# Screening Strategies for Biosurfactant Discovery

Marla Trindade, Nombuso Sithole, Sonja Kubicki, Stephan Thies, and Anita Burger

Contents



Abstract The isolation and screening of bacteria and fungi for the production of surface-active compounds has been the basis for the majority of the biosurfactants discovered to date. Hence, a wide variety of well-established and relatively simple methods are available for screening, mostly focused on the detection of surface or interfacial activity of the culture supernatant. However, the success of any biodiscovery effort, specifically aiming to access novelty, relies directly on the

M. Trindade (✉), N. Sithole, and A. Burger

Institute for Microbial Biotechnology and Metagenomics, University of the Western Cape, Cape Town, South Africa
e-mail: ituffin@uwc.ac.za; alburger@uwc.ac.za

S. Kubicki and S. Thies
Institute of Molecular Enzyme Technology, Heinrich-Heine-Universität Düsseldorf, Düsseldorf, Germany
e-mail: s.kubicki@fz-juelich.de; s.thies@fz-juelich.de

characteristics being screened for and the uniqueness of the microorganisms being screened. Therefore, given that rather few novel biosurfactant structures have been discovered during the last decade, advanced strategies are now needed to widen access to novel chemistries and properties. In addition, more modern Omics technologies should be considered to the traditional culture-based approaches for biosurfactant discovery. This chapter summarizes the screening methods and strategies typically used for the discovery of biosurfactants and highlights some of the Omics-based approaches that have resulted in the discovery of unique biosurfactants. These studies illustrate the potentially enormous diversity that has yet to be unlocked and how we can begin to tap into these biological resources.

Keywords Bioprospecting, High-throughput, Functional properties, Microbial, OMICS, OSMAC, Sequence-based, Surface-active

## 1 Introduction

Diverse microbial genera, from all domains of life, Bacteria, Archaea, and Eukaryota, have been identified as biosurfactant producers. To this end biodiscovery efforts have primarily focused on the screening of culturable isolates and generally included (1) the enrichment of microorganisms from a wide range of environments; (2) high-throughput screening using numerous assays suitable for the evaluation of large numbers of isolates; followed by (3) isolation and structural determination of the biosurfactant (Fig. 1). To increase the chance of identifying a novel compound, several culturing considerations have been applied in the biodiscovery stage. These include sampling from exotic (extreme) and underexplored environments [1]; targeting environments with prior exposure to hydrocarbon pollutants which have naturally selected for biosurfactant-producing microorganisms [2–4]; utilizing hydrophobic compounds in the culture media to enrich for the most capable producers [5]; and focusing on microorganisms from underrepresented phyla [6]. Considering that the compound identification and structural determination entails a time-consuming investigation, strain prioritization and dereplication are normally applied after the primary screening. Factors that are typically considered to select the most superior and novel producers include the novelty of the strain, the biosurfactant yield (based on the initial screening), and the activity range across property-based assays [7]. However, despite all these considerations, the structural diversity of the commercially available biosurfactants remains limited, and literature continues to report the rediscovery of structurally similar biosurfactants. Therefore, to identify truly novel biosurfactants, discovery efforts need rejuvenated approaches that critically assess the aspects that limit access to the novelty that is sought.

Indicated by Omics studies, it has become widely accepted that we have only scraped the tip of the iceberg in terms of accessing the biotechnological potential harbored in microorganisms. It can be reasonably expected, therefore, that there is yet much biosurfactant diversity to be discovered by investigating non-cultivable

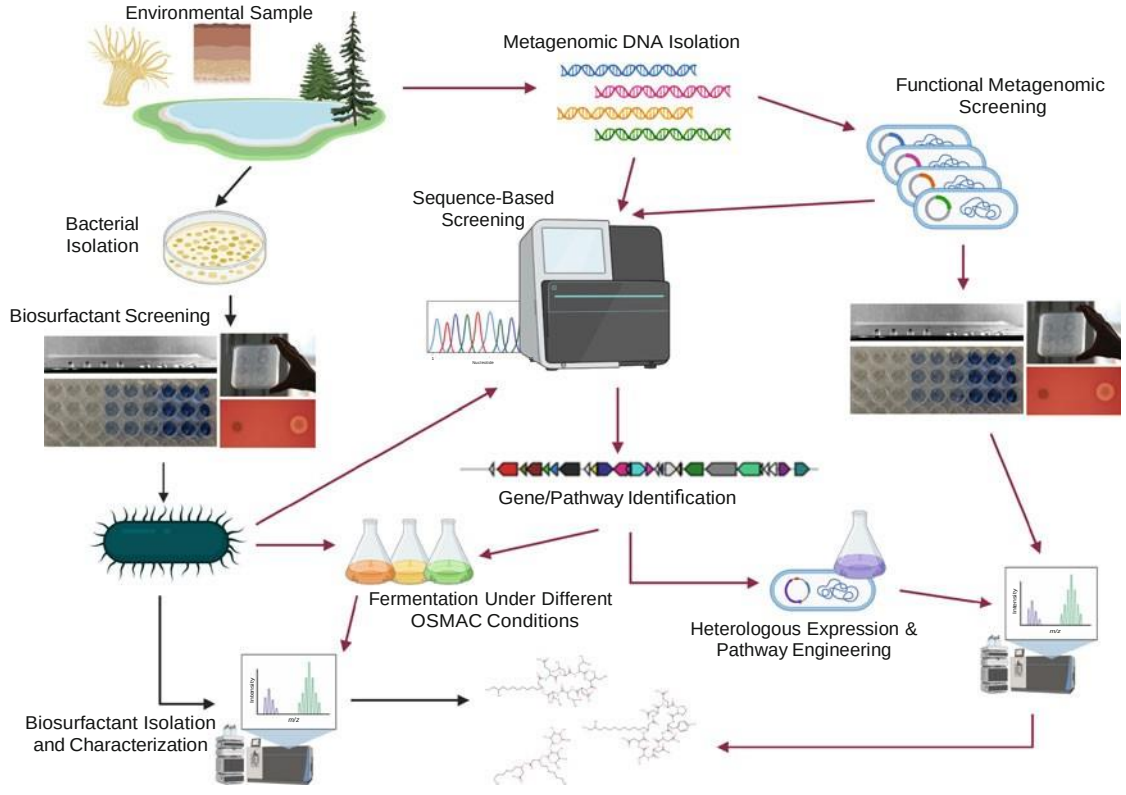


Fig. 1 The biosurfactant discovery pipeline. Biosurfactant discovery has largely been conducted through the isolation and screening of bacteria and fungi using traditional microbiological techniques (black arrows). Given that rather few novel biosurfactant structures have been discovered during the last decade, advanced strategies are needed to widen access to novel chemistries and properties. Omics technologies (red arrows) are beginning to yield unique biosurfactants and offer powerful tools to explore the expansive potential harbored in natural environments

microorganisms using culture-independent screening approaches, followed by the assessment of the activity through heterologous expression (Fig. 1). Moreover, the increased sequencing of culturable bacterial genomes has revealed great disparity between the number of putative secondary metabolite gene clusters identified based on bioinformatics analyses vs. the actual number of secondary metabolites detected from culture fermentations [8]. This not only points to an astounding level of as yet undiscovered metabolites for the majority of cultured organisms, but it also provides the opportunity to employ sequence-based analyses to inform on strategies to elicit the biosynthetic pathways that remain silent during the growth of culturable isolates under standard laboratory conditions. The level to which genome mining (vs. screening for biosurfactant activity) can already contribute to biosurfactant discovery will be discussed in this chapter (Fig. 1).

Regardless of whether culture-based or culture-independent approaches are employed for biosurfactant discovery, assessment of the activity constitutes a crucial component of each biodiscovery approach. Focused on the detection of novel biosurfactants with specific physicochemical characteristics and performance properties, the selection of a strategy to screen for biosurfactant-producing microorganisms is of vital importance. The strategy sets the scene for the likelihood and the level of success in the identification of novel and structurally diverse biosurfactants with potential commercial value in different sectors of bioeconomy. In the interest of product development, the strategy should not only incorporate methods to screen large collections of microorganisms rapidly, easily and with a high level of sensitivity, but should also facilitate the detection of specific biosurfactant characteristics and the quantification of key parameters for the selection of the most promising biosurfactant candidates. Therefore, this chapter will provide an exhaustive summary of the wide range of screening methods available, addressing specific factors that need to be considered and revised in the prospecting efforts to ensure that novel properties and structures are identified. Another major obstacle to the development of biosurfactants for commercial application is the yield, which may be factored into the screening process. However, some bioengineering approaches to improve strain yields are available, therefore, this chapter will not incorporate yield as a screening criterium.

## 2 Screening Methods Based on Physical Properties

A wide range of screening methods, based on the physicochemical properties displayed by surfactants, are available for the identification of biosurfactants produced by microorganisms. These are mostly dependent on direct and indirect measures of surface and interfacial tension activity, with a few methods that assess specific physicochemical features of specific groups of biosurfactants. Screening for surface activity, using a single or a combination of assays, can be performed on purified biosurfactants, on whole cells, or culture supernatants, where both qualitative and quantitative data can be obtained. The principal aim in such screenings is to

identify structurally new biosurfactants with effective surface and interfacial tension reduction, low critical micelle concentration (CMC), high emulsion capacity, good solubility and retained activity at a broad pH, salinity, and temperature range [10]. Notably, the screening methods can also be employed to prioritize cultures that could offer a commercial competitiveness by demonstrating a high production rate/yield at low production costs.

This section provides an overview of all the methods reported to date and highlights the screening strategies most often employed. The screening methods are categorized here as "Universal" – assays for the initial screening of large numbers or collections for general biosurfactant activity; or, "Targeted" – which includes either indirect assays to detect specific biochemical or performance properties or the more sophisticated direct assays to identify biosurfactants based on physicochemical properties. The described methods are compared in terms of necessary processing efforts, skill level, and equipment requirement, and suitability for high-throughput screening and quantification (Table 1). Notably, many of these methods lack specificity, therefore the initial results, in particular from universal screening methods, should be confirmed with complementary methods up to structural elucidation. A guideline for screening and characterization approaches for biosurfactants was recently suggested by Twigg et al. [29].

## *2.1 Universal Screening Assays*

Included in this category are screening methods based on an indirect measure of the ability to reduce surface and interfacial tension, the phenotypic characteristics shared by surfactants. They are easy, rapid, sensitive, do not normally require expensive equipment (Table 1), and are often suitable for the screening of large numbers of cultured strains or metagenomic library clones. Hence, such assays are typically used as the first line of screening for the isolation of positive biosurfactant-producing strains/clones. Of all the assays described hereafter, the drop collapse, oil spread test, and the atomized oil spray have been the most widely applied in biodiscovery and screening studies [30, 31].

### 2.1.1 The Drop Collapse Test

This method assesses the stability of drops of culture broth, culture supernatant or solutions of pure biosurfactant when they encounter an oil-coated or hydrophobic surface (Fig. 2) [32]. The underlining principle is that the drop collapse occurs as a result of the reduction of interfacial tension within the liquid and the surface tension reduction on the hydrophobic surface caused by biosurfactant activity [33, 34]. The control drops without biosurfactant activity remain stable and do not collapse due to the hydrophobic surface repelling the polar water. The stability of the drop depends

Table 1 Comparison of the ease of use of the methods available for the screening of biosurfactants[a]

| Screening method | Sample type | Sensitivity | Level of difficulty | Analysis speed | Special equipment required | Qualitative (QL), semi - quantitative (S-QN), quantitative (QN) | Applicability for high throughput screening [a] Can be adapted to be | Recent biodiscovery examples employing the respective method |
|---|---|---|---|---|---|---|---|---|
| Atomized Spray Method | Colonies | H | Easy | Sec | N | S-QN | Y | [11] |
| Drop Collapse Test | Culture supernatant, relatively pure compound | M | Easy | Min | N | S-QN | Y | [7] |
| Oil Spread Test | Culture supernatant, relatively pure compound | H | Easy | Sec | N | S-QN | N | [12] |
| Micro Plate Assay | Culture supernatant, relatively pure compound | H | Easy | Min | N | QL | Y | [13] |
| Penetration Assay | Culture supernatant | M | Easy | Min | N | QN | Y | [14] |
| Tilting Slide Test | Culture supernatant, relatively pure compound | M | Easy | Min | N | QL | N | [15] |
| VPBO-Assay | Culture supernatant, relatively pure compound | H (>CMC) | Easy | Sec | N | QN | Y | [16] |
| EC 24 | Culture supernatant | M | Easy | Hours | N | QN | N | [13] |
| Hemolytic Assay | Colonies | M | Easy | Days | N | S-QN | N[a] | [7] |
| CTAB-Methylene Blue | Colonies | H | Easy | Days | N | S-QN | N[a] | [17] |
| CPC-Bromothymol Blue | Culture supernatant, crude extract | H | Easy | Min | N | S-QN QN | N[a] depends on the need for extraction | [18] |
| TLC | Crude extract | H | Medium | Hours | N | S-QN | N | [19] |
| Hydrocarbon Overlay Agar | Colonies | M | Easy | Days | N | QL | N | [20] |
| BATH | Culture with cells | H | Easy | Hours | Y | QL | N | [20] |
| HIC | Relatively pure compound | H | Challenging | Hours | Y | QL | N | [21] |
| Replica Plate Test Assay | Colonies | H | Easy | Min | N | QL | N | |
| Salt aggregation | Culture supernatant | H | Easy | Min | N | QN | N | [22] |
| Solubilization of Crystalline Anthracene | Culture supernatant, relatively pure compound | H | Challenging | Min | N | QN | N | |
| MALDI-TOF/MS | Crude extract | H | Advanced | Hours | Y | QN | N | [23] |
| Du-Nouy-Ring Method | Culture supernatant, relatively pure compound | H | Medium | Hours | Y | QN | N | [24] |
| Wilhelmy Plate Method | Culture supernatant, relatively pure compound | H | Medium | Hours | Y | QN | N | [25] |
| Stalagmometric Method | Culture supernatant | H | Medium | Hours | Y | QN | N | [26] |
| Axisymmetric Drop Shape Analysis by Profile | Culture supernatant, relatively pure compound | H | Challenging | Hours | Y | QN | N | [27] |
| Pendant Drop Shape Technique | Culture supernatant, relatively pure compound | H | Medium | Hours | Y | QN | N | [28] |

[a]The methods are compared in terms of necessary processing efforts, skill level, and equipment requirement, and suitability for high-throughput screening and quantification. The methods are presented in four categories (yellow) “Universal” preliminary screening assays; (gray) methods that screen for specific biochemical or physicochemical characteristics and performance of the biosurfactant; and (green) those that directly measure surface and interfacial tension reduction

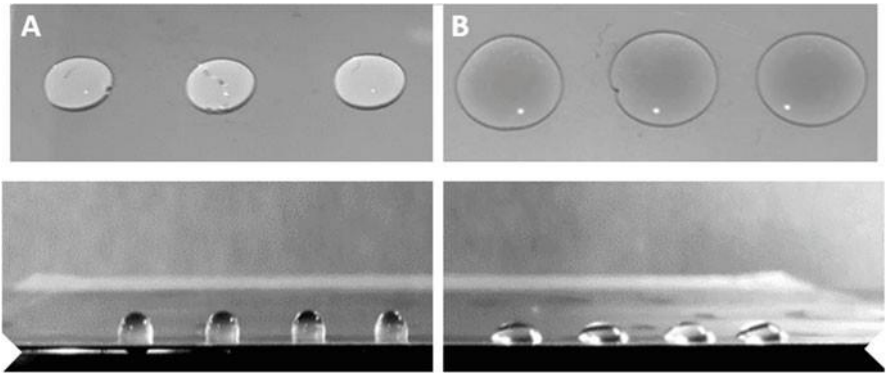


Fig. 2 The drop collapse method is used to screen for biosurfactant activity by observing the stability of drops containing biosurfactant on a hydrophobic surface. (a) Pure water samples without the presence of biosurfactant. (b) Mono-rhamnolipid containing supernatants of recombinant *P. putida*

on the concentration and surface tension reduction capabilities of the biosurfactant being screened.

One of the advantages of this method is its sensitivity and ease of use, with just a small volume of sample needed and no special equipment required [34]. It can be applied in large sample screening, and modifications to enable automated screening in microplates for high-throughput screening have been successfully employed [33, 35]. Staining of the droplets is an additional modification to enhance the visualization of the drops [36]. Disadvantageously, hydrophobic biosurfactants, and those that possess surface reduction capabilities but do not necessarily result in droplet collapse, cannot be detected using this method [37]. Activity relies obviously on a detectable concentration of the compound in the droplet and the hydrophobicity of the applied surface. Notably, there is actually no conclusive evidence that links surface tension reduction to the method, thus reducing its reliability. Lastly, the method is only qualitative, although it can be adapted to be quantitative for relative measurement of biosurfactant concentration for pure surfactants by measuring the drop size or the contact angle [7].

### 2.1.2 Oil Spread Test

The oil spread test is a rapid and easy method that does not require any special equipment [38]. A volume of crude oil is added to the surface of distilled water in a petri dish, resulting in the formation of a thin layer of oil on top of the water. The culture or culture supernatant is then placed on the oil layer, and where a biosurfactant is present, it displaces the oil creating a clear zone. This occurs as a result of the pressure formed upon contact of the hydrophobic part of the oil and that of the biosurfactant; the interface tension is reduced and the oil layer breaks resulting in a zone of clearing. The assumption is that the displacement of oil is directly

proportional to the concentration of the biosurfactant in the sample tested, and therefore provides both qualitative and semi-quantitative measurements [39]. A correlation between the oil spread test and the drop collapse method suggests that both could be used for preliminary screening; however, due to the disadvantages associated with the drop collapse method, perhaps the oil spread test represents a more appropriate assay for generalized screening.

### 2.1.3 The Atomized Oil Spray Method

In this assay a thin mist of paraffin is sprayed over bacterial colonies cultivated on agar plates, revealing the formation of droplets as a halo around a biosurfactant-producing colony (Fig. 3), the radius of which can be measured for a semi-quantitative analysis [35]. The method rapidly detects activity and does not require sample preparation, making it suitable for high-throughput screening of thousands of colonies at once, and therefore ideal for metagenomic library screening [31, 40]. Furthermore, the atomized spray method (also known as the oil vaporization assay)

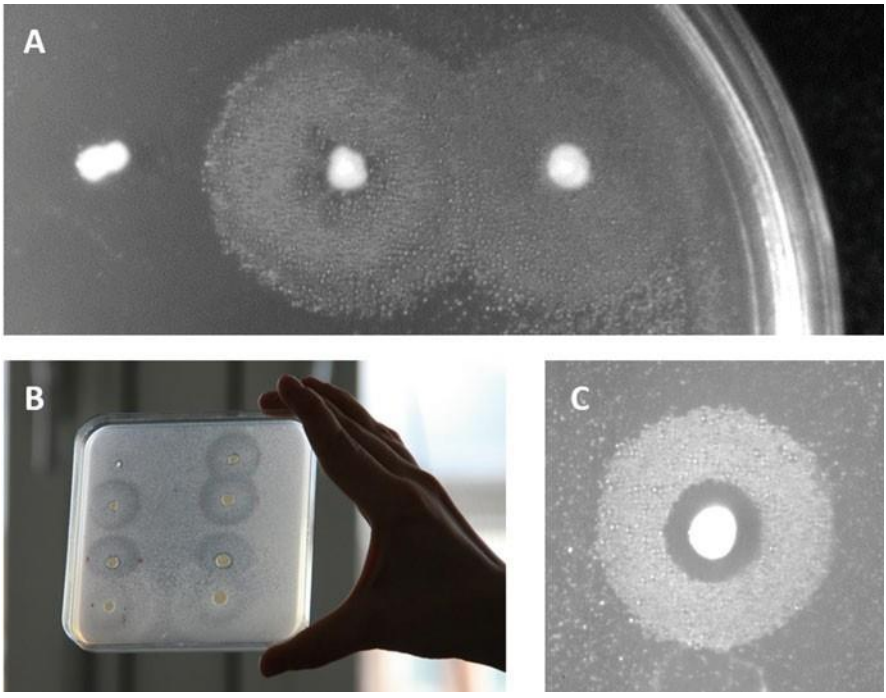


Fig. 3 The atomized oil spray method showing biosurfactant activity as a light-diffractive halo via formation of uniformed droplets surrounding microbial growth on agar. (a) Recombinant *E. coli* producing Serrawettin W1; (b) Recombinant *P. putida* producing mono-rhamnolipid; (c) Recombinant *Erwinia billingiae* producing Serrawettin W1

provides greater sensitivity than the drop collapse method, detecting 10–100-fold lower surfactant concentrations and allows the detection of biosurfactants with low water solubility [35]. Due to the versatility and sensitivity of the atomized oil assay, it is considered a more superior screening method than most of its indirect screening method counterparts.

### 2.1.4 Microplate Assay

The microwell plate assay has been patented as a qualitative measure to screen culture supernatant for surface tension reduction abilities [41]. The assay involves assessing optical changes of gridded paper placed under the 96 well plate containing the culture supernatant being tested (Fig. 4). Pure water in a hydrophobic well has a flat surface and no optical distortion, whereas the presence of a biosurfactant results in optical distortion. As a consequence, the fluid changes the surface brought about by the wetting of the edge of the well, subsequently becoming concave and taking the shape of the diverging lens. The assay is easy, rapid, sensitive, allowing instantaneous detection of surface-active compounds from a small volume [42] and is suitable for automated high-throughput screening, therefore appropriate as a method for functional metagenomic library screening [31]. Proper imaging is required to capture the correct optical distortion to remove subjective bias. Besides, the sample must be clear with no turbidity or intense color to observe the underlying grid changes [33].

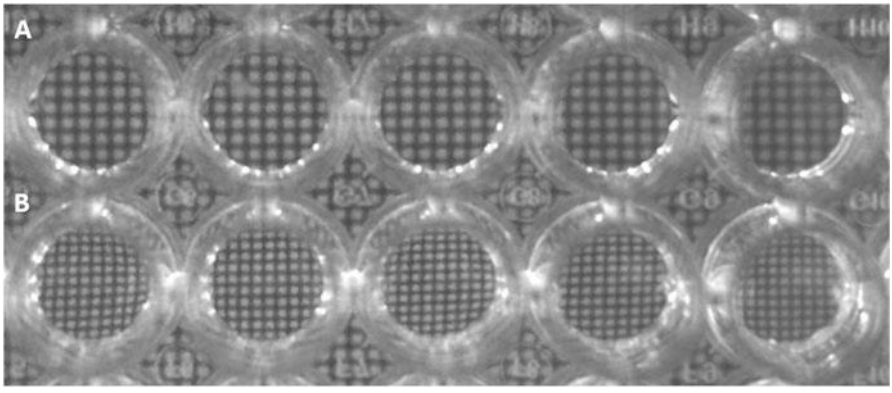


Fig. 4 Microwell plate assay measures the surface activity of biosurfactants in a solution. (a) Pure water in a hydrophobic well which has a flat surface; (b) optical distortion that is caused by mono-rhamnolipid recombinantly expressed in *P. putida*

### 2.1.5 Penetration Assay

This colorimetric qualitative assay is based on the infusion of two insoluble phases in a 96 well plate that results in a color change [43]. A hydrophobic paste mixture consisting of oil and silica gel applied to the wells is then covered with oil. The culture supernatant or sample, colored by adding a red staining solution, for example 1% safranin, is then placed on the surface of the oil mixture. In the presence of biosurfactant, the hydrophilic liquid breaks through the oil film barrier into the paste causing the stain to be absorbed by the silica, and the color of the upper phase changes from clear red to cloudy white within 15 min. The effect is based on the ability of the silica gel to enter the hydrophilic phase from the hydrophobic paste much more quickly in the presence of a biosurfactant. In the case of no biosurfactant activity, the upper layer will turn cloudy but remain red. The assay is simple, and with the help of tools such as a Cybi-Disk robot, it can be applied in high-throughput screening [43].

### 2.1.6 Tilting Slide Test

This test examines the flow of water droplets over a glass slide surface and can be applied in preliminary screenings [44]. A single test colony picked from an agar plate is applied onto a sterilized glass slide near the glass edges and mixed with 1% saline water. The glass slide is gradually tilted to observe the flow of the water droplet over the glass surface, and activity is recorded if it flows. The method is easy to apply and does not require any expensive or specialized equipment [25], but serves only as a preliminary screening method and must be supported by secondary screening.

### 2.1.7 Victoria Pure Blue BO (VPBO) Assay

This assay is based on the surfactant-dependent solubilization of Victoria Pure Blue BO, a hydrophobic blue dye typically used in ballpoint pens. Assay plates are prepared before the screening by immobilizing the dye on 96-well polystyrene plates. The surfactants in aqueous solutions applied to the wells, e.g., supernatants of bacterial cultures can re-solubilize the dye into the liquid [16, 45]. The solubilized dye can be quantified, if desired, after the transfer of the liquid to a clean plate (Fig. 5) via the specific absorption at 625 nm. The method, initially used to determine residual detergent levels in medical preparations, offers a broad range of applications beyond the qualitative high-throughput screening for biosurfactant production, including biosurfactant quantification, e.g., for the comparative evaluation of different cultivation conditions and assessment of the CMC and solubilization properties of isolated surfactants [16, 46]. The VPBO Assay has been shown to be suitable for chemically different ionic and non-ionic biosurfactants; however, like

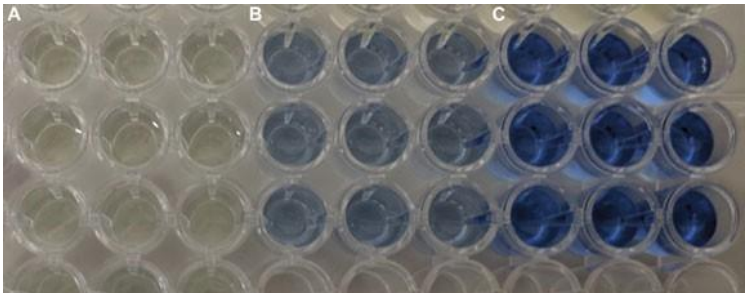


Fig. 5 Biosurfactant solutions after VPBO Assay in a microwell plate. The presence of surfactant is indicated by the blue dye Victoria Pure Blue BO that is released from the surface of the plate well by surfactant activity. This response (b + c) is concentration dependent, whereas in the absence of surface-active agents (or in amounts below CMC) the solution remains uncolored (a)

for the drop collapse assay, water solubility of the biosurfactant and a concentration above the CMC appear to be a prerequisite.

## *2.2 Targeted Screening Assays*

The assays described here encompass two parts: methods that assess specific biochemical or physicochemical characteristics and performance of the biosurfactant; and those that directly measure surface and interfacial tension reduction.

### 2.2.1 Emulsification After 24 h (EC24)

The emulsification capacity index (EC24) measures the ability of an emulsifier to stabilize the emulsion of immiscible liquids over 24 h. After the mixing of two immiscible phases, for example, water and oil, the unstable emulsion divides into separate phases depending on the respective densities, whereas in the presence of a biosurfactant with emulsification capabilities, the emulsion is stabilized through interfacial tension reduction between the immiscible phases allowing them to readily mix (Fig. 6). This method, therefore, assesses the capability of a culture (or supernatant) to form an emulsion with a hydrocarbon such as paraffin, kerosene, and hexadecane after they are mixed [47]. The EC24 is measured as the height of the emulsification over the total height of the two-phased mixture, 24 h after mixing by agitation to form emulsions. Alternatively, emulsification can be detected by quantifying the turbidity from the emulsion using a turbidimeter [48].

However, the ability of a biosurfactant to form an emulsion is rarely associated with its surface and interfacial tension reduction potential [31, 37]. Therefore, good emulsion does not necessarily equate to surface and interfacial tension reduction [9],

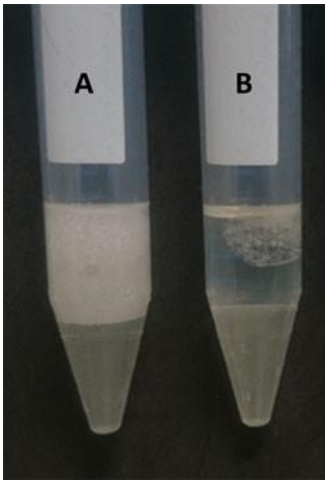


Fig. 6 Emulsification after 24 h, after mixing paraffin with culture supernatant. Following the mixing of two immiscible phases an unstable emulsion divides into separate phases (b), whereas a stabilized emulsion is formed due to the presence of recombinantly expressed lyso-ornithine lipid (LOL) in the cell-free culture media (a)

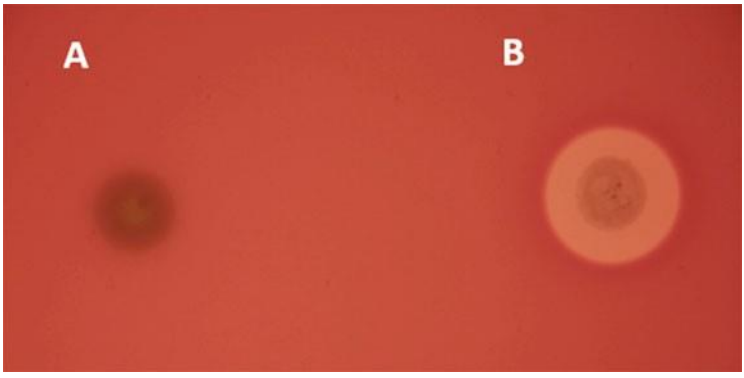


Fig. 7 The blood agar method detects hemolytic capabilities of some biosurfactants (b) through lysis of erythrocytes cells, producing a colorless transparent ring around the agar well, compared to no clear zone for a control solution (a)

and vice versa, biosurfactants capable of reducing the surface and interfacial tension are not necessarily good emulsifiers.

### 2.2.2 Hemolytic Assay

The blood agar method is widely used to detect biosurfactant production through the lysis of erythrocytes cells producing a colorless transparent ring around the colonies (Fig. 7) [49, 50]. It is a semi-quantitative method as the concentration of biosurfactant correlates to the linear increase of the diameter of lysis on the blood agar. The assay is also suitable for a 96-well and liquid format where the amount of hemoglobin released into the solution is determined photometrically [51]. However, hemolysis is not a universal property of biosurfactants. Furthermore, hemolysis may

also be evoked by pore-forming proteins or phospholipases [52] which can yield false-positive results. Hence, hits from this assay must be treated with care and maybe not as the method of choice for primary screenings.

### 2.2.3 Colorimetric Complex Release Assays

The complex formation of biosurfactants with cationic detergents, resulting in the displacement of a dye molecule, can be applied to determine the presence of biosurfactants in supernatants photometrically at 96 well scale.

#### CTAB-Methylene Blue Plate Assay

The cetyltrimethylammonium bromide (CTAB)-methylene blue method, also referred to as the "blue agar plate" method, serves as a semi-quantitative assay for the preliminary detection of extracellular anionic surfactants [17]. The positive detection of biosurfactants on agar containing 0.5 mg/ml CTAB and 0.2 mg/ml methylene blue is seen by the formation of a blue halo surrounding a bacterial colony or sample (Fig. 8). The blue halo is formed through the binding and forming of a complex of anionic surfactant with the cationic surfactant of CTAB. Not all bacteria can be screened using the method because CTAB is toxic to some bacteria including *E. coli* [31].

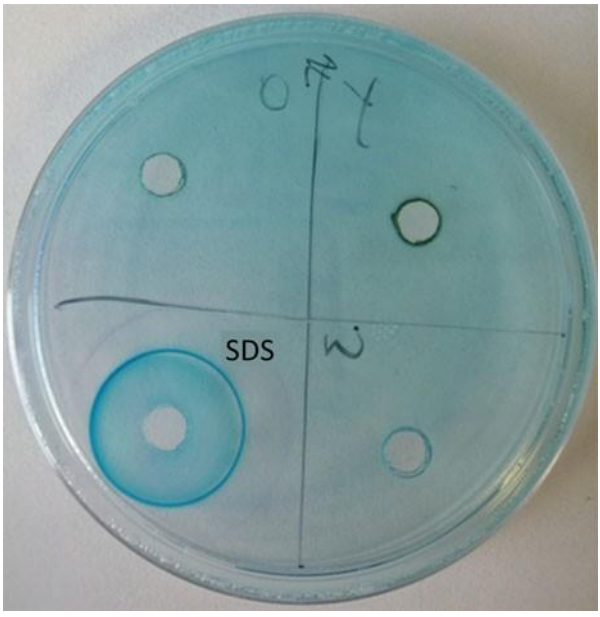


Fig. 8 The CTAB-methylene blue method for the detection of extracellular glycolipids or other anionic surfactants. SDS shows the positive detection of surfactants as seen by the formation of a blue halo

CPC-Bromothymol Blue Assay

Cetylpyridinium chloride (CPC) in combination with bromothymol blue (BTB) or fluorescein has been successfully applied in a similar manner as the CTAB-methylene blue assay [18, 53]. Biosurfactant presence and concentration can be determined via the color/fluorescence shift evoked by the displacement of the dye components. This strategy was described as a very reliable method to even quantify the anionic lipopeptide surfactin [18] but it may interfere with media components or primary metabolites [54] and may therefore require extraction procedures before the assay. Furthermore, it is most likely that both complex release assays are restricted to anionic biosurfactants.

### 2.2.4 Detection of Biosurfactant Production by Thin Layer Chromatography (TLC)

Thin layer chromatography (TLC) is mainly applied in the characterization of the chemical nature of the produced biosurfactants by using selective reagents and manipulating the polarity of solvents when separating crude extracts on a silica gel plate. For the detection of functional groups of biosurfactants, different staining reagents can be used, e.g. ninhydrin stains lipopeptides red whereas α-naphthol stains glycolipids purple (Fig. 9). Hydrophobic moieties like aromatic ring systems or lipid chains can be visualized applying iodine vapor or primuline. This method is not suitable for high-throughput screening.

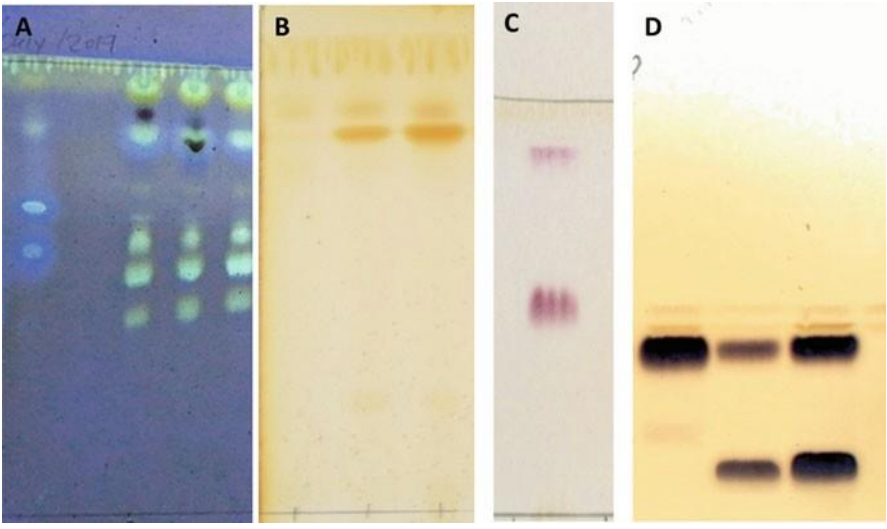


Fig. 9 TLC analyses of different biosurfactant extracts, (a) stained with primuline for lipid detection; (b) stained with iodine for lipid detection; (c) stained with α-naphthol for sugar detection; (d) stained with orcinol/sulfuric acid for sugar detection

### 2.2.5 Screening Methods Based on Cell Surface Hydrophobicity

Several indirect methods are suitable to screen for differences in cell surface hydrophobicity. The cell-bound biosurfactant production by a microorganism is associated with high hydrocarbon uptake and therefore high surface hydrophobicity, whereas microorganisms that release biosurfactant extracellularly are associated with low surface hydrophobicity. Many other factors influence the hydrophobicity of bacteria, such as physiological aspects like growth conditions and cellular age. Therefore, these methods are generally used for rapid identification during the isolation stages and are followed by secondary screening.

#### Hydrocarbon Overlay Agar

Microbial isolations on oil-coated agar plates can identify strains that degrade hydrocarbons and produce biosurfactants by the production of an emulsified halo surrounding the colony [36, 55]. The method is easy to set up and does not require special equipment, but it cannot be applied for strains that do not degrade hydrocarbons. The method is used for initial screening purposes only and requires further confirmation of positive results.

#### Bacterial Adhesion to Hydrocarbon Test (BATH)

The photometrical bacterial adhesion to hydrocarbons (BATH) assay measures the hydrophobicity of a cell surface by measuring the degree of adhesion of washed microbial cells to different hydrocarbon compounds, such as hexadecane or octane [56]. Hydrophobicity of the cells is measured by assessing changes in absorbance at 550 nm of the lower aqueous phase before and after the mixing procedure, expressed as a percentage. The basic principle is that a decrease in the turbidity of the aqueous phase correlates to the hydrophobicity of the cell; however, it is considered one of the least reliable methods [30].

#### Hydrophobic Interaction Chromatography (HIC)

This chromatography screening method is based on the hydrophobic interaction between non-polar group regions of particles and the non-polar groups on a hydrophobic chromatographic resin. It was initially employed to purify and separate biomolecules based on differences in their surface hydrophobicity [27] and was modified for biosurfactant screening. A bacterial suspension in a high-salt buffer is allowed to flow through a gel bed of hydrophobized Sepharose to which hydrophobic cells adhere. Turbidity and bacterial counting in the elute are measured to obtain the degree of adsorption of the cells to the gel. Stepwise or continuous decrease of

the ionic strength for desorption of the adherent microbes promotes their elution. This way, microbes of different surface hydrophobicity can be separated. The main advantage of applying HIC for screening biosurfactant production is that it is convenient because both isolation and screening of strains can be achieved simultaneously while also serving as a good comparative analysis of the hydrophobic properties of microorganisms.

#### Replica Plate Test Assay: Adhesion of Bacteria to Hydrophobic Polystyrene

This simple assay is used to identify and isolate hydrophobic microorganisms by their adhesion to hydrophobic polystyrene by pressing a flat sterile disk of polystyrene onto agar containing the colonies [57]. The underlying principle is that the affinity of bacteria to polystyrene strongly correlates to the hydrophobicity of the cell surface [58]. The advantage of this method is that hydrophobic strains can be simultaneously isolated and identified.

#### Salts Aggregation Assay

This assay involves the precipitation of cells by increasing salt concentration, the same principle used in salting out proteins [59]. The underlining principle is that the more hydrophobic the surface of the cell, the lower the salt concentration that is required to aggregate the cells. A bacterial suspension is mixed with various ammonium sulfate concentrations on glass depression slides and monitored for the formation of a white aggregate.

#### Solubilization of Crystalline Anthracene

This quantitative assay is based on the solubilization of anthracene, a highly hydrophobic crystalline compound, when added to the culture supernatant [60]. The production of biosurfactant is determined by measuring the concentration of the solubilized anthracene at 354 nm with a photometric device. Important to note is that the cell-free supernatant of the culture is used to conduct the assay, since bacteria could metabolize the anthracene, which may be mistakenly interpreted as biosurfactant activity [61, 62].

### 2.2.6 Structure-Based Screening as a Recent Advance in Physicochemical Screening Methods

The typically low biosurfactant titers in broth cultures not only impact the efficiency of the screening process, but also impede the structural determination of the isolated biosurfactants which relies on a large amount of purified compound and

time-consuming methods. To overcome these bottlenecks, Sato and co-authors [23] recently demonstrated a structure-based screening method in the early stages of the screening process. The approach they developed relies on the use of matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF/MS), a technique that is commonly used to identify organic molecules based on precise mass-to-charge ratio measurements. Although MALDI-TOF/MS has been applied for the structural determination of purified biosurfactants [63–67], this study has been the first to employ it for screening for biosurfactant producers and on crude samples. Currently, this method has only been applied for the screening of glycolipid-type producers, and therefore its application to other classes of biosurfactants has yet to be ascertained. Since the technique is premised on identifying mass to charge ratios of known compounds, its ability to identify completely novel structures is most likely very limited. However, this does represent a rapid and reliable tool to detect variants of a class of compound, as was demonstrated by Sato et al. [23]. Additional advantages of this approach are that MALDI-TOF/MS requires a small amount of sample for measurement and can distinguish structural differences in mixtures because of the precise measurement of the mass-to-charge ratio corresponding to the molecular weights of the compounds. Furthermore, it can be used to screen unidentified microbial cultures in addition to new biosurfactant variants. The fact that the structure can already be ascertained in the primary screening of the crude extract is an obvious advantage for accelerating the screening process.

### 2.2.7 Quantitative Screening Methods Based on the Direct Measure of the Surface and Interfacial Tension

Surface and interfacial tension reduction is the common measure of the physical property of surfactants, defined in two ways; the tension force per unit length exerted by a liquid in all directions at an interface of a solid or another liquid; or, explained in terms of energy, as the amount required to decrease the interior forces of bulk liquid molecules and molecules at the interface of the liquid in contact with other surfaces [68, 69]. The surface tension is measured as dimensions force/length in the units dyne/cm or mN/m. At a value of 72 mN/m, water is known to be one of the organic liquids with the highest surface tension value [10]. An effective surfactant should interrupt the forces per unit length of water and lower the surface tension from 72 to 30 mN/m [9]. Interfacial tension (IFT) is the intermolecular attractive force of the molecule in a liquid and represents the emulsion capacity of a surfactant. A high emulsion relates to a low IFT [10]. A surface-active biomolecule must lower the interfacial tension for water against *n*-hexadecane from 40 to 1mN/m.

Tensiometers can be universally used to quantify the activity of any biosurfactant by measuring the change in surface and interfacial tension at their air/water and oil/water interfaces. Force or optical tensiometry are techniques that are commonly used to measure the surface and interfacial tension. Force tensiometry involves a direct measure of the surface or interfacial force exerted on a probe, whereas the

optical tensiometry unit measure is calculated from theoretical equations whereby an image profile of a drop or bubble is extracted and fitted to an equation.

The reduction of the interfacial tension in air/water or oil/water systems increases with (bio)surfactant concentration but only up to a critical point, above which no further surface tension reduction occurs, instead, surfactants aggregate to form structures like micelles, bilayer, and vesicles. The value at the critical point is known as the critical micelle concentration (CMC) [9]. The efficiency of the surfactant is determined by how low its critical micelle concentration is, the lower the CMC, the less product needed to reduce the surface and interfacial tension. In most industrial processes, ranging from drug delivery systems to agricultural remediation technologies, the CMC value is considered particularly important for determining the biosurfactants' suitability in the respective application [70]. Discussed below are some of the direct screening techniques:

### Du-Nouy-Ring Method

This is a traditional technique used for the direct measurement of surface or interface tension change generally using an automated tensiometer [36]. The method involves measuring the force required to pull through a fully submerged ring or loop of platinum wire from an interface or surface of the liquid of interest. The detachment force is measured relative to the surface or interfacial tension. A biosurfactant-containing solution is noted as one that reduces the tension of pure water to 40 mN/m or less [68, 69]. This measure shows a direct relationship to the drop collapse, oil spreading, and surface tension assays. It is the most widely applied method as it is accurate and easy to use; however, it requires specialized equipment [71].

### Wilhelmy Plate Method

This is a universal method for measuring surface or interfacial tension at an air–water or water–water interface. A thin Wilhelmy plate is submerged perpendicular to the air–liquid or liquid–liquid interface and the force applied on it is measured [72]. The Wilhelmy plate is often made from filter paper, glass, or platinum with a rough surface to ensure wetting. The choice of material is not particularly important, but the material must have the capacity to be wetted by a liquid. The advantages of this method include that one can use disposable papers; it is considered the simplest and most accurate measure; it does not require correction factors when calculating surface tension, because the Wilhelmy plate assumes a zero-contact angle with the liquid; and there is no need to measure or know the density of liquids, only mass of plate and wetting force is considered [36]. The disadvantage of the application is that a large volume of liquid is needed.

### Stalagmometric Method

This is one of the most common methods adopted to screen for biosurfactant production, using a traube stalagmometer to measure surface tension activity [73]. A pipette with a broad flattened tip is used as the capillary system, with which large drops of reproducible size are suspended from the tip, drop once a maximum weight/ volume is reached. The volume is calibrated by the stalagmometer and the weight of the drop is dependent on the characteristics of the liquid being tested. The underlying principle is that the weight of volume is in equilibrium with the surface tension. Biosurfactant production is measured by counting the number of each drop that falls per volume from the glass capillary tube, and by measuring the density of the sample and the surface tension of the control sample liquid used, which is normally water. The main disadvantage of this screening method is the large variability usually obtained in the results, suggested being due to drop formation being too fast and not allowing the complete adsorption of the surfactant to the newly generated drop surface [10]. Another disadvantage is that consecutive measurements are not possible.

### Axisymmetric Drop Shape Analysis by Profile (ADSA-P)

This optical method is used to simultaneously measure liquid surface tension and contact angle from the profile of a droplet resting on a solid surface [74]. The underlying principle is that the shape of a liquid droplet depends greatly on the liquid surface tension; biosurfactant solutions with low surface tension tend to minimize the surface area of the drop causing the droplet to deviate from a perfectly spherical shape when compared to those with high surface tension, as indicated by the drop collapse test (Sect. 2.1.1). The circumference of a liquid on a solid surface is captured as an image and the measurements are subsequently fitted to the capillary Laplace equation to calculate the surface tension [68, 69, 75]. The advantage of the ADSA-P is the small volume needed for the drop shape analysis. However, the shortcomings of using this method include the requirement for a camera and software; and it involves complex calculations; complex computational routine and samples cannot be measured in parallel [10, 36, 75].

### Pendant Drop Shape Technique

This is the most common optical tensiometry method used to screen for biosurfactant activity and is considered an excellent screening technique for a quick analysis of surface and interfacial tension and measurement of contact angle [74]. The surface and interfacial tension properties are measured from a drop of liquid allowed to hang from the end of a capillary. The drop adopts an equilibrium profile based on the tube radius, the interfacial tension, its density, and the gravitational field [76].

## 3 In Silico Screening of Sequence Datasets for Novel Biosurfactants

The development and continuous improvement of next-generation sequencing (NGS) platforms and the subsequent scalability, cost reductions, and development of in silico tools have enabled the advancement of sequence-based screening for the discovery of novel biomolecules. The availability of genome sequences for over 800,000 bacteria and 4,000 fungi [77], as well as enormous metagenomic sequence datasets in public databases, representing largely untapped resources from diverse organisms from almost every environment imaginable, makes sequence-based screening particularly attractive. Not only can researchers bypass the sampling and isolation of strains and/or (meta)genomic DNA and sequencing costs, it enables also the convenient de novo synthesis of the identified genes and pathways, cloned into a plasmid vector of choice, through a number of service providers [78]. Without question DNA sequencing technologies have reinvigorated the discovery of new microbial enzymes and secondary metabolites [79, 80], but it has had rather limited application for the discovery of novel biosurfactants. However, several genome-guided efforts involving biosurfactant-producing strains have reinforced the notion that targeted sequence-based approaches have the potential to contribute to biosurfactant discovery. The following sections will outline the sequence-based screening process and discuss the potential for biosurfactant discovery.

### *3.1 Gene/Pathway Identification*

Assembled genomes and/or contigs are queried to identify protein-coding sequences based on homology to reference sequence data in curated sequence databases where open reading frames (ORFs) or conserved protein motifs (for example active sites/domains) are identified through similarity search algorithms (e.g., BLAST [81], COG [82], KEGG [83, 84]. In many cases, expression of secondary metabolites, which encompass biosurfactants, involves more than one gene for biosynthesis. One of the most popular tools for the identification of biosynthetic gene clusters (BGCs) in general is antiSMASH (antibiotic and secondary metabolite analysis shell) [85]. This tool is continuously developed and currently provides researchers with an easy-to-use, up-to-date collection of state-of-the-art annotated BGC data. It identifies all BGCs present in the query sequence as well as facilitating cross-genome analyses. In addition to cluster predictions, more complex searches can be implemented via the graphical query builder from which researchers can gauge the novelty of the clusters, and by extension, the novelty of the compound they encode. Therefore, in addition to discovery, sequence-based mining can also serve as a dereplication tool that can be used to prioritize strains most likely to produce a novel biosurfactant. Advantageously, sequence-based screenings can be conducted in an ultra-high-throughput manner provided suitable bioinformatics and

computational capacity is available. Another advantage of sequence-based mining pertains to the realization that there is great disparity between the number of putative secondary metabolite gene clusters identified based on bioinformatics analyses vs the actual number of secondary metabolites detected from culture fermentations [86]. Such BGCs are regarded as cryptic, where either the genes are not expressed under standard laboratory cultivation conditions or they encode compounds that are produced in yields insufficient for direct isolation and characterization [80]. This not only points to an astounding level of as yet undiscovered metabolites for the majority of cultured organisms, but it also provides the opportunity to employ genome-guided strategies to identify biosurfactant BGCs in organisms that may have initially tested negative in a functional screen [87].

Given the structural and biosynthetic diversity of biosurfactants, there is no universal sequence-based screen to detect biosurfactant genes or pathways from datasets a priori [31]. For example, glycolipid discovery using a sequence-based approach is currently not viable due to the lack of a specific conserved domain to distinguish glycolipid associated enzymes from the wealth of glycosyltransferases performing a multitude of functions in bacteria [88, 89]. In consequence, glycolipid operon prediction is not implemented e.g., in antiSMASH. However, for lipopeptide biosurfactants which are encoded by nonribosomal synthase (NRPS) gene clusters, the conserved adenylation domain of the synthase gene could be targeted to identify NRPS BGCs, followed by a secondary screen of the initiation domains to discern for lipopeptide-specific NRPS BGCs [31]. For example, holrhizin A, a novel linear lipopeptide from the *Burkholderia rhizoxinica*, an endosymbiont of the rice seedling blight fungus *Rhizopus microspores*, was discovered through such a genome mining approach [90]. The identification of a conserved cryptic NRPS gene cluster among all sequenced Rhizopus endosymbionts led to the isolation and characterization of holrhizin A as a biosurfactant. It was further demonstrated to influence the formation of mature biofilms and thus cell motility behavior, typical for biosurfactants, and thus ultimately supports the colonization and invasion of the fungal host, furthering the understanding of the mechanism behind the exceptional *Burkholderia-Rhizopus* symbiosis relationship.

The discovery of holrhizin A represents an elegant example of how a genomics-led discovery not only resulted in the description of a novel biosurfactant, but also provided a functional link between orphan NRPS genes and a chemical mediator that promotes bacterial invasion into the fungal host. But this example also serves to highlight that sequence alone could not have predicted the biosurfactant properties of the NRPS-encoded metabolite. Therefore, it can be reasonably expected that a purely sequence-based screening approach will have limited success for biosurfactant discovery. A much higher success rate can, however, be expected when employing sequence-based screening for strains with confirmed biosurfactant activity, especially in the case where the primary or secondary screening already points to a specific class of biosurfactant which can inform the sequence-based search [91–93].

Another limitation of sequence-based screening pertains to the level of novelty that can be identified. Since sequence-based searches rely on similarity algorithms that score based on sequence identity, the discovery of completely novel

biosurfactants will be limited; and true novelty may be overlooked as very distantly related sequences are often unlikely to be found by homology-based searches [94]. However, a sequence-based approach could have enormous value in identifying variants of certain classes of biosurfactants. For example, rhamnolipids encompass a wide diversity of congeners and homologues [95], the synthesis of which are encoded by the *rhlA*, *rhlB*, and *rhlC* genes. Perhaps, sequence variations in the genes could be a proxy for predicting novel homologues and therefore sequence-based screening could identify new targets to validate by culturing the native host or through heterologous expression of the genes (discussed further below). Especially the products of BGCs that are not readily expressed by the natural producers under the chosen growth parameters may be accessed using heterologous expression and/or genetic engineering approaches to bypass the strict regulation systems in the natural hosts [96].

## *3.2 Heterologous Expression of Putative Biosurfactant-Encoding Genes/Pathways Identified Through In Silico Mining*

Once identified, the genes or pathways can be cloned and heterologously expressed in a suitable host, to produce the biosurfactants for purification and characterization. The transfer of complete biosynthetic pathways is a considerable challenge because the respective genes may be dispersed over the chromosome (as for example the genes necessary for di-rhamnolipid biosynthesis in *P. aeruginosa*) [68, 69] or organized in very large BGCs. For the NRPS-synthesized lipopeptides, where BGCs can span >50 kb in size, standard cloning procedures involving polymerase chain reaction (PCR) amplification are not suitable and de novo synthesis approaches would not be feasible [97, 98]. However, if identified from genomes of culturable organisms, a large collection of tools has become available for generating conventional genome libraries and/or capturing even large BGCs in clones which can then be screened (in the case of a genome library) or assessed for biosurfactant activity [5, 99].

Even if complete BGCs are transferred to suitable host strains, the successful expression and biosynthesis for a specific biosurfactant is to a large extent dependent on the chosen host strain that must be suitable to produce a respective biosurfactant in amounts at least sufficient for detection and structural elucidation. This typically requires promoter recognition (or a suitable promoter sequence on the applied vector backbone), efficient translation of the foreign genes, supply of accessory proteins and cofactors, supply of precursor metabolites, and tolerance toward the surface-active and often also bioactive product itself. For example, Gram-positive bacteria like *Bacillus* or *Staphylococcus* are very susceptible to surface-active compounds in general, most likely because of the lack of a protective LPS-containing outer membrane [100]. Hence, although Gram-positive organisms are often pronounced

lipopeptide producers, they appear to not be favorable hosts to produce recombinant biosurfactants at high levels. The dependence of active production of NRPS machinery on a phosphopantetheinyl transferase (PPT) for posttranslational modification exemplifies the importance of suitable enzymatic capabilities of a host strain [101]. PPTs are often not part of BGCs and therefore must be supplied by the host in these cases, preferably with a broad substrate spectrum. Another consideration for host selection is for the host strain to lack biosurfactant/bioemulsification activity itself, to facilitate the isolation and characterization of the recombinantly expressed biosurfactant.

Approaches toward recombinant biosurfactant production are so far mainly focused on different proteobacteria like *E. coli* [102, 103], *Burkholderia* sp., and several non-pathogenic members of the *Pseudomonas* genus [103] as host organisms. In this context, the well-explored production of rhamnolipids in *P. putida* exemplifies the potential of recombinant production in a strain that combines high tolerance, a versatile metabolism, sufficient precursor supply, and low background activity with effective expression to enable high-yield production and, besides that, tailoring of biosynthetic pathways [104, 105]. With respect to recombinant lipopeptide production, *Bacillus* sp. and *Streptomyces* sp. as naturally potent lipopeptide producers have been additionally applied as hosts [5].

Recombinant production strategies have been largely based on prokaryotic host organisms so far. However, fungi represent a technologically important class of biosurfactant producers well-known for the production of sophorolipids, mannosylerythritol lipids, cellobiose lipids or hydrophobines and functional expression of related BGCs of fungal origin can often not be achieved in prokaryotic hosts. To this respect, there is a need to complement the established set of microorganisms for recombinant biosurfactant production with eukaryotic expression systems. Accordingly, a few initial studies report successful functional heterologous expression of (partial) biosynthetic pathways for biosurfactants in *Saccharomyces cerevisiae*, *Starmerella bombicola,* or *Pichia pastoris* [84, 106–108].

## 4 Metagenomic Biodiscovery: Unlocking Hidden Diversity

It is acknowledged that diverse and complex microbial communities inhabiting many unique niches remain undiscovered; yet could represent the source of enormous biosurfactant novelty. Moreover, researchers have for decades appreciated the difficulty of bringing the abundant microbial diversity to culture in the laboratory. To overcome the culturing limitations and to explore the natural wealth beyond the minority of microorganisms that is culturable, metagenomic screening approaches have been established in the hope of accelerating biodiscovery, and to specifically tap into novel chemical space [96, 109]. Metagenomic approaches for the identification of novel biomolecules utilize both approaches that have been explained in the previous sections, functional, activity-based screenings of metagenomic libraries constructed in a heterologous host; and sequence-based screening via bioinformatic

analysis of next-generation sequencing data of environmental DNA (eDNA) [21, 84]. For reasons that have already been eluded to, functional metagenomics (as opposed to sequence-based) has been considered the more promising approach for the discovery of novel biosurfactants [31, 84, 110]. However, only few studies have so far employed metagenomic screening successfully for surface-active metabolite discovery, in which novel genes encoding the synthesis of N-acylated amino acids are largely reported. N-acyl amino acid synthases (Nas) catalyzing the synthesis of acylated aromatic amino acids appeared rather frequently in functional screens for active compounds against Gram-positive bacteria [111]. However, aromatic N-acylated amino acids show pronounced surface activity and were, hence, detected in functional screenings for biosurfactants [112]. Considering the susceptibility of Firmicutes toward surfactants, it would be reasonable to assume that the observed antibiotic effect of many biosurfactants is connected to their surface activity. A recent study showed the advantage of different expression hosts even for such simple molecules by indicating that the expression of the same *nas* gene in different Proteobacteria led to different products [113]. Whereas *E. coli* extracts contained predominantly N-acyl tyrosine, *P. putida* produced mainly N-acyl-phenylalanine. In *P. koreensis* extracts, N-acyl-leucine was detected additionally.

Recent functional screens for surface activity revealed a structurally different biosurfactant from the family of acylated amino acids, namely lactamized lyso-ornithine lipids and ornithine lipids [40]. Key to success in this study was the simultaneous screening in different hosts, because the respective library clone showed surface activity only in the *P. putida* screening, whereas it was not detectable in the *E. coli* library, although later experiments proofed the functionality of the responsible metagenomic acyl-ACP-ornithine acyl transferase in *E. coli.* Here again, the different strains produced biosurfactants of different composition, with only *P. putida* producing ornithine lipids. Interestingly, a chromosomal integration shuttle vector was applied here for screening in *P. putida*, which might be a useful strategy to stabilize large eDNA fragments in the host cells.

The potential of especially functional metagenomics for the detection of completely novel biosurfactants was recently illustrated by the discovery of MBSP1, a protein with emulsifying properties [114]. Remarkably, this protein is putatively of archaeal origin whereas the known classes of emulsifying proteins, hydrophobins and cerato-platanines, are produced by fungi [115, 116]. Hence, MBSP1 represents the first example of a prokaryotic homologue. Moreover, sequence searches revealed that homologues of MBSP1 are common among *Halobacteriaceae* but were previously unassigned to a function. Although advances are being made in computational tools to improve predictions of hypothetical proteins which represent a significant fraction of the sequences in public databases [117], this study elegantly exemplifies functional metagenomics as the most reliable method for determining the functionality of novel protein sequences, a task which is currently beyond the capabilities of existing in silico annotation technologies [94].

The available studies on functional metagenomic identification of biosurfactants commonly reported surface-active molecules retractable to the activity of one protein, whereas the biosynthesis of the majority of known biosurfactants is encoded

in operons of several genes or even in large BGCs. This bias may, in part, be related to the challenges associated with maintaining a complete biosynthetic pathway during library construction. In particular, with regard to lipopeptides, it is unlikely to achieve clones encoding a complete NRPS gene cluster in a plasmid, cosmid, or fosmid library with typical average fragment sizes of 5–40 kb [97, 98]. Although there are examples for very compact NRPS machineries (e.g., Serrawettin W1 synthesized in an iterative mode by only one NRPS module [118]), to successfully access this class of biosurfactant from uncultured representatives, metagenomic libraries need to ensure fragment sizes greater than 40 kb. This not only presents an enormous challenge to the handling and maintenance of DNA fragments of this size from environmental samples, there are also very few vectors and expression hosts available for the construction of such libraries [31, 119]. Alternatively, lipopeptide gene clusters can be reassembled from sequence-based screenings and cloned for recombinant expression as discussed earlier; however, metagenomic sequencing datasets are overly complex and prediction and correct reassembly of gene clusters from several contigs remain a challenge [120, 121].

Occasional reports on the discovery of novel lipopeptides through metagenomic approaches illustrate that sequence-based approaches targeting this group may be feasible despite the mentioned challenges. Nonribosomal peptides in general are well-known for their bioactivities, e.g., as antibiotics or anticancer agents. Hence, many studies were conducted with this focus to identify novel nonribosomal peptides and the related biosynthetic machinery using sequence-based screening approaches to explore novel pharmaceutic lead structures [122]. Several of those studies revealed lipopeptides which were, however, not tested for surface activity yet most likely exhibit it because of the amphiphilic structure of the molecules. Recently discovered examples are the antibiotics cadaside A and malacidin A with massively charged cyclic peptide headgroups [123, 124]. Here, the adenylation modules incorporating the charged amino acids were used as target for sequence-based screenings for novel calcium-dependent antibiotics. Another likely biosurfactant candidate is humimycin A, a linear lipopeptide with activity against *Staphylococcus* that was resynthesized according to a NRPS machinery architecture discovered in metagenomic sequence data [125].

Strikingly, most of the successful recombinant biosurfactant production examples described utilize biosynthetic genes from related strains, e.g., *P. aeruginosa* rhamnolipid genes were used to establish rhamnolipid production in *P. putida*. Such a strategy appears advantageous to avoid issues of promoter/RBS recognition or differing codon usage. In this light, it appears as a strong limitation that *E. coli* is still overwhelmingly applied as host strain for functional metagenomic library screens, probably because well-established protocols and commercial kits for library construction are based on *E. coli* vectors. A number of studies reported accordingly the successful application of shuttle vectors to enable screening for natural product formation in different hosts, specifically in a range of proteobacteria including Pseudomonads and in actinomycetes [97]. Their results illustrated the huge advantage of considering screening strains other than *E. coli* or even parallel screening in different strains [31, 122, 126]. Different hosts did not only vary in their capabilities

to realize a specific biosynthetic pathway encoded by the introduced DNA, even the compounds produced from the same BGC differed sometimes in dependence of the host, as described before [40, 113, 127]. Furthermore, if the recombinant production of a certain biosurfactant is detrimental for the applied host, these novel biosurfactants will obviously remain undiscovered using only this specific host for the screening.

In this context the application of functional metagenomics using eukaryotic hosts remains comparably unexplored; probably because the reassembly of BGCs from intron-free metagenomic cDNA libraries, which are usually used to uncover single biocatalysts, appears challenging [128]. However, the bacteria-similar organization of biosurfactant-related genes in *S. bombicola* or *U. maydis* as a distinct gene cluster without or with just a few introns [129–131] suggests that yeast-based screening might be a useful tool to identify novel fungi-borne biosurfactants within classical eDNA libraries in the future.

## 5 Coming Full Circle: Culturing Considerations to Unlock Novel Biosurfactant Potential

Due to the "Great Plate Count Anomaly," referring to the orders of magnitude difference between the number of organisms that can be cultured on laboratory media vs. the numbers countable by microscopic examination [132], together with the availability of well-established culture-independent technologies with revolutionary impact, the merits of continued traditional culture-based approaches for the discovery of novel biosurfactants could be questioned. This is especially pertinent given the dearth in novel structures despite ongoing culture-based screening efforts, whereas metagenomics has more recently revealed novel biosurfactant classes [114]. Furthermore, genomics presents a number of advantages for overcoming the limitations associated with the culturing of microorganisms for biosurfactant discovery; both in terms of the inability to establish pure cultures of representatives of all bacterial divisions and accessing the number of biosynthetic pathways that remain "silent" in the native host. These will undoubtedly open new possibilities for biosurfactant discovery.

However, despite the genomic revolution, bringing a microorganism into culture is still essential for realizing its full potential [133], especially toward designing and operating stable and resilient high-performing systems required for industrial scale production. This is particularly relevant for biosurfactant discovery since in many cases the biosurfactant production is tightly linked to the physiology of the microorganism. For example, culture-based studies continue to bring new understanding of the various roles that biosurfactants play in quorum sensing and swarming motility and how this contributes to bacteria co-ordinating virulence and pathogenesis [134, 135]. The value of having the organism in culture to query experimentally provides unmatched opportunities to understand the microorganism in question

which may be crucial to the downstream aspects concerning the development of the biosurfactant for industrial application [136]. This highlights two major considerations for biosurfactant discovery:

1. Many biosurfactants will likely remain undiscovered if the producing microorganisms are not brought into culture. In other words, the success of discovering novel biosurfactants is as much dependent on the employment of novel isolation approaches as on the aspects concerning screening. Several advances have been made over the last decade to improve the culturability of rare and novel microorganisms and the reader is referred to a number of recent reviews on this topic [133, 136–140]. Although very much needed, not all currently proposed alternative isolation protocols are feasible with the screening technologies available; for example, where the isolation involves diffusion chambers due to a dependence on metabolic consortia [133].
2. Biosurfactant production may be strongly influenced by cell culture conditions and therefore the screening process needs to take cognizance of this. For example, the production of surfactants has been shown to be conditional on whether the bacteria are grown on a surface or cultured planktonically [141], while in others expression is influenced by growth stage [142, 143]. Therefore, the traditional approach to assess culture supernatants for biosurfactant activity may completely miss the discovery of those that are only produced when cultured on agar. While a move to more high-throughput screening methods is advocated as being key to the discovery of new biosurfactants [10], it needs to take into consideration the more tedious and slower One Strain Many Compounds (OSMAC) principles. OSMAC is a culture-based approach that involves the manipulation of easily accessible culturing conditions to induce the expression of all the biosynthetic pathways encoded by a single microorganism [144]. A major advantage provided by OSMAC is that it eliminates bias during biosurfactant screening because the stimuli responsible for the activation of the many biosurfactant pathways differ between microorganisms. This approach exploits the fact that microorganisms produce secondary metabolites as a defense mechanism against other organisms in nature [145] and that under stressful conditions, microorganisms tend to produce secondary metabolites either to adapt to the environment for self-defense or intercellular communication [146, 147]. Moreover, OSMAC is aimed at stimulating the expression of the “silent” biosynthetic pathways and should form an integral part of the screening process, to unlock expression of untapped microbial-derived biosurfactants [148]. Biotic and abiotic parameters such as nutrient content (carbon, phosphate, nitrogen sources, and trace elements), aeration levels and shape of the culturing flasks, physical parameters (i.e., pH, temperature, salinity, heat shock treatments), the effect of ethanol or organic compounds, the presence of precursors of secondary metabolites and co-culturing can be easily changed and may alter the global physiology of a microbial strain, and in turn significantly affect biosurfactant expression [80, 149]. It is important to note that the expression could be subject to regulation that might not be immediately linked to biosurfactant production and rather

associated with central physiological features and/or dependent on the coordinated expression of different pathways [80]. Furthermore, the triggers are organism and pathway specific [150], therefore systematic alterations to culture conditions need to be conducted. The empirical nature of the OSMAC approach is therefore time-consuming, laborious, and challenging from a practical standpoint, as the number of cultivation parameters that can be changed is virtually limitless. However, the rewards could be enormous as it offers the potential to further diversify the biosurfactant repertoire and improve the hit-rate to feed the biosurfactant pipeline, essential toward creating eco-friendly and cost-effective industrial scale production processes.

## 6 Concluding Remarks

There is a need and a gap to finding new biosurfactants with structural diversity suitable for specifically tailored applications in different industries beyond the three main products surfactin, sophorolipids, and rhamnolipids that are currently available commercially. In this chapter, we have highlighted the main areas that with concerted effort could help uncover much diversity that is undoubtedly still hidden in nature.

Firstly, increased effort in the application of a broad range of different screening methods has been highlighted as one of the solutions to finding new biosurfactant products. The screening methods over the years have been dominated by those mainly relying on the detection of surface or interfacial tension reduction and emulsion activity. One of the biggest challenges of using well-established assays is that it places a substantial restriction on the level of novelty that can be acquired, thus the same groups of biosurfactants will continue to be isolated. There has been little to no effort in developing alternative methods that assess properties other than the ones already screened for and therefore this is an area that calls for technological innovation to accelerate the discovery of novel biosurfactants. Sequence based screening has been presented in this chapter as an alternative approach to the more traditional activity-based screening of culture fermentations. Although the heterologous expression of biosurfactant pathways identified through in silico screening opens a number of opportunities for biosurfactant discovery and development, especially in the case of lipopeptide biosurfactants and for those encoded by “silent” BGCs, it remains to be seen the level to which novel chemistry can be revealed through homology dependent screening. The added advantages of employing an in silico screen followed by heterologous expression for biosurfactant discovery is that the cloning strategy can be designed to maximize the biosurfactant yield, and by using metabolically characterized hosts, the biosurfactant purification and characterization steps can be substantially accelerated in comparison with conducting this from the native host [151]. An important aspect to its success will be the ability to tackle some of the challenges associated with heterologous expression, most

notably, the development of a wider range of bacterial and fungal hosts and corresponding genetic tools to maximize the expression and production.

Secondly, the cultivation of microorganisms poses a significant limitation on biosurfactant discovery. On the one hand, metagenomic biodiscovery has the potential to contribute to biosurfactant discovery without the need to culture microorganisms, which holds much promise since only less than 1% of the microbial diversity is currently accessible through the traditional laboratory-based culturing [111]. Therefore, if this vast genetic repertoire is to be exploited and novel microbial-derived biosurfactants uncovered, it is clear that culture-independent approaches will need to be employed. On the other hand, it is recognized that the pure culture of microorganisms to gain insight into their physiological and cell-biological properties is essential to progressing biosurfactant discovery. The application of new isolation protocols has demonstrated that the number of species isolated can be doubled and allows the culturing of microorganisms corresponding to sequences previously not assigned [137], therefore we are far from having exhausted all culturing options. Even for already cultured isolates, extensive experimentation is needed to ensure that the right culturing conditions are used to trigger biosurfactant production, as there are potentially many undiscovered biosurfactants from previously isolated and screened microorganisms. Therefore, greater attention should be given to the isolation and culturing and not just the screening aspects of biosurfactant discovery, even though these may sometimes result in an overall long and costly route to undertake [80]. However, mature technologies such as micro-fermentation and experimental design represent promising strategies that in combination should streamline the discovery processes.

Finally, as with many other biodiscovery programs aimed to develop novel biocatalysts to improve industrial pipelines, the overall notion is that integrated strategies that include genomic and synthetic biology approaches have the potential to fast-track the discovery and subsequent improvement of a new generation of biosurfactants [152]. We have without question only scratched the tip of the iceberg with respect to biosurfactant diversity, and there is no single approach that will ensure that we fully realize all that Nature has to offer. Bottlenecks exist in both culture-based and culture-independent approaches; however, the continuous development of more efficient and powerful tools to explore the expansive potential harbored in natural environments will undoubtedly deliver novel biosurfactants.

Acknowledgements The authors thank Sabrina Linden, Lisa-Marie Kirschen, and Phillip Venter for their contributions to the figures. The scientific activities of ST and SK were financially supported by the Ministry of Culture and Research within the framework of the NRW-Strategieprojekt BioSC (No. 313/323-400-002 13) and by Federal Ministry of Education and Research in the Project GlycoX (grant number 031B0866A); MT, NS, and AB were supported through grants (UID 87326 and 105876) by the National Research Foundation.

# References


1. Schultz J, Rosado AS (2020) Extreme environments: a source of biosurfactants for biotechnological applications. Extremophiles 24(2):189–206
2. Mahjoubi M, Jaouani A, Guesmi A et al (2013) Hydrocarbonoclastic bacteria isolated from petroleum contaminated sites in Tunisia: isolation, identification and characterization of the biotechnological potential. N Biotechnol 30:723–733
3. Cai Q, Zhang B, Chen B et al (2014) Screening of biosurfactant producers from petroleum hydrocarbon contaminated sources in cold marine environments. Mar Pollut Bull 86:402–410
4. Varjani SJ, Upasani VN (2017) A new look on factors affecting microbial degradation of petroleum hydrocarbon pollutants. Int Biodeter Biodegr 120:71–83
5. Kubicki S, Bollinger A, Katzke N et al (2019) Marine biosurfactants: biosynthesis, structural diversity and biotechnological applications. Mar Drugs 17:408
6. Kurtböke I (2010) Biodiscovery from microbial resources: actinomycetes leading the way. Microbiol Aust 31(2):53
7. Kurniati TH, Rahayu S, Sukmawati D et al (2019) Screening of biosurfactant producing bacteria from hydrocarbon contaminated soil. J Phys Conf Ser 1402(5)
8. Challis G (2008) Mining microbial genomes for new natural products & biosynthetic pathways. Microbiology 154(6):1555–1569
9. Sourav D, Susanta M, Aniruddha G et al (2015) A review on natural surfactants. RSC Adv 5:65757–65767
10. Walter V, Syldatk C, Hausmann R (2010) Screening concepts for the isolation of biosurfactant producing microorganisms. In: Sen R (ed) Biosurfactants. Springer, New York, pp p1–p13
11. Domingues PM, Oliveira V, Serafim LS et al (2020) Biosurfactant production in sub-oxic conditions detected in hydrocarbon-degrading isolates from marine and estuarine sediments. Int J Environ Res Public Health 17(5):1746
12. Phulpoto IA, Yu Z, Hu B et al (2020) Production and characterization of surfactin-like biosurfactant produced by novel strain *Bacillus nealsonii* S2MT and it's potential for oil contaminated soil remediation. Microb Cell Fact 19:145
13. Rani M, Weadge JT, Jabaji S (2020) Isolation and characterization of biosurfactant-producing bacteria from oil well batteries with antimicrobial activities against food-borne and plant pathogens. Front Microbiol 11:64
14. Chittepu O (2019) Isolation and characterization of biosurfactant producing bacteria from groundnut oil cake dumping site for the control of foodborne pathogens. GOST 2(1):15–20
15. Sohail R, Jamil N (2020) Isolation of biosurfactant producing bacteria from Potwar oil fields: Effect of non-fossil fuel based carbon sources. Green Process Synth 9(1):77–86
16. Kubicki S, Bator I, Jankowski S et al (2020) A straightforward assay for screening and quantification of biosurfactants in microbial culture supernatants. Front Bioeng Biotechnol 8:958
17. Fenibo EO, Douglas SI, Stanley HO (2019) A review on microbial surfactants: production, classification, properties and characterization. J Adv Microbiol 18(3):1–22
18. Yang H, Yu H, Shen Z (2015) A novel high-throughput and quantitative method based on visible color shifts for screening *Bacillus subtilis* THY-15 for surfactin production. J Ind Microbiol Biotechnol 42:1139–1147
19. Joy S, Rahman P, Sharma S (2017) Biosurfactant production and concomitant hydrocarbon degradation potentials of bacteria isolated from extreme and hydrocarbon contaminated environments. Chem Eng J 317:232–241
20. Nayarisseri A, Singh P, Singh S (2018) Screening, isolation and characterization of biosurfactant producing *Bacillus subtilis* strain ANSKLAB03. Bioinformation 14(06):304–314
21. Sharma D, Ansari M, Al-Ghamdi A, Adgaba N, Khan K, Pruthi V, Al-Waili N (2015) Biosurfactant production by *Pseudomonas aeruginosa* DSVP20 isolated from petroleum

hydrocarbon-contaminated soil and its physicochemical characterization. Environ Sci Pollut Res 22(22):17636–17643
22. Nishanthi R, Kumaran S, Palani P, Chellaram C, Prem Anand T, Kannan V (2010) Screening of biosurfactants from hydrocarbon degrading bacteria. J Ecobiotechnol 2(5):47–53
23. Sato S, Fukuoka T, Saika A, Koshiyama T, Morita T (2019) A new screening approach for glycolipid-type biosurfactant producers using MALDI-TOF / MS. J Oleo Sci 1294(12):1287–1294
24. Sun W, Cao W, Jiang M et al (2018) Isolation and characterization of biosurfactant-producing and diesel oil degrading *Pseudomonas* sp. CQ2 from changing oil field. China RSC Adv 69 (8):39710–39720
25. Varadevenkatesan T, Murty RV (2013) Production of a lipopeptide biosurfactant by a novel *Bacillus* sp. and its applicability to enhanced oil recovery. Int Sch Res Notices 2013:621519
26. Londhe M, Khambe D, Govindvar D (2012) Isolation, peliminary screening and process optimization for production of surface active agent from *Chlorella pyrenoidosa* by non-disruptive method. Int J Sci Res 3(6):399–402
27. Rodrigues LR, Teixeira JA, Van der Mei HC et al (2006) Physicochemical and functional characterisation of a biosurfactant produced by *Lactococcus lactis* 53. Colloids Surf B Biointerfaces 49(1):79–86
28. Satpute S, Mone N, Das P et al (2019) Inhibition of pathogenic bacterial biofilms on PDMS based implants by *L. acidophilus* derived biosurfactant. BMC Microbiol 19:39
29. Twigg MS, Baccile N, Banat IM et al (2020) Microbial biosurfactant research: time to improve the rigour in the reporting of synthesis, functional characterization and process development. J Microbial Biotechnol:1751–7915
30. Thavasi R, Sharma S, Jayalakshmi S (2011) Evaluation of screening methods for the isolation of biosurfactant producing marine bacteria. J Pet Environ Biotechnol S1:001
31. Williams W, Trindade M (2017) Metagenomics for the discovery of novel biosurfactants. In: Charles T, Liles M, Sessitsch A (eds) Functional metagenomics: tools and applications. Springer, Cham, pp 95–117
32. Jain DK, Collins-Thempson DL, Trevors JT (1991) A drop-collapsing test for screening surfactant-producing microorganisms. J Microbiol Methods 13(4):271–279
33. Saruni NH, Razak SA, Habib S et al (2019) Comparative screening methods for the detection of biosurfactant-producing capability of antarctic hydrocarbon-degrading *Psuedomonas* Sp. J Environ Microbiol Toxicol 7(1):44–47
34. Youssef NH, Dencane KE, Nagle DP et al (2004) Comparison of methods to detect biosurfactant production by diverse microorganisms. J Microbiol Methods 56:339–347
35. Burch AY, Shimada BK, Browne PJ et al (2010) Novel high-throughput detection method to assess bacterial surfactant production. Appl Environ Microbiol 76(16):5363–5372
36. Mnif I, Ghribi D (2015) Microbial derived surface-active compounds: properties and screening concept. World J Microbiol Biotechnol 31:1001–1020
37. Balan SS, Kumar CG, Jayalakshmi S (2017) Aneurinifactin, a new lipopeptide biosurfactant produced by a marine *Aneurinibacillus aneurinilyticus* SBP-11 isolated from gulf of Mannar: purification, characterization and it is biological evaluation. Microbiol Res 194:1–9
38. Morikawa M, Hirata Y, Imanaka T (2000) A study on the structure-function relationship of lipopeptide biosurfactants. Biochim Biophys Acta 1488(3):211–218
39. Cipinyete V, Grigiskis S, Sapokaite D et al (2011) Production of biosurfactants by Arthrobacter sp. N3, a hydrocarbon degrading bacterium. In: Noviks G, Ansone V (eds) Proceedings of the 8th international scientific and practical conference, Latvia, June 2011, vol 1. RA Izdevniecība, pp 68–75
40. Williams W, Kunorozva L, Klaiber I et al (2019) Novel metagenome-derived ornithine lipids identified by functional screening for biosurfactants. Appl Microbiol Biotechnol 103 (11):4429–4441
41. Cottingham M, Bain C, Vaux D (2003) Rapid method for measurement of surface tension in multiwell plates. Lab Invest 84:523–529

42. Kavuthodi B, Thomas SK, Sebastian D (2015) Co-production of pectinase and biosurfactants by the newly isolates strain *Bacillus subtilis* BKDS1. Br Microbiol Res J 10(2):1–2
43. Maczek J, Junne S, Götz P (2007) Examining biosurfactant producing bacteria – an example for an automated search for natural compounds. Appl Note CyBio AG
44. Person A, Molin G (1987) Capacity for biosurfactant production of environmental *Pseudomonas* and *Vibrionaceae* growing on carbohydrates. Appl Microbiol Biotechnol 26(5):439–442
45. Roosloot R, Schoen P (2011) A colorimetric assay for determination of residual detergent levels in reconstituted membrane protein preparations. Anal Biochem 413:72–74
46. Vulliez-Le Normand B, Eiselé JL (1993) Determination of detergent critical micellar concentration by solubilization of a colored dye. Anal Biochem 208:241–243
47. Cooper DG, Goldenberg BG (1987) Surface active agents from two *Bacillus* species. Appl Environ Microbiol 53(2):224–229
48. Reddy SR, Fogler HS (1981) Emulsion stability: determination from turbidity. J Colloid Interface Sci 79(1):101–104
49. Bernheimer AW, Avigad LS (1970) Nature and properties of a cytolytic agent produced by Bacillus subtilis. J Gen Microbiol 61(3):361–369
50. Carrillo PG, Mardaraz C, Pitta-Alvarez SJ et al (1996) Isolation and selection of biosurfactant-producing bacteria. World J Microbiol Biotechnol 12:82–84
51. Johnson M, Boese-Marrazzo D (1980) Production and properties of het-stable extracellular hemolysin from *Pseudomonas aeruginosa*. Infect Immun 29(3):1028–1033
52. Shimuta K, Ohnishi M, Iyoda S et al (2009) The hemolytic and cytolytic activities of *Serratia marcescens* phospholipase A (PhlA) depend on lysophospholipid production by PhlA. BMC Microbiol 9:261
53. Pinzon NM, Ju L-K (2009) Improved detection of rhamnolipid production using agar plates containing methylene blue and cetyl trimethylammonium bromide. Biotechnol Lett 31:1583–1588
54. Heuson E, Etchegaray A, Filipe SL et al (2019) Screening of lipopeptide-producing strains of *Bacillus* sp. using a new automated and sensitive fluorescence detection method. Biotechnol J 14:1–8
55. Satpute SK, Bhawsar BD, Dhakephalkar PK et al (2008) Assessment of different screening methods for selecting biosurfactant producing marine bacteria. Indian J Mar Sci 37(3):243–250
56. Rosenberg M, Gutnick D, Rosenberg E (1980) Adherence of bacteria to hydrocarbons—a simple method for measuring cell-surface hydrophobicity. FEMS Microbiol Lett 9(1):29–33
57. Rosenberg M (1981) Bacterial adherence to polystyrene – a replica method of screening for bacterial hydrophobicity. Appl Environ Microbiol 42(2):375–377
58. Pruthi V, Cameotra SS (1997) Rapid identification of biosurfactant producing bacterial strains using a cell surface hydrophobicity technique. Biotechnol Tech 11(9):671–674
59. Lindahl M, Faris A, Wadstrom T et al (1981) A new test based on salting out to measure relative surface hydrophobicity of bacterial cells. Biochim Biophys Acta 677:471–476
60. Willumsen PA, Karlson U (1997) Screening of bacteria isolated from PAH-contaminated soils for production of biosurfactants and bioemulsifiers. Biodegradation 7:415–423
61. Ghosal D, Ghosh S, Dutta TK et al (2016) Current state of knowledge in microbial degradation of polycyclic aromatic hydrocarbons (PAHs): a review. Front Microbiol 7(386):1369–1396
62. Safitri R, Handayani S, Surono W et al (2019) Biodegradation of phenol, anthracene and acenaphthene singly and consortium culture of indigenous microorganism isolates from underground coal gasification area. In: IOP conference series: earth and environmental science. Conference on sustainability science, Indonesia, October 2018, vol 306, p 012026
63. Fanaei M, Emtiazi G (2018) Microbial assisted (*Bacillus mojavensis*) production of biosurfactant lipopeptide with potential pharmaceutical applications and its characterization by MALDI-TOF-MS analysis. J Mol Liq 268:707–714

64. Fukuoka T, Morita T, Konishi M et al (2007) Structural characterization and surface-active properties of a new glycolipid biosurfactant, mono-acylated mannosylerythritol lipid, produced from glucose by *Pseudozyma antarctica*. Appl Microbiol Biotechnol 76:801–810
65. Fukuoka T, Morita T, Konishi M et al (2007) Characterization of new glycolipid biosurfactants, tri-acylated mannosylerythritol lipids, produced by *Pseudozyma* yeasts. Biotechnol Lett 29:1111–1118
66. Imura T, Kawamura D, Morita T et al (2013) Production of sophorolipids from non-edible jatropha oil by *Stamerella bombicola* NBRC 10243 and evaluation of their interfacial properties. J Oleo Sci 62:857–864
67. Morita T, Fukuoka T, Imura T et al (2013) Accumulation of cellobiose lipids under nitrogen-limiting conditions by two ustilaginomycetous yeasts, *Pseudozyma aphidis* and *Pseudozyma hubeiensis*. FEMS Yeast Res 13:44–49
68. Satpute SK, Banpurkar AG, Dhakephalkar PK et al (2010) Methods for investigating biosurfactants and bioemulsifiers: a review. Crit Rev Biotechnol 30(2):127–144
69. Satpute SK, Bhuyan SS, Pardesi KR et al (2010) Molecular genetics of biosurfactant synthesis in microorganisms. In: Sen R (ed) Biosurfactants. Advances in experimental medicine and biology. Springer, New York, pp 14–41
70. Schramm LL, Stasiuk EN, Marangoni DG (2003) Surfactants and their applications. Annu Rep Prog Chem Sect C Phys Chem 99:3–48
71. Pereira JFB, Gudiña EJ, Costa R et al (2013) Optimization and characterization of biosurfactant production by *Bacillus subtilis* isolates towards microbial enhanced oil recovery applications. Fuel 111:259–268
72. Tuleva BK, Ivanov GR, Christova NE (2002) Biosurfactant production by a new *Pseudomonas putida* strain. Z Naturforsch 57:356–360
73. Dilmohamud B, Seeneevassen J, Rughooputh S et al (2005) Surface tension and related thermodynamic parameters of alcohols using the Traube stalagmometer. Euro J Physics 26 (6):1079–1084
74. Van der Vegt W, Vander Mei HC, Noordmans J et al (1991) Assessment of bacterial biosurfactant production through axisymmetric drop shape analysis by profile. Appl Microbiol Biotechnol 35:766–770
75. Berry JD, Neeson MJ, Dagastine RR et al (2015) Measurement of surface and interfacial tension using pendant drop tensiometry. J Colloid Interface Sci 454:226–237
76. Tadros T (2005) Adsorption of surfactants at the air/liquid and liquid/liquid interfaces. In: Applied surfactants: principles and applications. Wiley VCH, Weinheim, pp 81–82
77. Mac Aogáin M, Chaturvedi V, Chotirmall SH (2019) MycopathologiaGENOMES: the new 'Home' for the publication of fungal genomes. Mycopathologia 184:551–554
78. Hughes RA, Ellington AD (2017) Synthetic DNA synthesis and assembly: putting the synthetic in synthetic biology. Cold Spring Harb Perspect Biol 9(1):a023812
79. Kurtböke I (2017) Revisiting biodiscovery from microbial sources in the light of molecular advances. Microbiol Aust 38(2):58
80. Romano S, Jackson S, Patry S et al (2018) Extending the "One Strain Many Compounds" (OSMAC) principle to marine microorganisms. Mar Drugs 16(244):1–29
81. Altschul SF, Gish W, Miller W et al (1990) Basic local alignment search tool. J Mol Biol 215:403–410
82. Tatusov RL, Galperin MY, Natale DA et al (2000) The COG database: a tool for genome-scale analysis of protein functions and evolution. Nucleic Acids Res 28:33–36
83. Kanehisa M, Goto S (2000) KEGG: Kyoto encyclopedia of genes and genomes. Nucleic Acids Res 28:27–30
84. Jackson S, Borchert E, O'Gara F, Dobson A (2015) Metagenomics for the discovery of novel biosurfactants of environmental interest from marine ecosystems. Curr Opin Biotechnol 33:176–182
85. Blin K, Shaw S, Steinke K et al (2019) antiSMASH 5.0: updates to the secondary metabolite genome mining pipeline. Nucleic Acids Res 47:81–87

86. Rutledge PJ, Challis GL (2015) Discovery of microbial natural products by activation of silent biosynthetic gene clusters. Nat Rev Microbiol 13:509–523
87. Wang X, Zhou H, Chen H et al (2018) Discovery of recombinases enables genome mining of cryptic biosynthetic gene clusters in Burkholderiales species. Proc Natl Acad Sci U S A 115 (18)
88. Coutinho PM, Deleury E, Davies GJ et al (2003) An evolving hierarchical family classification for glycosyltransferases. J Mol Biol 328:307–317
89. Rabausch U, Juergensen J, Ilmberger N et al (2013) Functional screening of metagenome and genome libraries for detection of novel flavonoid-modifying enzymes. Appl Environ Microbiol 79:4551–4563
90. Niehs SP, Scherlach K, Hertweck C (2018) Biomolecular chemistry genomics-driven discovery of a linear lipopeptide promoting host colonization by endofungal bacteria. Org Biomol Chem 16:8345–8352
91. Gerc AJ, Stanley-Wall NR, Coulthurst SJ (2014) Role of the phosphopantetheinyltransferase enzyme PswP, in the biosynthesis of antimicrobial secondary metabolites by *Serratia marcescens* Db10. Microbiology 160:1609–1617
92. Retamal-Morales G, Heine T, Tischler JS et al (2018) Draft genome sequence of *Rhodococcus erythropolis* B7g, a biosurfactant producing actinobacterium. J Biotechnol 20(280):38–41
93. Waghmode S, Suryavanshi M, Dama L et al (2019) Genomic insights of halophilic *Planococcus maritimus* SAMP MCC 3013 and detail investigation of its biosurfactant production. Front Microbiol 10:235
94. Tuffin M, Anderson D, Heath C et al (2009) Metagenomic gene discovery: how far have we moved into novel sequence space? Biotechnol J 4(12):1671–1683
95. Abdel-Mawgoud AM, Lépine F, Déziel E (2010) Rhamnolipids: diversity of structures, microbial origins and roles. Appl Microbiol Biotechnol 86:1323–1336
96. Zhang X, Hindra EMA (2019) Unlocking the trove of metabolic treasures: activating silent biosynthetic gene clusters in bacteria and fungi. Curr Opin Microbiol 51:9–15
97. Nora LC, Westmann CA, Martins-Santana L et al (2019) The art of vector engineering: towards the construction of next-generation genetic tools. J Microbial Biotechnol 12:125–147
98. Uchiyama T, Miyazaki K (2009) Functional metagenomics for enzyme discovery: challenges to efficient screening. Curr Opin Biotechnol 20:616–622
99. Lin Z, Nielsen J, Liu Z (2020) Bioprospecting through cloning of whole natural product biosynthetic gene clusters. Front Bioeng Biotechnol 8:526
100. Sotirova AV, Spasova DI, Galabova DN et al (2008) Rhamnolipid-biosurfactant permeabilizing effects on gram-positive and gram-negative bacterial strains. Curr Microbiol 56:639–644
101. Beld J, Sonnenschein EC, Vickery CR et al (2014) The phosphopantetheinyl transferases: catalysis of a post-translational modification crucial for life. Nat Prod Rep 31:61–108
102. Thies S, Santiago-Schübel B, Kovačić F et al (2014) Heterologous production of the lipopeptide biosurfactant serrawettin W1 in *Escherichia coli*. J Biotechnol 181:27–30
103. Tiso T, Thies S, Müller M et al (2017) Rhamnolipids: production, performance, and application. In: Lee SY (ed) Consequences of microbial interactions with hydrocarbons, oils, and lipids: production of fuels and chemicals. Springer, Cham, pp 587–622
104. Loeschcke A, Thies S (2020) Engineering of natural product biosynthesis in *Pseudomonas putida*. Curr Opin Biotechnol 65:213–224
105. Wittgens A, Rosenau F (2018) On the road towards tailor-made rhamnolipids: current state and perspectives. Appl Microbiol Biotechnol 102:8175–8185
106. Roelants SLKW, Saerens KMJ, Derycke T et al (2013) *Candida bombicola* as a platform organism for the production of tailor-made biomolecules. Biotechnol Bioeng 110:2494–2503
107. Bahia FM, De Almeida GC, De Andrade LP et al (2018) Rhamnolipids production from sucrose by engineered *Saccharomyces cerevisiae*. Sci Rep 8:1–10
108. Wang Z, Feng S, Huang Y et al (2010) Expression and characterization of a *Grifola frondosa* hydrophobin in *Pichia pastoris*. Protein Expr Purif 72:19–25

109. Streit WR, Schmitz RA (2004) Metagenomics- the key to the uncultured microbes. Curr Opin Microbiol 7:492–498
110. Kennedy J, O'Leary ND, Kiran GS et al (2011) Functional metagenomic strategies for the discovery of novel enzymes and biosurfactants with biotechnological applications from marine ecosystems. J Appl Microbiol 111:787–799
111. Brady SF, Chao CJ, Clardy J (2004) Long-chain N-acyltyrosine synthases from environmental DNA. Appl Environ Microbiol 70:6865–6870
112. Thies S, Rausch SC, Kovacic F et al (2016) Metagenomic discovery of novel enzymes and biosurfactants in a slaughterhouse biofilm microbial community. Sci Rep 6:27035
113. Lee C-M, Kim S-Y, Yoon S-H et al (2019) Characterization of a novel antibacterial N-acyl amino acid synthase from soil metagenome. J Biotechnol 294:19–25
114. Araújo SC, Silva-Portela RCB, de Lima DC et al (2020) MBSP1: a biosurfactant protein derived from a metagenomic library with activity in oil degradation. Sci Rep 10:1–13
115. Cox PW, Hooley P (2009) Hydrophobins: new prospects for biotechnology. Fungal Biol Rev 23:40–47
116. Pitocchi R, Cicatiello P, Birolo L et al (2020) Cerato-Platanins from marine fungi as effective protein biosurfactants and bioemulsifiers. Int J Mol Sci 21:2913
117. Jeong H, Qian X, Yoon B-J (2016) Effective comparative analysis of protein-protein interaction networks by measuring the steady-state network flow using a Markov model. BMC Bioinformatics 17:395
118. Li H, Tanikawa T, Sato Y et al (2005) *Serratia marcescens* gene required for surfactant serrawettin W1 production encodes putative aminolipid synthetase belonging to nonribosomal peptide synthetase family. Microbiol Immunol 49:303–310
119. Trindade M, van Zyl L, Navarro-Fernández J, Abd Elrazak A (2015) Targeted metagenomics as a tool to tap into marine natural product diversity for the discovery and production of drug candidates. Front Microbiol 6:1–14
120. Ayling M, Clark MD, Leggett RM (2020) New approaches for metagenome assembly with short reads. Brief Bioinform 21:584–594
121. Meleshko D, Mohimani H, Tracanna V et al (2019) BiosyntheticSPAdes: reconstructing biosynthetic gene clusters from assembly graphs. Genome Res 29:1352–1362
122. Stevenson LJ, Owen JG, Ackerley DF (2019) Metagenome driven discovery of nonribosomal peptides. ACS Chem Biol 14:9b00618
123. Hover BM, Kim S-H, Katz M et al (2018) Culture-independent discovery of the malacidins as calcium-dependent antibiotics with activity against multidrug-resistant gram-positive pathogens. Nat Microbiol 3:415–422
124. Wu C, Shang Z, Lemetre C et al (2019) Cadasides, calcium-dependent acidic lipopeptides from the soil metagenome that are active against multidrug-zesistant bacteria. J Am Chem Soc 141:3910–3919
125. Chu J, Vila-Farres X, Inoyama D et al (2016) Discovery of MRSA active antibiotics using primary sequence from the human microbiome. Nat Chem Biol 12:1004–1006
126. Craig JW, Chang F-Y, Kim JH et al (2010) Expanding small-molecule functional metagenomics through parallel screening of broad-host-range cosmid environmental DNA libraries in diverse proteobacteria. Appl Environ Microbiol 76:1633–1641
127. Wang G, Zhao Z, Ke J et al (2019) CRAGE enables rapid activation of biosynthetic gene clusters in undomesticated bacteria. Nat Microbiol 4:2498–2510
128. Marmeisse R, Kellner H, Fraissinet-Tachet L et al (2017) Discovering protein-coding genes from the environment: time for the eukaryotes? Trends Biotechnol 35:824–835
129. Hewald S, Linne U, Scherer M et al (2006) Identification of a gene cluster for biosynthesis of mannosylerythritol lipids in the basidiomycetous fungus *Ustilago maydis*. Appl Environ Microbiol 72:5469–5477
130. Teichmann B, Linne U, Hewald S et al (2007) A biosynthetic gene cluster for a secreted cellobiose lipid with antifungal activity from *Ustilago maydis*. Mol Microbiol 66:525–533

131. Van Bogaert IN, Holvoet K, Roelants SL et al (2013) The biosynthetic gene cluster for sophorolipids: a biotechnological interesting biosurfactant produced by *Starmerella bombicola*. Mol Microbiol 88:501–509
132. Staley JT, Konopka A (1985) Measurements of in situ activities of nonphotosynthetic microorganisms in aquatic and terrestrial habitats. Annu Rev Microbiol 39:321–346
133. Joint I, Mühling M, Querellou J (2010) Culturing marine bacteria- an essential prerequisite for biodiscovery. J Microbial Biotechnol 3:564–575
134. Wang S, Yu S, Wei Q et al (2014) Coordination of swarming motility, biosurfactant synthesis, and biofilm matrix exopolysaccharide production in *Pseudomonas aeruginosa*. Appl Environ Microbiol 80(21):6724–6732
135. Yang A, Tang WS, Si T et al (2017) Influence of physical effects on the swarming motility of *Pseudomonas aeruginosa*. Biophys J 112(7):1462–1471
136. Thrash JC (2019) Culturing the uncultured: risk versus reward. mSystems 4:e00130-19
137. Lagier J, Khelai S, Alou MT et al (2016) Culture of previously uncultured members of the human gut microbiota by culturomics. Nat Microbiol 1:16203
138. Lewis WH, Ettema TG (2019) Culturing the uncultured. Nat Biotechnol 37:1278–1279
139. Oberhardt MA, Zarecki R, Gronow S et al (2015) Harnessing the landscape of microbial culture media to predict new organism–media pairings. Nat Commun 6:8493
140. Stewart EJ (2012) Growing unculturable bacteria. J Bacteriol 194(16):4151–4160
141. Burch AY, Browne PJ, Dunlap CA et al (2011) Comparison of biosurfactant detection methods reveals hydrophobic surfactants and contact-regulated production. Environ Microbiol 13(10):2681–2691
142. Lin SC, Carswell K, Sharma M et al (1994) Continuous production of the lipopeptide biosurfactant of *Bacillus licheniformis* JF-2. Appl Microbiol Biotechnol 41:281–285
143. Ochsner UA, Reiser J (1995) Autoinducer-mediated regulation of rhamnolipid biosurfactant synthesis in *Pseudomonas aeruginosa*. Proc Natl Acad Sci U S A 92:6424–6428
144. Bode HB, Bethe B, Höfs R et al (2002) Big effects from small changes: possible ways to explore nature's chemical diversity. Chembiochem 3(7):619–627
145. Yoon V, Nodwell JR (2014) Activating secondary metabolism with stress and chemicals. J Ind Microbiol Biotechnol 41(2):415–424
146. Bhatnagar I, Kim SK (2010) Immense essence of excellence: marine microbial bioactive compounds. Mar Drugs 8(10):2673–2701
147. Hutchinson CR (2003) Polyketide and non-ribosomal peptide synthases: falling together by coming apart. Proc Natl Acad Sci U S A 100(6):3010–3012
148. Craney A, Ahmed S, Nodwell J (2013) Towards a new science of secondary metabolism. J Antibiot 66(7):387–400
149. Dusane DH, Matkar P, Venugopalan VP et al (2011) Cross-species induction of antimicrobial compounds, biosurfactants and quorum-sensing inhibitors in tropical marine epibiotic bacteria by pathogens and biofouling microorganisms. Curr Microbiol 62:974–980
150. Xue Y, Sherman DH (2000) Alternative modular polyketide synthase expression controls macrolactone structure. Nature 403(6769):571–575
151. Huo L, Hug JJ, Fu C et al (2019) Heterologous expression of bacterial natural product biosynthetic pathways. Nat Prod Rep 36(10):1412–1436
152. Parages ML, Gutiérrez-Barranquero JA, Reen FJ et al (2016) Integrated (meta) genomic and synthetic biology approaches to develop new biocatalysts. Mar Drugs 14(3):62