Review

Biosurfactants: Recent Advances

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Abstract: Surfactants find applications in a wide variety of industrial processes. Biomolecules that are amphiphilic and partition preferentially at interfaces are classified as biosurfactants. In terms of surface activity, heat and pH stability, many biosurfactants are comparable to synthetic surfactants. Therefore, as the environmental compatibility is becoming an increasingly important factor in selecting industrial chemicals, the commercialization of biosurfactant is gaining much attention. In this paper, the general properties and functions of biosurfactants are introduced. Strategies for development of biosurfactant assay, enhanced biosurfactant production, large scale fermentation, and product recovery are discussed. Also discussed are recent advances in the genetic engineering of biosurfactant production. The potential applications of biosurfactants in industrial processes and bioremediation are presented. Finally, comments on the application of enzymes for the production of surfactants are also made.

Key words: biosurfactant, glycolipid, lipopeptide, rhamnolipid, surfactin, ELISA.

INTRODUCTION

Surfactants are amphiphilic molecules that tend to partition preferentially at the interface between fluid phases of different degrees of polarity and hydrogen bonding (such as oil/water or air/water interfaces). The formation of such an ordered molecular film at the interface lowers the interfacial energy (interfacial tension) and is responsible for the unique properties of surfactant molecules. In addition to lowering the interfacial tension, the molecular layer can also dominate the interfacial rheological behavior and mass transfer. Because of these properties, surfactants find applications in an extremely wide variety of industrial processes including emulsification for emulsion polymerization, foaming for food processing, detergency for household and industrial cleaning, wetting and phase dispersion for cosmetics and textiles, or solubilization for agrochemicals. The total sales volume of specialty surfactants in the USA in 1992 was estimated at \$1·7 billion, and was expected to rise at a rate of 3–5% annually.[1] Examples of commercially available ionic surfactants include fatty acids, ester sulfonates or sulfates (anionic) and quaternary ammonium salts (cationic).

One of the most widely used indexes for evaluating surfactant activity is the critical micelle concentration (CMC). The CMC is in effect the solubility of a surfactant within an aqueous phase or the minimum surfactant concentration required for reaching the lowest interfacial or surface tension values. At concentrations above the CMC, amphiphilic molecules associate readily to form supramolecular structures such as micelles, bilayers and vesicles. The interfacial tension between the aqueous and oleic phases changes very little above the critical micelle concentration because all additional surfactant molecules form micellar structures since the oil/water interface already has a monomolecular layer of amphiphiles. The forces that hold these structures together include hydrophobic, van der Waals', electrostatic and hydrogen bonding interactions. Since no chemical bonds are formed, these structures are fluid-like and are easily transformed from one state to another as conditions such as electrolyte concentration and temperature are changed.

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Lipids can form micelles (spherical or cylindrical) or bilayers based mainly on the area of the hydrophilic head group and the chain length of the hydrophobic tail. Molecules with small chain lengths and large head groups generally form spherical micelles. Those with smaller head groups tend to associate into cylindrical micelles, while those with long hydrophobic chains form bilayers which, in turn, under certain conditions form vesicles.[2] The formation of micelles can result in the solubilization of oil or water in the other phase, giving rise to a microemulsion. The unique properties of micelles are being explored for applications such as the extraction of proteins from fermentation broth and the removal of organics and metal ions from aqueous streams for environmental applications.[3–5]

Another parameter frequently used for predicting surfactant behavior is the hydrophilic and lipophilic balance (HLB) value. Generally, surfactants with HLB values less than 6 are more soluble in the oil phase; those with HLB values between 10 and 18 have the opposite characteristics.[6]

Many biological molecules are amphiphilic and partition preferentially at interfaces. Those compounds which exhibit particularly high surface activity are classified as biosurfactants. The physicochemical properties, such as decreases in interfacial tension, heat and pH stability, of many biosurfactants have been shown to be comparable to synthetic surfactants.[7] In addition, the chemical diversity of naturally produced amphiphiles offers a wider selection of surface active agents with properties closely tailored to specific applications. However, biosurfactants have not yet been employed extensively in industry because of technical and/or economic reasons. This is beginning to change as environmental compatibility is becoming an increasingly important factor for the selection of industrial chemicals. Unlike synthetic surfactants, microbially-produced compounds are easily biodegradable and thus particularly suited for environmental applications such as bioremediation and the dispersion of oil spills.[8–11]

This work is intended to be a general review of biosurfactants with recent progress in biosurfactant assay, biosurfactant production, and genetic engineering of biosurfactant-producing microorganisms. The advantages and disadvantages of employing microorganisms and enzymes for the production of surfactants are also compared.

2 PROPERTIES AND FUNCTIONS OF BIOSURFACTANTS

Microbial biosurfactants include a wide variety of chemical structures, such as glycolipids, lipopeptides, polysaccharide–protein complexes, phospholipids, fatty acids and neutral lipids.[7] It is, therefore, reasonable to expect diverse properties and physiological functions for different families of biosurfactants. The structures of biosurfactants have been extensively reviewed[6,7,12–15] and will not be covered in this review. It is enough to mention that most biosurfactants consist of distinct hydrophilic and hydrophobic moieties. The former can be either ionic or non-ionic and consist of mono-, di-, or polysaccharides, carboxylic acids, amino acids, or peptides. The hydrophobic moieties are usually saturated, unsaturated or hydroxylated fatty acids. For some high molecular weight surfactant molecules, such as protein–polysaccharide complexes, the hydrophobic and hydrophilic moieties are contributed by different molecules.

2.1 Properties

A comprehensive list of biosurfactants which reduce the surface tension of the fermentation broth to less than 30 mN m^{-1} and the interfacial tension against *n*-alkanes to values below 1 mN m^{-1} has been compiled.[7] The interfacial properties of surfactants depend on the ionic composition of the aqueous phase. For example, high NaCl concentrations inactivate the glycolipids of *Torulopsis apicola*. On the other hand, the interfacial tension of the fermentation broth of *Bacillus licheniformis* JF-2 decreases by more than an order of magnitude in the presence of 10% (w/v) NaCl but is not affected by calcium salts.[16,17] Interestingly, this microorganism grows and produces biosurfactant under both aerobic and anaerobic conditions and in the presence of up to 8% NaCl.[18]

The glycolipids produced by *Rhodococcus* sp. H13[19] and the biosurfactant from *Bacillus licheniformis* JF-2[16,20–22] have been shown to reduce the surface tension of aqueous solutions to 26–27 mN m^{-1} and the interfacial tension against decane or octane to 10^{-2} mN m^{-1}. These values compare favorably with those obtained with commercial synthetic surfactants.

Some biosurfactants also exhibit good thermal and chemical stability characteristics. For example, the lipopeptides from *B. licheniformis* JF-2 are stable at a temperature up to 75°C for at least 140 h.[17,23] The biosurfactant is stable at pH values between 5·5 and 12 but slowly loses activity under more acidic conditions.

2.2 Physiological functions

The physiological functions of biosurfactants are not clear. Although most biosurfactants are considered as secondary metabolites, some may play essential roles for the survival of the producing-microorganisms either through facilitating nutrient transport or microbe–host interactions, or as biocides. Almost all of these biological *in-vivo* functions are related to the amphipathic properties of the biosurfactants.

It has been suggested that the production of biosurfactants can enhance the emulsification and solu-

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bilization of hydrocarbon substrates, and therefore facilitate the growth of microorganisms on hydrocarbons. Considering the kinetics of microorganism growth, it is easy to understand the relationship between microbial growth rate and nutrient concentrations. By secreting biosurfactants into the growth medium, microorganisms relying on non-polar substrates as sole carbon sources ensure the timely supply of carbon source to maintain their survival and growth. This hypothesis is supported by the fact that some hydrocarbon-utilization microorganisms produce reduced amounts of biosurfactants when grown on water-soluble substrates.[24] It has been demonstrated that the growth of *Pseudomonas aeruginosa* on *n*-alkane could be accelerated by adding a very small amount of a growth stimulant, a rhamnolipid, into the growth medium.[25] The growth of a *P. aeruginosa* mutant, which produced a reduced amount of rhamnolipid and was deficient in utilizing *n*-paraffin,[26] on hydrocarbon was enhanced significantly with added rhamnolipid.[27] However, a mutant unable to grow on hexadecane produced twice as much rhamnolipid as the wide-type strain when grown in glucose-containing media, where the emulsification of hydrocarbon is not needed. *T apicola* also produces glycolipids, which do not stimulate its own growth on hydrocarbon.[24] The hypothesis is also contrasted by the facts that *Bacillus subtilis* produces biosurfactant only with water-soluble substrates[28] and that some mutant microorganisms produce elevated levels of biosurfactants with water-soluble substrates.[26] Some *Streptococcus thermophilus* strains were shown to produce biosurfactants as anti-adhesives with glucose as the main carbon source.[29]

Some cell-bound biosurfactants may be responsible for hydrocarbon transport and the attachment of the cells to interfaces.[30–32] This mechanism is supported by the observation that 2·5% fatty acid was isolated in the polysaccharide moiety from the cell surface of *Candida tropicola* grown on alkanes, while only a trace amount of fatty acid was detected in the corresponding polysaccharide fraction from the cells grown on glucose. This indicated that the cell-bound polysaccharide–fatty acid complex might be involved in the direct transportation of hydrocarbon substrates into the cells. Biosurfactants produced by *Serratia marcescens* presumably modulate the hydrophobicity of the cell surface, which appears to be an important factor for cell adhesion and colonization of various interfaces.[32] Anionic phospholipids are believed to play a critical role in the membrane insertion of proteins. It has been recently demonstrated that anionic phospholipids might be responsible for mediating the membrane insertion of protein toxin.[33]

Various biosurfactants, mainly lipopeptides and glycolipids, have been shown to have biocidic activities.[34,35] As mentioned above, the biocidic activities of biosurfactants may have a direct connection with their amphiphathic properties. Most of the antibiotic biosurfactants, such as rhamnolipids produced by *P. aeruginosa*[35] and surfactin produced by *B. subtilis*,[34] function as antibiotics by solubilizing the major components of cell membranes. By releasing antibiotics into the culture medium, microorganisms have a better chance of survival in an altered environment.

3 BIOSYNTHESIS AND GENETICS OF BIOSURFACTANTS

The biosynthesis and genetics of secondary metabolites, such as biosurfactants, are generally complex and not as well characterized as those of proteins for several reasons, such as the diverse structures of biosurfactants, the possible involvement of various biosynthetic pathways, and the poor understanding of microbial genetics for industrial microbes except for *B. subtilis*. Only some biosynthetic pathways involved in the synthesis of hydrophobic and hydrophilic domains of biosurfactants and a few developments in the genetics of surfactin production by *B. subtilis* have been reported.

3.1 Biosynthesis

As may be expected from the wide variety of biosurfactant structures that have been determined so far,[7] their formation involves an equally diverse range of biosynthetic pathways. For simplicity, three classes of pathways can be distinguished depending on whether the hydrophobic domain, the hydrophilic domain, or both, are synthesized *de novo*.[14] Obviously, this classification does not reflect the many different biosynthetic routes that are involved in the formation of the lipid and hydrophilic domains. Those components that are not synthesized *de novo* are produced by modification of the carbon source, i.e. sugars, alkanes, etc. Often, a variety of different carbon substrates can be incorporated into the biosurfactant, giving rise to a family of related molecules.

In lipopeptides such as herbicolin A and surfactin, both the lipid and the peptide domains are directly synthesized from carbohydrates. Addition of amino acids or fatty acids in the growth medium can affect the yield but not the structure of the product.[36] The trehalose lipids formed by *Rhodococcus erythropolis* are typical of compounds in which the hydrophilic component, in this case the disaccharide trehalose, is not affected by the carbon substrate, whereas the fatty acid domain depends on the chain length of the alkane feed.[37] In an enzymatic step that is probably rate limiting, the fatty acid (corynomycolic acid) is esterified to trehalose-6-phosphate which is subsequently subject to dephosphorylation and further modification. Finally, the surfactants produced by *Arthrobacter paraffineus* represent an example of the class of compounds in which the

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hydrophilic (sugar) moiety is influenced by the carbon source. Fructose lipids are produced when this microorganism is grown on fructose as the carbon source, whereas glucose and sucrose lipids predominate in sucrose-grown cultures.[38,39]

The fatty acid components of biosurfactants are synthesized by the rather well characterized pathways of lipid metabolism.[40] The hydrophilic moieties, on the other hand, exhibit a greater degree of structural complexity which is the outcome of a wide variety of biosynthetic mechanisms. Recent studies have begun to shed light on the formation of the amino acyl part of lipopeptide antibiotics, many of which display interesting surface active characteristics.[41,42] Lipopeptides are synthesized non-ribosomally by large multifunctional enzyme complexes exemplified by gramicidin S synthetase. The first step in the formation of the decapeptide antibiotic gramicidin S is the activation of amino acids via adenylation by ATP. The activated intermediates are attached to specific sites on the gramicidin S synthetase complex by thioether linkages. The amino acid intermediates are arranged on the enzyme in a linear fashion corresponding to the sequence with which they will be incorporated into the growing peptide. Assembly of the peptide involves a pantetheine cofactor having a reactive–SH group. The cofactor functions as an internal swinging arm to mediate the transport of the growing peptide between the sites of attachment of the activated amino acids. This mode of synthesis is called the thiotemplate mechanism. The antibiotic tyrocidine is formed by a similar process except that the tyrocidine synthetase complex consists of three rather than two enzymes.

The synthesis of the surface active lipopeptide surfactin has been investigated in detail. Genetic evidence has indicated that two putative components of the surfactin-synthesizing enzyme from the *Bacillus subtilis* complex share homology with tyrocidine synthetase 1 and gramicidin S synthetase 1.[43] Recent biochemical studies demonstrated that surfactin synthesis occurs via a thiotemplate-based process. *In-vitro*, the synthesis of surfactin by a cell free system requires ATP, Mg^{2+} precursors and sucrose, the latter presumably because of the need to stabilize the enzyme complex. Even though the peptide contains D-Leu, only the L-isomer of the amino acid can serve as a precursor. The fatty acid component is incorporated only as an acetyl-CoA derivative.[42,44]

3.2 Genetics

Although the genetic analysis of biosurfactant production is currently at an early stage, the use of recombinant DNA techniques for the manipulation of biosurfactant production is slowly gaining ground and could become instrumental in future efforts for both scientific research and commercial development. Metabolic engineering, i.e. the application of genetic engineering to improve the synthesis of non-ribosomal products,[45–49] has been exploited effectively in the antibiotics area. So far the only example of metabolic engineering for biosurfactant production is the expression of the lactose utilization genes in *Pseudomonas aeruginosa* to allow growth and rhamnolipid production on lactose or cheese whey.[50]

DNA transfer systems including shuttle vectors and transducing phages are available for many biosurfactant-producing microorganisms such as *Bacillus* and *Rhodococcus* sp. To successfully implement genetic studies for enhanced biosurfactant production, suitable plate assays for the screening of mutants is indispensable. To this end, direct colony thin-layer chromatography and blood agar plate assay, to be discussed in the next section, have been developed and successfully used to isolate mutants with the desired properties.[51,52] Similar approaches were taken to isolate null mutants of *B. subtilis* for surfactin production.[53] A Tn5 mutagenized population of *P. aeruginosa* was screened for defective growth on hydrocarbon minimal media and two variants were characterized in detail. One strain was found to be defective for biosurfactant production whereas the second exhibited a two-fold higher production when grown in minimal media with glucose as the carbon source.[27]

Recent studies on the genetics of *B. subtilis* development and surfactin production have shed light on the complexities involved in the molecular-level regulation of biosurfactant synthesis,[41,43,53–55] which has been previously reviewed[6] and therefore will not be covered in this review. Nevertheless, it is noteworthy that the *srfA* genes, required for surfactin production, have been placed under the control of an inducible promoter so that the production of surfactin is only dependent on the addition of the inducer isopropyl-β-galactoside (IPTG) in the growth medium.[43] Other recombinant microorganisms with enhanced biosurfactant production have also been constructed.[56,57] The construction of a recombinant strain, *Bacillus subtilis* MI113 with a plasmid-containing gene related to surfactin production from a wild-type surfatin producer, *B. subtilis* RB14, has been reported. Under optimal conditions, the amount of surfactin production was eight times as high as that of the wild-type strain.[57]

4 BIOSURFACTANT ASSAYS

The development of an effective biosurfactant assay is critical to the success in optimizing biosurfactant production by medium optimization and/or fermentation technology and in the selection of biosurfactant-producing microbes and/or their mutants. However, the

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lack of common reactive groups or chromophores in most biosurfactant molecules has impeded the development of universal chemical or spectral assays for biosurfactants. Recent developments in biosurfactant assays for medium optimization and strain selection will be discussed in this section.

4.1 Biosurfactant assays for optimization studies

The effective biosurfactant assays for optimization studies should have the capability of handling large amount of samples with relatively good specificity and sensitivity. So far, the most widely used methods for the detection of biosurfactants have been interfacial/surface tension measurements and thin-layer chromatography (TLC). However, these methods are inappropriate for quantitative studies for their lack of sensitivity and are also time-consuming. For example, interfacial/surface tensions of cell-free culture against organic phases are generally strongly affected by factors such as pH and ionic strength, which excludes their utility as a quantitative assay to investigate the effects of these factors on biosurfactant production. Therefore, interfacial/surface tension measurements are only good for the preliminary screening of biosurfactant-producing microbes.[58] On the other hand, although TLC can provide reasonably good resolution and semi-quantitative information provided with appropriate solvent systems, it is not appropriate for analyzing large amount of samples obtained in optimization studies, because time-consuming prepurification procedures such as precipitation and extraction are necessary for sample preparation. The determination of wetting activity by measuring the droplet diameter and contact angle have similar problems to interfacial/surface tension measurements.[59,60] Therefore, to effectively implement an optimization program, a new assay for biosurfactants must be developed.

High performance liquid chromatography (HPLC) has been widely used for the detection, quantification and purification of biomolecules. However, its application in biosurfactant analysis was not achieved until the development of a reverse phase HPLC method with a C18 column for the analysis of a biosurfactant produced by *Bacillus lichenifomis* JF-2.[61] The assay has the advantages of high specificity and sensitivity, and the capability of handling large amounts of samples. Furthermore, the amount of sample required for accurate analysis is small, without the need for tedious prepurification. This assay was successfully used to monitor the unique biosurfactant production profile, to be discussed later, which eventually led to the development of a procedure for the continuous production of *Bacillus licheniformis* biosurfactant.[62]

The specific interactions between biomolecules, such as enzymes and substrates or substrate analogs and antigens and antibodies, have been extensively utilized for the development of novel purification and diagnostic techniques, such as affinity chromatography and enyzme-linked immunosorbent assay (ELISA). An ELISA procedure for lipopeptide biosurfactant by *Bacillus licheniformis* JF-2 was reported recently.[22] Such a biosurfactant ELISA is extremely specific and sensitive (at least as low as 0·01 mg dm^{-3}) and capable of handling large numbers of samples simultaneously. However, this approach may not be applied to other types of biosurfactants, because not all biosurfactants are immunogenic even upon conjugation with carrier proteins such as bovine serum albumin (BSA) or keyhole limpet hemocyanin (KLH).

4.2 Biosurfactant assays for strain selection

An efficient screening strategy is the key to the success in isolating the desired microbes or their variants, because a large number of strains need to be characterized. The *ex-situ* biosurfactant assays described above are not appropriate for this purpose. Fortunately, some *in-situ* techniques utilizing the physiological and chemical properties of biosurfactants have been developed.

A modified version of TLC with bacterial colonies for screening biosurfactant-producing variants has been reported.[51] Instead of spending days for TLC sample preparation, this technique involves the direct application of bacterial mass on a TLC plate and predevelopment of the plate. The plate containing the bacterial extracts was subsequently developed following the removal of adhering bacterial mass and drying. This assay was employed successfully to identify and isolate the bacteria variants defective in biosurfactant production. However, this direct colony thin-layer chromatography may not be applied to microbes producing low levels of biosurfactant, because of its low sensitivity.

Some biosurfactants possess antibiotic activity or hemolytic activity probably because of their amphiphilic properties. These activities have been utilized for the development of *in-situ* biosurfactant assays. For example, surfactin, a lipopeptide biosurfactant produced by *B. subtilis*, can rupture erythrocytes, although it is not a hemolytic enzyme *per se*.[34] This hemolytic activity has enabled the development of a blood agar assay for surfactin.[63] The sizes or diameters of colorless hemolytic zones around the colonies correspond well to the ability of the microbes in biosurfactant production. This assay was successfully employed to select for microbes capable of producing biosurfactants in media without hydrocarbons and later to isolate *Bacillus subtilis* mutants with enhanced biosurfactant productivity.[52] This approach can be expanded to develop *in-situ* assays for biosurfactants with antibiotic activity such as the lipopeptide biosurfactant by *Bacillus licheniformis*.[64,65] However, it is important to keep in mind

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