mM) in phosphate buffer, pH 7.2 with an equal volume of cell-free culture medium. Color change is determined spectrophotometrically at 410 and 616 nm. This method can be used for both culture broth and purified lipopeptide-containing biosurfactants, such as surfactin, iturin, and fengycin. Color changes are determined as yellow for iturin, light green for fengycin, and green for surfactin (Ong and Wu 2018). The advantages of this method are that it is suitable for rapid, simple, quantitative analysis, and screening of lipopeptide type biosurfactant-producing species. In addition, similar to this test, cetylpyridinium chloride-bromothymol blue (Yang et al. 2015) and polydiacetylene (Zhu et al. 2014) methods were also used for surfactin determination.

Recently, Kubicki et al. (2020) have reported a useful colorimetric method with the potential to detect biosurfactant in culture supernatants. Victoria Pure Blue BO, a hydrophobic blue dye, can also be used in the comparative assessment of biosurfactant quantification in supernatants of bacterial cultures (Kubicki et al. 2020). The quantity of dye released is measured spectrophotometrically at 625 nm.

3.3 CTAB Agar (Blue Agar) Plate Assay

Siegmund and Wagner (1991) described this method for the detection of rhamnolipids. The blue agar plate assay is another name for the CTAB (cetyltrimethylammonium bromide) agar plate assay. This method is used for screening of extracellular glycolipids or anionic surfactants, which have a polar head and a nonpolar tail. Microorganisms that produce glycolipid-type biosurfactant, such as *P. aeruginosa*, form a clear halo around the colony as shown in Fig. 1.

For screening, microbial isolates are spot-inoculated on mineral salt agar medium supplemented with 0.005 g/L methylene blue and 0.2 g/L CTAB and grown for 1 to 2 days. Formation of the clear blue zone surrounding the streaks on dark blue agar plates is attributed to the secretion of anionic biosurfactant. CTAB assay is a semiquantitative approach because the size of the zone is related to the amount of biosurfactant released.

CTAB screening assay provides fast, simple, and accurate results, but CTAB itself has been shown to inhibit the development of several bacteria. Furthermore, its specificity for anionic biosurfactants like glycolipids limits its use in screening microorganisms producing other types of biosurfactants. Therefore, this approach has often been used to evaluate producer of glycolipid biosurfactants (Eldin et al. 2019; Rani et al. 2020).

3.4 Drop-Collapse Assay

Drop-collapse assay works on the idea that biosurfactant destabilize or collapse the liquid droplets on hydrocarbon surface. In drop-collapse screening assay, described by Jain et al. (1991), drops of culture supernatant are deposited on a surface coated

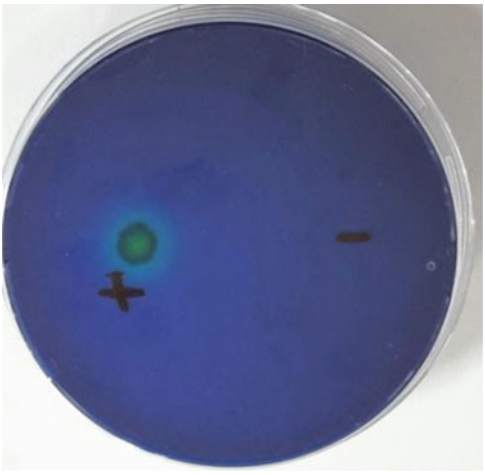

Fig. 1 Clear halo formation by *P. aeruginosa* (+) on mineral salt agar plates supplemented with 0.005 g/L methylene blue and 0.2 g/L CTAB. No zone formation is observed in microorganisms that cannot produce biosurfactant (−)

with oil. In the lack of biosurfactant, the hydrophobic surface repels the polar water molecules, keeping the drop stable on oil-coated surface. Drops from the culture supernatant of biosurfactant-producing colonies collapse and spread as a result of the decrease in the interfacial tension between the oil-coated surface and the liquid droplet. The consistency of drops is connected with surface tension but not with emulsifying activity, and it is dependent on biosurfactant concentration.

Bodour and Miller-Maier (1998) developed the drop-collapse assay on 96-well microtiter plate cover. Two microliters of oil is placed on 96-well microplate lid and left to equilibrate at room temperature for 24 h. A drop of supernatant is applied to the surface coated with oil and monitored after 1 min by the use of a magnifying glass. In the absence of biosurfactant, the drops remained stable and rounded; but in its presence, they spread and became flat.

Drop-collapse assay is a reliable technique often used in screening as it offers a quick and simple approach to evaluate large numbers of samples simultaneously, does not require the use of special equipment, and only a minimal volume of sample is required for examination (Sohail and Jamil 2020; Rani et al. 2020; Oliveira et al. 2021; Balakrishnan et al. 2022).

3.5 *Emulsification Assay*

Emulsification assay precedes the most commonly used methods for screening biosurfactant-producing colonies (Nayarisseri et al. 2018; Rani et al. 2020; Sohail and Jamil 2020; Carolin et al. 2021). Assay, described by Rosenberg et al. (1979), evaluates the emulsification of a hydrocarbon by spectrophotometric measurements.

Culture supernatant, suspended in Tris buffer (pH 8), is mixed with equal volume of crude oil and vortexed for 1 min. The emulsion is allowed to stand for about 20 min, and the optical density in the aqueous phase is then measured in the spectrophotometer at 400 nm. The formula below is used to calculate emulsification activity (EU/mL).

$$\mathrm{EU/mL} = 0.01\ \mathrm{OD}_{400} \times \mathrm{Dilution\ Factor}$$

3.6 *Emulsification Index Assay (E_{24})*

Emulsification index approach described by Cooper and Goldenberg (1987) is another frequently used method for screening of the biosurfactant producers (Zarinviarsagh et al. 2017; Chebbi et al. 2017; Almansoory et al. 2019; Trudgeon et al. 2020; Fariq and Yasmin 2020). It is simple to implement and requires minimal specialist instruments. Emulsifying activity is measured by calculating the emulsification index (E_{24}) for a crude oil.

In E_{24} examination, culture supernatant is mixed with equal volume of hydrocarbon in a test tube, vortexed thoroughly for at least 2 to 3 min, and allowed to stand for 24 h at room temperature. Emulsification index is calculated by using the formula as given below:

$$\text{Emulsification index } (E_{24}) = (\text{Height of the emulsion layer}/\text{Total height}) \times 100.$$

3.7 Foam Test

Biosurfactants have foaming properties due to their amphiphilic (hydrophobic and hydrophilic groups) nature (Gurkok and Ozdal 2021a). The foaming properties of biosurfactants can be used to screen for biosurfactant producers. Foaming is related to the reduction of surface tension by surfactants. Foam, in the presence of surfactant, reduces the surface tension between an aqueous solution and air, resulting in the mixing of the two different phases and, consequently, the formation of bubbles.

The foaming capacity is determined by transferring 10 mL of the cell-free culture broth into a 50 mL graduated measuring cylinder and vigorously shaking or vortexing for 1 to 2 min (El-Sheshtawy and Doheim 2014). To calculate the foaming capacity, the foaming height and the total height are measured. The foaming capacity is determined according to the following equation.

$$\text{Foaming capacity} = (\text{Height of foam}/\text{Total height}) \times 100.$$

This method is an easy and simple test to screen biosurfactant production (Hamzah et al. 2020).

3.8 Hemolysis Test

The basic idea behind the hemolysis test invented by Mulligan et al. (1984) is that biosurfactants can lyse erythrocytes. In this assay, isolates to be screened are streaked on blood agar plates containing 5% blood and incubated for 2 days at 25 °C. Formation of hemolysis halos surrounding the colonies due to the blood cells lysis indicates the production of biosurfactant as shown in Fig. 2.

The fairly easy to implement hemolysis assay is often used as a preliminary screening for biosurfactant producers (Astuti et al. 2019; Sohail and Jamil 2020; Rani et al. 2020; Balakrishnan et al. 2022), but the method has some limitations, such as giving false-positive and false-negative results. In the absence of biosurfactant, lytic enzymes also cause the formation of a transparent hemolysis zone; in the presence of some biosurfactants, clear zone formation may not be

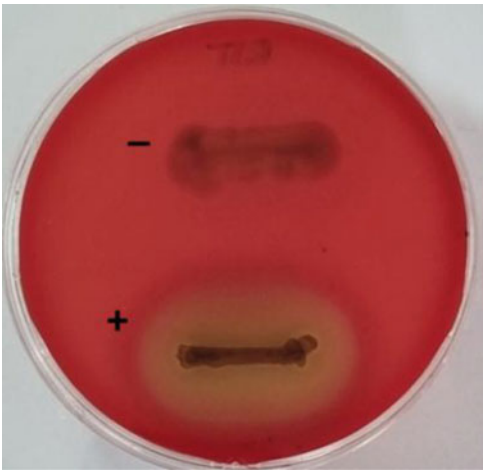

Fig. 2 Hemolysis zone formation by *P. aeruginosa* (+) after incubation on blood agar plates with 5% blood at 25 °C for 2 days

observed because they do not have hemolytic activity (Schulz et al. 1991). Therefore, this test needs to be validated by other screening methods.

3.9 Hydrocarbon Overlay Method

Hydrocarbon overlay assay uses Zobell Marine Agar (ZMA) coated with a hydrocarbon, such as benzene, toluene, and hexadecane, for qualitative screening of biosurfactants producers. Isolates are spot-inoculated on ZMA plates and incubated at 25 °C for 3 to 5 days. The formation of an emulsified halo surrounding the colonies suggests the production of biosurfactants (Nayarisseri et al. 2018; Eldin et al. 2019).

3.10 Hydrophobic Interaction Chromatography (HIC)

Hydrophobic interaction chromatography (HIC) allows the rapid and reliable screening for biosurfactant producer isolates depending on the hydrophobicity of the cell surface, which has a direct relationship with biosurfactant production (Smyth et al. 1978). Since, HIC is a chromatographic separation technique that separates molecules based on differences in their surface hydrophobicity, it can simultaneously purify these isolates in addition to screening.

The column resin with hydrophobic moieties like butyl, phenyl or octyl, is stabilized with a buffer containing salt to increase hydrophobic interaction. Cells are suspended in this buffer, and suspension is applied to the column. Non-retained cells are eluted whereas organisms having hydrophobic cell surface are retained by

the column. The initial cell suspension is compared to the elution by cell count or spectrophotometer measurement to determine the hydrophobic index as the percentage of retained bacteria.

3.11 Lipase Assay

The principle of using the lipase assay to screen biosurfactant-producing bacteria is that there is a correlation between production of lipases and biosurfactants (Colla et al. 2010). Lipase production is the characteristic feature of the biosurfactant-producing organisms (Kalyani and Sireesha 2014). Qualitative lipase assay is performed on tributyrin agar (TBA) plate (Gurkok and Ozdal 2021b). Isolate is streaked on TBA plate, and lipolytic clear zone formation is monitored after 3 to 5 days of incubation at 25 °C (Fig. 3). This assay is used as prescreening approach and should be confirmed by additional screening methods (Kalyani and Sireesha 2014; Chittepu 2019).

3.12 Oil-Displacement (Oil-Spreading) Assay

Oil-spreading test also known as oil-displacement test has been described by Morikawa et al. (2000) and is widely used for screening of biosurfactant-producing microorganisms (Zarinviarsagh et al. 2017; Garg and Chatterjee 2018; Trudgeon et al. 2020; Othman et al. 2022; Wang et al. 2022; Balakrishnan et al. 2022). It is a reliable, fast, and simple method and requires only a small amount of sample, and therefore, it is one of the most commonly used tests.

Fig. 3 Lipolytic zone formation by *B. cereus* on TBA plate after 3 days at 25 °C

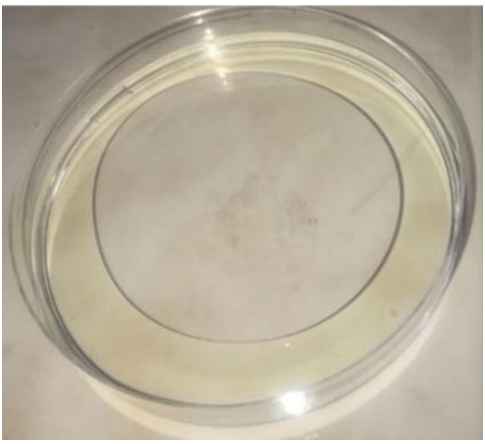

Fig. 4 Oil-spreading assay of *P. aeruginosa* cell-free culture supernatant by using diesel motor oil

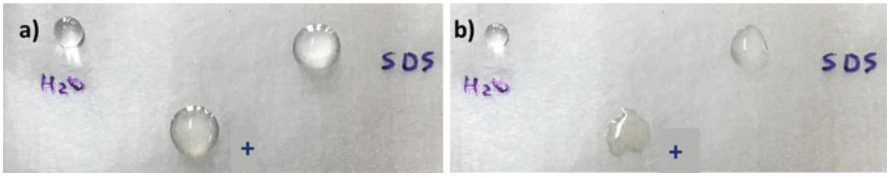

Fig. 5 Parafilm M test by *P. aeruginosa* cell-free culture supernatant deposited on parafilm after 1 min (**a**) and 5 min (**b**). SDS is the positive control; H_2O is the negative control

In this screening method, 1 mL of oil is deposited on the surface of 20 to 30 mL distilled water in a Petri plate to create a fine coating of crude oil. Ten microliter of culture supernatant is deposited in the center of the oil layer surface. In the presence of biosurfactant, a distinct zone surrounding the supernatant is observed as shown in Fig. 4. There is a linear correlation between the size of the zone and the amount of biosurfactant.

3.13 Parafilm M Test

Parafilm M test is fairly simple and rapid test requiring modest amount of sample. This approach is often used for qualitative preliminary screening of biosurfactant producer organisms in conjunction with other procedures (Eldin et al. 2019; Sharma et al. 2022; Othman et al. 2022). A droplet of supernatant is deposited with a micropipette on parafilm M, which is used as a hydrophobic surface. After a minute, the shape of the droplet on the parafilm is monitored. The droplet spreads on the surface when biosurfactant is present; otherwise, it remains dome-shaped (Fig. 5).

3.14 Penetration Assay

Penetration test, developed by Maczek et al. (2007), is another assay appropriate for high throughput screening of biosurfactant producers (Chittepu 2019). This test is based on the color change that occurs when two insoluble phases come into contact.

The wells of 96-well microplates are filled with 200 μL of hydrophobic paste containing oil and silica gel. Hydrophobic paste is covered by 10 μL of crude oil. Ninety microliter of supernatant stained with 10 μL safranin is deposited to the wells, and color change is monitored. Oil cover is destroyed in the presence of biosurfactant, and silica gel reaches to the hydrophilic phase within 10 to 15 min, and the color of the supernatant shifts from bright red to hazy white. The color of the supernatant still gets cloudy but stays red in the lack of biosurfactant.

3.15 Replica Plate Assay

Rosenberg (1981) developed the replica plate experiment on the idea that cell producing biosurfactant binds to hydrophobic polystyrene because of its hydrophobic cell surface. This method allows both screening and isolation of biosurfactant-producing colonies.

Cells to be examined are incubated on agar medium. A sterile and flat polystyrene disc is pressed on colonies, and the replicas of the colonies are formed. The disc is rinsed with water to eliminate the loosely attached colonies. Biosurfactant-producers due to their hydrophobic surfaces have affinity to polystyrene and firmly attach to disc. Remaining securely adhered colonies are fixed with methanol and dyed for visibility. The positive colonies can easily be obtained by isolation from the original plate.

3.16 Salt Aggregation Assay

Salt aggregation assay invented by Lindahl et al. (1981) is also a screening approach based on cell surface hydrophobicity of biosurfactant-producing cells. Salt aggregation testing, which requires no special equipment, provides an easy way to screen for bacteria-producing biosurfactants (Pruthi and Cameotra 1997; Walter et al. 2010). Depending on their cell surface hydrophobicity, various cell types precipitate at different salt concentrations. Cells that produce biosurfactants have more hydrophobic cell surfaces and precipitate at lower salt concentrations. Due to its great solubility, ammonium sulfate ($(NH_4)_2SO_4$) is widely employed in salt aggregation assays.

In the assay, an overnight culture is centrifuged, and the harvested cell pellets are dissolved in phosphate buffer. Increasing quantities of $(NH_4)_2SO_4$ (0.01–4.0 M) are

arranged in the same buffer. Equal amounts of cell suspension and salt solutions are combined at room temperature on a glass depression slide. The aggregation result, which produces a clear solution and white aggregates, is then evaluated on a black background.

3.17 Surface/Interfacial Tension Analysis

Determination of surface or interfacial activity of a cell culture supernatant is the easiest way of screening for biosurfactant-producing microorganisms. The surface tension is defined as the surface force between a liquid and air, while interfacial tension is the force between two liquids (Bodour and Miller-Maier 1998). Surface/interfacial activity is measured directly by the use of a digital tensiometer coupled with a Du Noüy platinum ring (Du Noüy 1925). Du Noüy platinum ring is positioned beneath the supernatant surface. The force needed to move the ring from the aqueous phase to the air is determined and used to determine surface tension using the equation below:

$$\mathrm{ST} = [(F\text{-}P_0)/4\Pi r] \times 1000$$

Where, F is the force measured, P_0 is the force measured prior to removing the ring, and r is the ring radius.

The surface/interfacial tension can also be measured by the stalagmometric method (Dilmohamud et al. 2005), pendant drop shape method (Nierderhauser and Bartell 1950; Tadros 2008), and axisymmetric drop shape method (Van der Vegt et al. 1991).

Although surface/interfacial tension analysis requires specialized equipment, it is a frequently used method due to ease of application and to get fast and accurate results (Astuti et al. 2019; Eldin et al. 2019; Oliveira et al. 2021).

3.18 Tilted Glass Slide Assay

In the tilted glass slide assay described by Persson and Molin (1987), a single colony to be screened is transferred to a drop of 0.9% NaCl placed at one end of a glass slide. The slide is tilted to the opposite side and the movement of a water droplet along its surface was monitored. In the presence of biosurfactant, the water runs across the surface. Tilting glass slide assay is a relatively less used method compared to the others (Sohail and Jamil 2020).

3.19 Determination of the Biochemical Composition of Biosurfactants

The biochemical composition of biosurfactants can be determined by total protein, total carbohydrate, and total lipid content analysis.

3.19.1 Phenol-Sulfuric Acid Test

Phenol sulfuric acid test applied for the screening of glycolipid surfactants was developed by Dubois et al. (1956). One milliliter of culture supernatant is mixed with 1 mL of 5% phenol. To this mixture, 3 to 5 mL of concentrated sulfuric acid (H_2SO_4) was added drop by drop. The presence of glycolipid biosurfactant is indicated by a color shift from yellow to orange. This method is generally used to quantify rhamnolipids by using a rhamnose standard curve (Ozdal et al. 2017; Eldin et al. 2019).

3.19.2 Biuret Test

In the presence of peptides, the copper (II) ion forms mauve in a basic solution and albumin is used as a standard. The biuret test is used to detect the presence of lipopeptide biosurfactants, such as lichenysin, fengycin, iturin, and pumilacidin produced by *Bacillus* genus (Kumar and Ngueagni 2021).

Two mL of crude extract solution is heated to 70 °C and 10 drops of 1 M NaOH solution are added. Next, 1% $CuSO_4$ is slowly added to the mixture to observe the violet or pink color change and measurements are made at 540 nm (Patel and Patel 2020).

3.19.3 Phosphate Test

This test is used to detect the presence of phospholipid biosurfactants. After adding 10 drops of 6 M HNO_3 to 2 mL of crude extract solution, it is heated to 70 °C. Five percent ammonium molybdate is added dropwise to the mixture. The formation of a yellow precipitate after the formation of a yellow color indicates the presence of phospholipids (Patel and Patel 2020).

In addition to these techniques, new screening approaches continue to be developed. The use of matrix-assisted laser desorption/ionization time-of-flight mass spectrometry to screen for glycolipid-type biosurfactant-producing organisms is an example to recent approaches (Sato et al. 2019). With the discovery of novel biosurfactants, new screening systems will undoubtedly be put into use in the near future.

4 Analytical Methods for Compound Detection of Biosurfactants

Characterization is generally performed with the purified biosurfactants obtained after extraction and purification steps. Some of the characterization methods used to analyze the biosurfactant properties are listed in the Table 2.

4.1 Extraction of Crude Biosurfactant

The main purpose of the extraction is to obtain crude biosurfactants that are free from other culture media components. The most common biosurfactant recovery method is acid precipitation followed by extraction with organic solvents (chloroform-methanol, ethyl acetate). As it is relevantly cheap method, acid precipitation is generally preferred. Concentrated HCl is widely added to culture supernatant for acidification until pH reaches 2.0 and the mixture is left overnight at 4 °C for precipitation. The biosurfactant is then extracted with chloroform:methanol (2:1 v/v) or ethyl acetate using a separatory funnel (Abdelli et al. 2019; Ratna and Kumar 2022). Different methods, such as ammonium sulfate precipitation, acetone

Table 2 Biosurfactant-producing microorganisms and analytical methods used for their characterization

Microorganism	Recovery process	Analytical method	References
B. safensis	Acid precipitation+ ethyl acetate	TLC, LC-MS, HPLC	Abdelli et al. (2019)
Ochrobactrum anthropi, Citrobacter freundii	Acid precipitation + chloroform: methanol (2:1)	TLC, FTIR	Ibrahim (2018)
Candida tropicalis	Chloroform: methanol (2:1)	TLC, FTIR, NMR LC-MS, GC-MS	Almeida et al. (2021)
B. velezensis	Ammonium sulfate	TLC, HPLC, GC-MS	Meena et al. (2021)
Metschnikowia-hurdharensis	Ethyl acetate	TLC, FTIR, LC-MS, GC-MS	Kumari et al. (2021)
B. altitudinis	Acid precipitation + ethyl acetate	TLC, FTIR, HPLC, GC-MS	Goswami and Deka (2019)
Gordonia sp.	Acid precipitation + chloroform: methanol (2:1)	TLC, FTIR, NMR, GC-MS, LC-MS	Zargar et al. (2022)
P. putida	Acid precipitation + chloroform: methanol (2:1)	TLC, FTIR, LC-MS	Mishra et al. (2020)
P. aeruginosa	Acid precipitation + chloroform: methanol (2:1)	TLC, GC-MS, HPLC	Hrůzová et al. (2020)
P. aeruginosa	Acid precipitation + chloroform: methanol (2:1)	TLC, FTIR, NMR, GC-MS, LC-MS	Ratna and Kumar (2022)

precipitation, centrifugation, crystallization, adsorption, and foam fractionation are the others methods for extraction of biosurfactants.

4.2 Chromatographic and Spectroscopic Methods

Thin layer chromatography (TLC), high-performance liquid chromatography (HPLC), liquid chromatography-mass spectrometry (LC-MS), gas chromatography-mass spectrometry (GC-MS), Fourier transform infrared spectroscopy (FTIR), and nuclear magnetic resonance spectroscopy (NMR) are recently preferred approaches used for detection of biosurfactants because of their automation, high sensitivity, and accuracy (Table 2). These methods have both advantages and disadvantages and can be chosen according to the properties of biosurfactants, including stability, solubility, molecular size, and charge.

TLC is a straightforward technique for determining the presence of substances such as lipids, peptides, and sugars. The purified biosurfactant (approximately 0.1 g) is dissolved in methanol or chloroform then aliquots (20 μL) are applied to silica gel TLC plate. The TLC plate is run with a mobile phase of chloroform:methanol:water (65:25:4) (Abdelli et al. 2019). Molish's reagents, iodine vapor, and 1% ninhydrin solution are sprayed on dry plate for staining of sugars, lipids, and free amino groups, respectively (Ibrahim 2018).

HPLC is a special type of column chromatography where it can separate the mixture of surfactant compounds, identify, quantify, and purify the biosurfactant components separately. HPLC applications have been reported for the purification, characterization, and quantification of biosurfactants (Twigg et al. 2021).

FTIR is a useful tool for rapid analysis and determination of functional groups of biosurfactants. This method determines hydroxyl, ester, and carboxylic groups in biosurfactants according to their IR absorption bands (Eslami et al. 2020; Sen et al. 2021).

GC-MS is widely used for structural analysis of biosurfactants. Frequently, it is used for quantitative or qualitative analysis of fatty acid structures. When combined with MS, information about the molecular mass and elemental composition, functional groups, and molecular geometry of each separated compound can be obtained. GC or GC-MS analysis is also used for the analysis of fatty acid chain derivatives (Biniarz et al. 2017).

LC-MS provides an excellent measurement for rapid, inexpensive, and quantitative measurements of organic molecules in a wide variety of applications. In general, LC-MS analyzes the hydrophilic (water-loving) part of the biosurfactant compound, while GC-MS identifies the hydrophobic (water-repellent) part (Jimoh and Lin 2019).

NMR gives information about the bond structures and functional groups in lipids and carbohydrates. NMR is a suitable technique to accurately correlate the chemical structure and position of the presence of biosurfactant compounds in a sample (Kim

et al. 2018). It is a suitable method for the chemical structure determination of the novel biosurfactants.

5 Future Directions

The vast majority of biosurfactants have been detected from microbial sources in a culture-dependent manner, leaving mostly unexplored supply of uncultured microorganisms producing possibly new biosurfactant structures. Recent progresses in understanding biosurfactants at the genome level have made screening of microorganisms much easier. Parallel to these developments, modern technologies rather than traditional culture-dependent approaches have begun to be considered and applied for the discovery of new microorganisms. Advanced techniques, such as metagenomic and meta transcriptomic analysis, have been used to investigate the potential of microorganisms for biosurfactant production (Jackson et al. 2015; Thies et al. 2016; Williams and Trindade 2017; Williams et al. 2019).

A new gene related to biosurfactant production and hydrocarbon degradation has been identified by da Araújo et al. (2020). They extracted environmental DNA from soil samples and constructed a metagenomic library. They identified a biosurfactant-positive clone by functional screening and an open reading frame with high similarity to sequences encoding a hypothetical protein. They purified the protein and observed biosurfactant activity. Also, they observed elevated hydrocarbon degradation in the *E. coli* cells transformed with the gene encoding this protein.

With these promising methods, environmental DNA samples as well as microorganisms can be genetically examined and rapidly screened without the need for culturing microorganisms, and enable the identification of novel biosurfactants with different and desired properties in the future (Perfumo et al. 2018).

6 Conclusion

The main barriers to large-scale biosurfactant production are still high production costs and low yields. In addition to using inexpensive substrates and waste or by-products in production and optimizing the production process, the isolation of strains that efficiently produce novel biosurfactants with diverse properties is a critical step to overcome the economic constraints of biosurfactant production. Currently, biosurfactants such as rhamnolipid, sophorolipid, and surfactin are commercially produced. It is necessary to find new biosurfactants with suitable structural diversity for specific purposes in different industries. One of the approaches to find new biosurfactants is the application of different screening methods. The shift to more precise, efficient screening methods is seen as the key to the discovery of new biosurfactants. In order to accelerate the discovery of new biosurfactants, technological devices should also be utilized. Although there are many screening methods,