# Chapter 1

# Screening Concepts for the Isolation of Biosurfactant Producing Microorganisms

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## Abstract

This chapter gives an overview of current methods for the isolation of biosurfactant producing microbes. The common screening methods for biosurfactants are presented.

Sampling and isolation of bacteria are the basis for screening of biosurfactant producing microbes. Hydrocarbon-contaminated sites are the most promising for the isolation of biosurfactant producing microbes, but many strains have also been isolated from undisturbed sites.

In subsequent steps the isolates have to be characterized in order to identify the strains which are interesting for a further investigation. Several techniques have been developed for identifying biosurfactant producing strains. Most of them are directly based on the surface or interfacial activity of the culture supernatant. Apart from that, some screening methods explore the hydrophobicity of the cell surface. This trait also gives an indication on biosurfactant production.

In recent years automation and miniaturization have led to the development of high throughput methods for screening. High throughput screening (HTS) for analyzing large amounts of potential candidates or whole culture collections is reflected in the end. However, no new principals have been introduced by HTS methods.

## Introduction

The overall establishment of biosurfactants is well-known to be impeded by a lack of availability of economic and versatile products. Currently there is only a very limited offer of commercially available biosurfactants, e.g., surfactin, sophorolipids and rhamnolipids. A variety of new biosurfactants respectively producing strains are the key issue in overcoming the economic obstacles of the production of biosurfactants. Therefore, increased efforts in the discovery of new biosurfactant producing microbes must be made by applying a broad range of different screening methods, which is the focus of this chapter.

The principle aim in screening for new biosurfactants is finding new structures with strong interfacial activity, low critical micelle concentration (cmc), high emulsion capacity, good solubility and activity in a broad pH-range. Besides these physicochemical properties, commercial viable biosurfactants have to be economically competitive. Therefore, the second aim in screening is the discovery of good production strains with high yields.

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Biosurfactants may be involved in pathogenesis due to their surface activity; however, for security and regulatory reasons, production strains should be nonpathogenic. In the above mentioned example of rhamnolipids this is not the case as *Pseudomonas aeruginosa*, the most common producing bacteria, is a pathogen.

A variety of methods for the screening of biosurfactant producing microbes has been developed and successfully applied. Since the 1970s there have been various trials in this field. These screenings have mostly been limited to a manageable number of samples. In recent years automation and miniaturization have led to the development of high throughput methods for screening of biosurfactant producing strains. A broad application of such methods could eventually lead to the desired upsurge of new commercially interesting strains.

An efficient screening strategy is the key to success in isolating new and interesting microbes or their variants, because a large number of strains needs to be characterized. A complete strategy for screening of new biosurfactants or production strains consists of three steps: sampling, isolation of strains and investigation of strains. Theses steps will be addressed in the next paragraphs. Bioinformatical approaches like homology search are not included herein.

## Sampling

According to Ron and Rosenberg,[1] biosurfactants can fulfill various physiological roles and provide different advantages to their producing strains:

- increase the surface area of water-insoluble substrates by emulsification,
- increase the bioavailability of hydrophobic substrates,
- bind heavy metals,
- be involved in pathogenesis,
- possess antimicrobial activity,
- regulate the attachment/detachment of microorganisms to and from surfaces.

According to these physiological roles, biosurfactant producing microbes can be found in different environments. Many biosurfactant producing microbes were isolated from soils or water samples which are contaminated with hydrophobic organic compounds like e.g., refinery wastes.[2-13] One biosurfactant producing microbe, *Cladosporium resinae*, which is also called the "kerosene fungus", was even isolated from an aircraft fuel tank.[14] In contrast, also undisturbed environments have yielded several interesting isolates, e.g., natural soils.[9] Marine environments have also been reported as successful sampling sites.[6,16-19] However, Bodour and Miller-Maier[15] showed that contaminated soils are more yielding than uncontaminated soils. One exceptional example is the discovery of biosurfactant producing strains which were originally isolated when investigating the food hygiene of meat.[20,21]

## Isolation

In natural environments, microbes occur almost always in a mixed population composed of a multitude of different strains and species. For analyzing the properties of a defined organism out of such a mixed population, a pure culture is required. Apart from direct isolation of strains by diluting and plating, enrichment cultures with hydrophobic substrates are very promising for the isolation of biosurfactant producing microbes. Additionally, hydrophobic interaction chromatography and the replica plate technique are also rewarding methods.

The principle of enrichment culture is to provide growth conditions that are very favorable for the organisms of interest and as unfavorable as possible for competing organisms. Hence, the microbes of interest are selected and enriched. For the screening of biosurfactant producing microbes, enrichment cultures utilizing hydrophobic compounds as the sole carbon source are applied.[3,5-7,11,12,22] This is an indirect screening method as the growth on hydrophobic compounds indicates the production of biosurfactants, but not always correlates with this trait.[3,5] Moreover, the applied screening medium and conditions will influence whether or not surfactants are produced.[9] Thus, it is possible that biosurfactant producing populations are present in the sample which are not enriched by the applied enrichment conditions.

Willumsen and Karlson[3] isolated biosurfactant producing bacteria from soil which was contaminated with polyaromatic hydrocarbons (PAHs). They used PAH-amended liquid minimal medium for enrichment culture. Furthermore, they used agar-plates coated with different PAHs and agar-plates with a PAH-soaked filter in the lid of the petri dish for the selection. The degradation of PAHs by the microorganisms then leads to a clearing zone agar around the colonies in the PAH coated agar. As result, they isolated 57 strains of which only 4 strains showed surface activity.

Mercadé et al.[5] isolated biosurfactant producing strains from petroleum-contaminated soil samples by using waste lubricating oil as the sole carbon source. They isolated 44 strains which were able to grow on hydrocarbons. Therefrom, five isolates produced biosurfactants.

Schulz and colleagues[6] isolated three bacterial strains of marine origin during a screening for biosurfactants among *n*-alkane degrading microorganisms. As enrichment medium, they used mineral media with $C_{14}$- and $C_{15}$-*n*-alkanes and also agar plates with an alkane-soaked filter in the lid. Yakimov and coworkers[17] isolated a biosurfactant producing bacterium of a new genus by using the same enrichment technique.

Rahman et al[7] isolated 130 oil-degrading isolates from hydrocarbon-polluted environments by enrichment techniques. A mineral salts medium containing crude oil as the sole carbon source was applied. Two of these strains were found to produce biosurfactants.

The degradation and consumption of hydrocarbons can also be visualized by the following colorimetric method developed by Hanson et al.[23] By adding a colored redox indicator, 2,6-dichlorophenol indophenol (DCPIP), to liquid cultures growing on hydrocarbons, a simple colorimetric assay results. The DCPIP is incorporated by bacteria that can degrade the hydrocarbons. It acts as electron acceptor and changes from blue (oxidized) to colorless (reduced). Thus, a decolorization of the culture shows degradation of hydrocarbons. However, the redox indicator DCPIP might be toxic to some organisms.

As a conclusion, sampling of contaminated sites combined with direct isolation or enrichment culture is an approved strategy for discovering new biosurfactant producing strains. However, as the proportion of positives is only in the range of a few percent, several dozen of isolates have to be tested for every hit.

## Screening Methods

Biosurfactants are structurally a very diverse group of biomolecules, e.g., glycolipids, lipopeptides, lipoproteins, lipopolysaccharides or phospholipids. Therefore, most methods for a general screening of biosurfactant producing strains are based on the physical effects of surfactants. Alternatively, the ability of strains to interfere with hydrophobic interfaces can be explored. On the other hand, specific screening methods like the colorimetric CTAB agar assay are suitable only to a limited group of biosurfactants. The screening methods can give qualitative and/or quantitative results. For a first screening of isolates, qualitative methods are generally sufficient.

### *Surface/Interfacial Activity*

The majority of screening methods for biosurfactant producing microbes are based on the interfacial or surface activity. Various methods have been developed for measuring this property. The methods which are applied for screening of biosurfactant producing microbes are reviewed in the next paragraph.

#### Direct Surface/Interfacial Tension Measurements

The direct measurement of the interfacial or surface activity of the culture supernatant is the most straightforward screening method and very appropriate for a preliminary screening of biosurfactant producing microbes.[24] This gives a strong indication on biosurfactant production. The interfacial or surface tension of a liquid can be measured by a variety of methods. However, there is a restriction in the range of measurement. The surface tension decreases with increasing surfactant concentration until the cmc is reached. If the concentration of biosurfactant is above the cmc, an increase in the concentration cannot be detected. Consequently, two cultures with very different concentrations of biosurfactant may display the same surface tension. This problem can be solved

by serial diluting until a sharp increase in surface tension is observed.[2,20,25-29] The corresponding dilution of the supernatant is called critical micelle dilution (cmd) and correlates to the concentration of biosurfactant. Furthermore, the measurements are strongly affected by factors such as pH and ionic strength. In addition, the measurement can be affected by plant oils as carbon sources because of the resulting fatty acids or mono/di-glycerids interfacial activity.

For screening purposes, the following methods are established. They can all be used for measuring the surface and interfacial tension of a liquid. Especially the Du-Nouy-Ring method is quite easy and most frequently applied.

### Du-Nouy-Ring Method

The Du-Nouy-Ring method is based on measuring the force required to detach a ring or loop of wire from an interface or surface.[30] The detachment force is proportional to the interfacial tension. It can be measured with an automated tensiometer which is available from many manufacturers. The ring must be free from contaminant, which is usually achieved by using a platinum ring that is flamed before use. Instead of a ring, a platinum plate, a so called Wilhelmy plate, can be applied in the same manner.[31-33]

The Du-Nouy-Ring assay is widely applied for screening of biosurfactant producing microbes.[3,5-7,15,20,27,34-36] Cooper considered a culture as promising if it reduces the surface tension of a liquid medium to 40 mN/m or less.[37] Willumsen and Karlson[3] give a similar definition: a good biosurfactant producer is defined as one being able to reduce the surface tension of the growth medium by ≥20 mN/m compared with distilled water.

The advantage of this method is the accuracy and the ease of use. However, it requires specialized equipment. A disadvantage is that measurements of different samples cannot be performed simultaneously. Other limitations of this assay include the volume of sample required for analysis, usually some milliliters and the restricted range of concentrations that can be analyzed without dilution.[15]

### Stalagmometric Method

The surface tension of a liquid can alternatively be measured with a Traube stalagmometer.[38] This device is essentially a pipette with a broad flattened tip, which permits large drops of reproducible size to form and finally drop under the action of gravity. The surface tension can be determined on the basis of the number of drops which fall per volume, the density of the sample and the surface tension of a reference liquid, e.g., water. According to Dilmohamud et al,[38] the surface tension is given by:

$$\sigma_L = \frac{\sigma_W \cdot N_W \cdot \rho_L}{N_L \cdot \rho_W}$$

where as $\sigma_L$ is the surface tension of the liquid under investigation, $\sigma_W$ is the surface tension of water, $N_L$ is the number of drops of the liquid, $N_W$ is the number of drops of water, $\rho_L$ is the density of the liquid and $\rho_W$ is the density of water.

Again, a disadvantage of this assay is that only consecutive measurements can be performed. Also, the method seems to be variability prone. Plaza et al[36] applied this method and conclude that it is not recommendable due to the large variability they obtained in their results. The reason is probably that the process of drop formation is too fast to allow the complete adsorption of the surfactants to the newly generated drop surface.

### Pendant Drop Shape Technique

The pendant drop shape technique is an optical method for measuring the interfacial tension. A drop of liquid is allowed to hang from the end of a capillary. It adopts an equilibrium profile that is a unique function of the tube radius, the interfacial tension, its density and the gravitational field.

According to Tadros,[30] the interfacial tension is given by the following equation:

$$\gamma = \frac{\Delta \rho g d_e^2}{H} \qquad H = f\left(\frac{d_s(t)}{d_e(t)}\right)$$

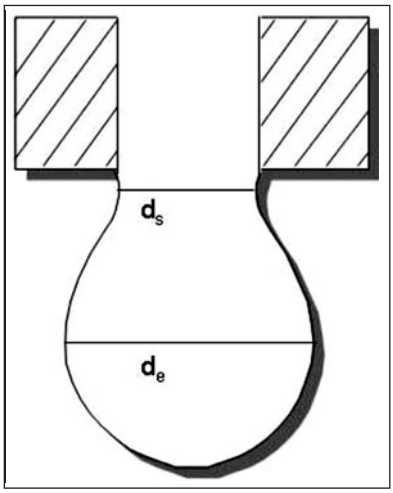


Figure 1. Shape of a pendant drop with the equatorial diameter $d_e$ and the smallest diameter $d_s$.

in which Δρ is the density difference between the two phases, $d_e$ is the equatorial diameter of the drop and $d_s$ is the smallest diameter of the hanging drop (see Fig. 1). H is a function of $d_s$ and $d_e$. Accurate values for H have been obtained by Nierderhauser and Bartell.[39]

A variant of this technique was applied by Chen et al[4] who measured in an inverse mode. A small volume of air was blown into a liquid and the shape of the air bubble in the liquid was measured. The disadvantage of the pendant drop shape technique is again that measurements cannot be performed simultaneously.

**Axisymmetric Drop Shape Analysis by Profile**

The drop shape analysis is another optical method for the determination of the surface tension. For screening purposes it was first applied by Van der Vegt et al[40] The underlying principle is that the shape of a liquid droplet depends greatly on the liquid surface tension. Droplets of liquids with a low surface tension are more apt to deviate from a perfectly spherical shape than droplets of liquids with a high surface tension.

According to Rotenberg et al,[41] the profile of a liquid droplet can be describes by the following equation:

$$\Delta p = \sigma\left(\frac{1}{r_1}+\frac{1}{r_2}\right)$$

in which $\Delta p$ is the pressure difference across the interface, $r_1$ and $r_2$ are the principal radii of curvature and σ is the surface tension (see Fig. 2).

For the drop shape analysis, a 100 µl droplet of a bacterial suspension is put on a FEP-Teflon surface. The profile of the droplet is determined with a contour monitor as a function of time up to 2 hours. The surface tension of the suspension can then be calculated from the droplet profiles with a solution scheme developed by Rotenberg et al.[41] As shown by Van der Vegt et al,[40] the drop shape analysis can be used to monitor bacterial biosurfactant production. For this assay, just small amounts of sample are needed. But a special camera and software are required. The calculation of the surface tension is rather complex. Furthermore, different samples cannot be measured in parallel.

**Measurements Based on Surface/Interfacial Tension**

Many screening methods have been developed that rely on the interfacial activity of the biosurfactants but that do not measure it directly. They are presented in the following.

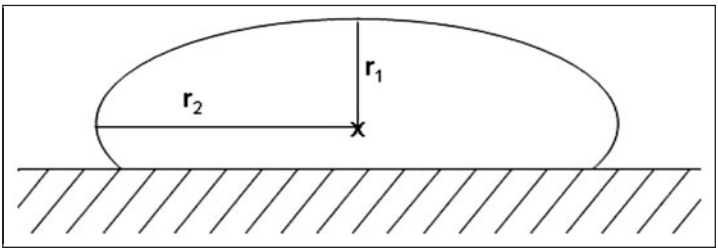


Figure 2. Shape of a sessile drop with the principal radii of curvature $r_1$ and $r_2$.

## Drop Collapse Assay

Jain et al[29] developed the drop collapse assay. This assay relies on the destabilization of liquid droplets by surfactants. Therefore, drops of a cell suspension or of culture supernatant are placed on an oil coated, solid surface. If the liquid does not contain surfactants, the polar water molecules are repelled from the hydrophobic surface and the drops remain stable. If the liquid contains surfactants, the drops spread or even collapse because the force or interfacial tension between the liquid drop and the hydrophobic surface is reduced. The stability of drops is dependent on surfactant concentration and correlates with surface and interfacial tension.

Persson and Molin[20] described a similar assay using a glass surface instead of the oil coated surface. Furthermore, Bodour and Miller-Maier[15] showed that for pure surfactant, this assay can even be quantitative by measuring the drop size with a micrometer. An important distinction of this assay is that it can be transferred to an automated screening in microplates, as it has been reported by Maczek et al.[42] They stained the culture supernatant to enhance the visual effect.

The drop collapse assay is rapid and easy to carry out, requires no specialized equipment and just a small volume of sample.[36] In addition, it can be performed in microplates.[43] This assay has been applied several times for screening purposes.[2,9,36,44] But it displays a relative low sensitivity since a significant concentration of surface active compounds must be present in order to cause a collapse of the aqueous drops on the oil or glass surfaces.

## Microplate Assay

The surface activity of individual strains can be determined qualitatively with the microplate assay developed and patented by Vaux and Cottingham.[45] This assay is based on the change in optical distortion that is caused by surfactants in an aqueous solution. Pure water in a hydrophobic well has a flat surface. The presence of surfactants causes some wetting at the edge of the well and the fluid surface becomes concave and takes the shape of a diverging lens. For this assay, a 100 µl sample of the supernatant of each strain is taken and put into a microwell of a 96-mircowell plate. The plate is viewed using a backing sheet of paper with a grid. If biosurfactant is present, the concave surface distorts the image of the grid below (see Fig. 3). The optical distortion of the grid provides a qualitative assay for the presence of surfactants.

The microplate assay is easy, rapid and sensitive and allows an instantaneous detection of surface-active compounds.[4] Just a small volume (100 µl) of sample is needed. Furthermore, the method is suitable for automated high throughput screening. Chen et al[4] demonstrated the efficiency of the microplate method for high throughput screening purposes.

## Penetration Assay

Maczek et al[42] developed another assay suitable for high throughput screening, the penetration assay. This assay relies on the contacting of two unsoluble phases which leads to a color change.

For this assay, the cavities of a 96 well microplate are filled with 150 µl of a hydrophobic paste consisting of oil and silica gel. The paste is covered with 10 µl of oil. Then, the supernatant of the

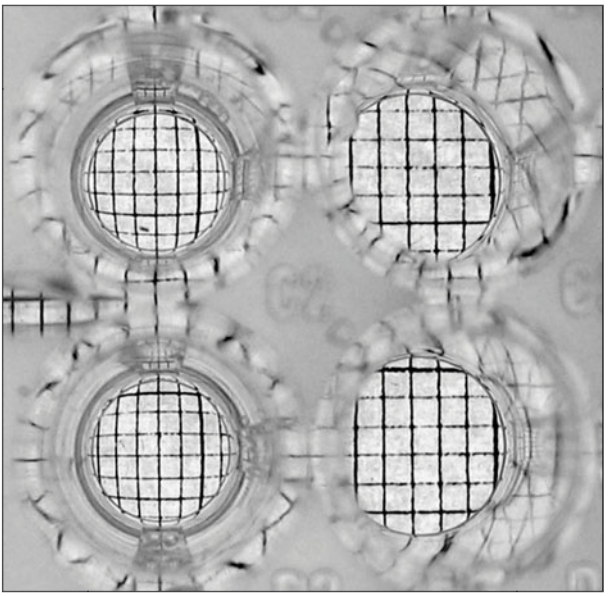

Figure 3. Microplate assay. Left) Biosurfactant rhamnolipid in water. Right) water.

culture is colored by adding 10 µl of a red staining solution to 90 µl of the supernatant. The colored supernatant is placed on the surface of the paste. If biosurfactant is present, the hydrophilic liquid will break through the oil film barrier into the paste. The silica is entering the hydrophilic phase and the upper phase will change from clear red to cloudy white within 15 minutes. The described effect relies on the phenomenon that silica gel is entering the hydrophilic phase from the hydrophobic paste much more quickly if biosurfactants are present. Biosurfactant free supernatant will turn cloudy but stay red.

The penetration assay is a simple, qualitative technique for screening large amounts of potential isolates. It can be applied in high throughput screening. The assay was described as recently as 2007 and to our knowledge there has been no further report of its application by now.

### Oil Spreading Assay

The oil spreading assay was developed by Morikawa et al.[28] For this assay, 10 µl of crude oil is added to the surface of 40 ml of distilled water in a petri dish to form a thin oil layer. Then, 10 µl of culture or culture supernatant are gently placed on the centre of the oil layer. If biosurfactant is present in the supernatant, the oil is displaced and a clearing zone is formed. The diameter of this clearing zone on the oil surface correlates to surfactant activity, also called oil displacement activity. For pure biosurfactant a linear correlation between quantity of surfactant and clearing zone diameter is given.

The oil spreading method is rapid and easy to carry out, requires no specialized equipment and just a small volume of sample.[36] It can be applied when the activity and quantity of biosurfactant is low. Plaza et al[36] and Youssef et al[44] demonstrated that the oil spreading technique is a reliable method to detect biosurfactant production by diverse microorganisms. The assay was also applied for screening by Huy et al.[12]

### Emulsification Capacity Assay

Another popular assay based on the emulsification capacity of biosurfactants was developed by Cooper and Goldenberg.[35] For measuring this trait, kerosene is added to an aqueous sample. The mixture is vortexed at high speed for 2 minutes. After 24 hours, the height of the stable emulsion

layer is measured. The emulsion index $E_{24}$ is calculated as the ratio of the height of the emulsion layer and the total height of liquid:[35]

$$E_{24} = \frac{h_{emulsion}}{h_{total}} \times 100\,\%$$

$E_{24}$ correlates to the surfactant concentration. Evaluating the emulsification capacity is a simple screening method suitable for a first screening of biosurfactant producing microbes. It is applied in many screenings,[3,4,6,10,11,13,26,27,36,46,47] whereas the kerosene can be replaced with other hydrophobic compounds, e.g., hexadecane. But surface activity and emulsification capacity do not always correlate.[3,26,35,36,48] Consequently, this method gives just an indication on the presence of biosurfactants.

### Solubilization of Crystalline Anthracene

Willumsen and Karlson[3] developed an assay based on the solubilization of crystalline anthracene. This screening method is based on the solubilization of a highly hydrophobic, crystalline compound, anthracene, by the biosurfactants. Therefore, crystalline anthracene is added to the culture supernatant and incubated on a shaker at 25 °C for 24 h. The concentration of the dissolved hydrophobic anthracene is measured photometrically at 354 nm and correlates to the production of biosurfactant.

This is a simple and rapid screening method, but the anthracene might be toxic to some microbes. To our knowledge there have been no further reports on its application.

## *Cell Surface Hydrophobicity*

The following screening methods are based on the hydrophobicity of the cell surface. Thus, they are indirect methods for the screening of biosurfactant producing microbes. Nevertheless, a rapid identification of biosurfactant producing strains can be achieved by assaying this trait.[46,49] A disadvantage is that the hydrophobicity of bacteria depends on physiological aspects like growth conditions or cellular age.[49]

### Bacterial Adhesion to Hydrocarbons Assay (BATH)

Rosenberg et al[50] developed the bacterial adhesion to hydrocarbons method, a simple photometrical assay for measuring the hydrophobicity of bacteria. The method is based on the degree of adherence of cells to various liquid hydrocarbons. For measuring this trait, a turbid, aqueous suspension of washed microbial cells is mixed with a distinct volume of a hydrocarbon, e.g., hexadecane or octane. After mixing for 2 minutes, the two phases are allowed to separate. Hydrophobic cells become bound to hydrocarbon droplets and rise with the hydrocarbon. They are removed from the aqueous phase. The turbidity of the aqueous phase is measured. The decrease in the turbidity of the aqueous phase correlates to the hydrophobicity of the cells. The percentage of cells bound to the hydrophobic phase ($H$) is calculated by:[40]

$$H = \left(1 - \frac{A}{A_0}\right) \cdot 100\,\%$$

whereas $A_0$ is the absorbance of the bacterial suspension without hydrophobic phase added and $A$ the absorbance after mixing with hydrophobic phase.

BATH is a simple but indirect screening method. Pruthi and Cameotra[49] showed that the ability of bacteria to adhere to hydrocarbons is a characteristic feature of biosurfactant producing microbes. This assay was applied several times for screening.[13,46,47] For example, Neu and Poralla[46] isolated 126 bacterial strains during screening for cell surface hydrophobicity. Forty-eight of the isolated strains produced an emulsifying agent.

### Hydrophobic Interaction Chromatography (HIC)

A method which allows the simultaneous isolation and screening of microbes was developed by Smyth et al.[51] They used hydrophobic interaction chromatography (HIC) for this purpose. HIC is a chromatographic procedure based on hydrophobic interaction between the nonpolar groups on a hydrophobic chromatographic resin and the nonpolar regions of a particle.

A bacterial suspension is drained into a gel bed of hydrophobized sepharose. Hydrophobic microbes are retained by the gel and the degree of adsorption of the cells to the gel can be measured by the turbidity of the eluate or by bacteria counting. For desorption of the adherent microbes, the ionic strength of the buffer is decreased.

HIC is very convenient because screening and isolation of potential strains can be combined in one step. Pruthi and Cameotra[49] reported that HIC is a reliable screening method for biosurfactant production. The technique is also valid for comparative analysis of the hydrophobic properties of microorganisms.

### Replica Plate Assay

A simple replica plate assay for the identification and isolation of hydrophobic microbes was developed by Rosenberg.[52] The principle of this assay is the adherence of bacterial strains to hydrophobic polystyrene which correlates to cell surface hydrophobicity. A flat, sterile disc of polystyrene is pressed on an agar containing the colonies to be screened. The replica of the colonies obtained on the polystyrene surface is washed under running water to remove all cells which are not firmly bound. To visualize the adherent colonies, they are fixed and stained. To isolate the hydrophobic strains the replica might be transferred to a new, sterile agar plate. Pruthi and Cameotra[49] demonstrated the strong correlation between cell surface hydrophobicity and affinity to polystyrene. They suggest that greater than 50% coverage of the disc by adherent cells can be scored as positive. This technique is an inexpensive way to identify an array of microbial strains for biosurfactant production simultaneously on readily available materials. Furthermore, the identification and isolation of potential strains might be combined in one step.

### Salt Aggregation Assay

A salt aggregation assay for exploring the hydrophobic surface properties was first described by Lindahl et al.[53] It is similar to the "salting out" of proteins. The cells are precipitated by increasing salt concentrations. The more hydrophobic the surface of the cells, the lower the salt concentration required to aggregate the cells. So, the most hydrophobic cells precipitate first, at low salt concentrations.

For this assay, a dilution series of ammonium sulfate in sodium phosphate buffer is used, ranging from 4 M to 0.02 M ammonium sulfate. The bacterial suspension is then mixed with an equal volume of salt solution on glass depression slides. The suspension is mixed for 2 minutes at 20°C, then visual reading against black background is carried out. A positive aggregation reaction shows a clear solution and white aggregates with a diameter of appr. 0.1 mm. As positive control, all readings are compared to the reaction at the highest molarity. A bacterial suspension mixed with 0.002 M sodium phosphate without addition of salt is used as negative control.

The salt aggregation test provides a simple means for identifying bacteria associated with the production of biosurfactants. No special equipment is needed. Pruthi and Cameotra[49] showed that this technique gives a good estimation of the degree of cell surface hydrophobicity.

## *Specialities*

This last section on screening methods deals with two special screening techniques: the CTAB agar plate assay and the hemolysis assay. They are exceptional because they are not suitable to a general screening for biosurfactant producing microbes.

### CTAB Agar Plate

The CTAB agar plate method is a semi-quantitative assay for the detection of extracellular glycolipids or other anionic surfactants. It was developed by Siegmund and Wagner.[54] The microbes of interest are cultivated on a light blue mineral salts agar plate containing the cationic surfactant cetyltrimethylammonium bromide and the basic dye methylene blue. If anionic surfactants are secreted by the microbes growing on the plate, they form a dark blue, insoluble ion pair with cetyltrimethylammonium bromide and methylene blue. Thus, productive colonies are surrounded by dark blue halos (see Fig. 4).

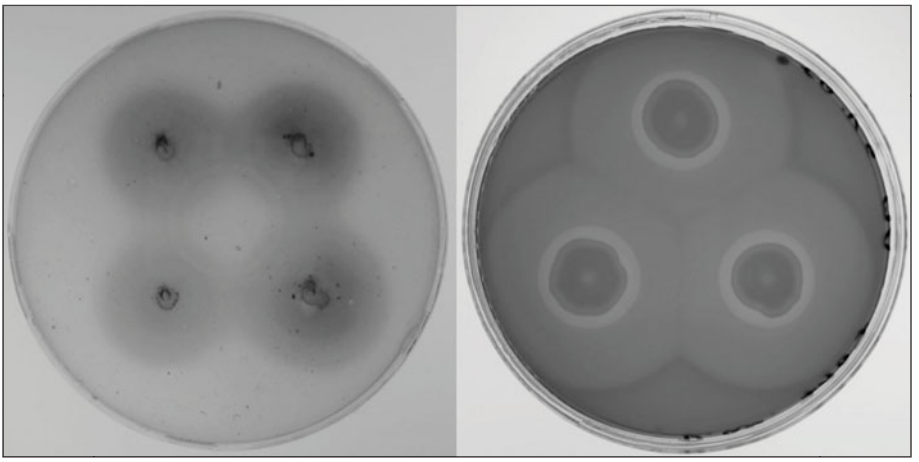

Figure 4. Left) *Pseudomonas sp.* grown on CTAB agar, dark blue halos around the 4 colonies indicate production of biosurfactant. Right) *Pseudomonas aeruginosa* grown on blood agar, lysis of erythrocytes is indicated by the lytic zones around the colonies. A color version of this image is available at www.landesbioscience.com/curie.

To strengthen the visual effect of this method, small wells can be melted into the agar surface with the heated point of a glass stick or pipette. The cultures are placed and incubated in the wells.

Even hydrophobic substrates like plant oils can be included in this test. Therefore, the oil droplets are stabilized with Gum Arabicum. Oil, agar and 1 g/L Gum Arabicum are mixed separately with ultrasound in a small volume of water. The homogenous mixture is added to the medium before sterilization.

The CTAB agar assay is a comfortable screening method, but it is specific for anionic biosurfactants. It has been applied in several screenings.[31,47,55-57] Different culture conditions can be applied directly on the agar plates, e.g., different substrates or temperature. Furthermore, it could be transferred to liquid culture conditions. The disadvantage is that CTAB is harmful and inhibits the growth of some microbes. But, as Siegmund et al[54] suggest, CTAB could be replaced by another cationic surfactant.

**Hemolysis**

Biosurfactants can cause lysis of erythrocytes. This principle is used for the hemolysis assay which was developed by Mulligan et al.[58] Cultures are inoculated on sheep blood agar plates and incubated for 2 days at 25˚C. Positive strains will cause lysis of the blood cells and exhibit a colorless, transparent ring around the colonies (see Fig. 4). Hemolysis can also be shown with purified biosurfactant.

The blood agar method is often used for a preliminary screening of microorganisms for the ability to produce biosurfactants on hydrophilic media.[6,36,44] Blood agar is a rich growth medium for many organisms. But the method has some limitations.[29] First, the method is not specific, as lytic enzymes can also lead to clearing zones. Second, hydrophobic substrates cannot be included as sole carbon source in this assay. Third, diffusion restriction of the surfactant can inhibit the formation of clearing zones. In addition, Schulz et al[6] showed that some biosurfactants do not show any hemolytic activity at all. Youssef et al[44] and Plaza et al[36] also confirmed the poor specificity of this method. It can give a lot of false negative and false positive results. Mulligan et al[58] recommend the blood agar method as a preliminary screening method which should be supported by other techniques based on surface activity measurements.

***Table 1. Comparison of the presented screening methods for biosurfactant production***

| Analytical Technique | Qualitative Analysis | Quantitative Analysis | Analysis Speed | Application in HTS |
|---|---|---|---|---|
| Direct surface/interfacial tension measurement | ++ | + | min | – |
| Drop collapse assay | ++ | – | min | + |
| Microplate assay | ++ | – | min | + |
| Penetration assay | ++ | – | min | + |
| Oil spreading assay | ++ | – | min | – |
| Emulsification capacity assay | + | – | d | – |
| Solubilization of crystalline anthracene | + | – | d | +/– |
| Bacterial adhesion to hydrocarbons assay | + | – | min | – |
| Hydrophobic interaction chromatography | + | – | h | – |
| Replica plate assay | + | – | d | – |
| Salt aggregation assay | + | – | min | +/– |
| CTAB agar assay | + | – | d | – |
| Hemolysis assay | + | – | d | – |

Qualitative analysis: ++ = very efficient, + = efficient; quantitative analysis (of surface activity): + = Yes,– = No; Analysis speed: (required time per sample) min = analysis within minutes, h = within hours, d = within days; Application in HTS: + = Yes,– = No, +/– = not reported but principally applicable.

## High Throughput Screening

The development of rapid and reliable methods for screening and selection of microbes from thousands of potentially active organisms and the subsequent evaluation of surface activity holds the key to the discovery of new biosurfactants or production strains. According to Chen et al,[4] a screening method for the isolation of biosurfactant producing microbes must fulfill three requirements:

- The ability to identify potential organisms
- The ability to assess quantitatively how effective the surfactant is
- The ability to screen many candidates quickly

The performance of the methods presented in this chapter according to these criteria is shown in Table 1.

The microplate assay, the penetration assay and the drop collapse assay can be performed in microplates. This is the basic requirement for high throughput screening. The solubilization of crystalline anthracene assay and the salt aggregation assay might as well be adopted for high throughput screening; however, this has not been reported yet. By now, there have been no other measurement principles adopted for high throughput screening.

## Conclusion and Perspectives

Interest in biosurfactants has led to the development of a multitude of methods for the screening of biosurfactant producing strains. As every method has its advantages and disadvantages, a combination of different methods is appropriate for a successful screening.

Some screening methods can be automated and used for HTS. By using these rapid screening methods and by screening many isolates or large culture collections, in the near future various new production strains or new biosurfactants may be found. Accordingly, if new production strains become available, the economic obstacle of biosurfactants may eventually be overcome.

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