Chapter 4

Microbial Surfactants and Their Potential Applications: An Overview

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Abstract

Biosurfactant or microbial surfactants produced by microbes are structurally diverse and heterogeneous groups of surface-active amphipathic molecules. They are capable of reducing surface and interfacial tension and have a wide range of industrial and environmental applications. The present chapter reviews the biochemical properties of different classes of microbial surfactants and their potential application in different industrial sectors.

Introduction

Surfactants are amphipathic molecules that partition preferentially at the interface between fluid phases such as oil/water or air/water interfaces. These properties of surfactants capable them of reducing surface and interfacial tension and make surfactant an excellent detergency, emulsifier, foaming and dispersing agents.

With increasing environmental awareness and emphasis on a sustainable society in harmony with the global environment, during the recent years, natural surfactants produced by living cells are getting much more attention as compared to the synthetic chemical surfactants. Among the natural surfactants, those produced by microbial origin, known as microbial surfactants or biosurfactants are the most promising. They are defined as "structurally diverse/heterogeneous groups of surface-active molecules synthesized by microorganisms".[1,2] Considering the important properties and a wide range of applications of biosurfactants, during recent years much more attention has been given to understand the biochemical properties and physiological role of different classes of biosurfactant on the producing microorganism as well as commercial application of biosurfactants.[3]

Classification of Biosurfactants

Based on their chemical composition and types of microbes producing them, biosurfactant are divided into five broad groups viz., glycolipids, lipopeptides and lipoproteins, phospholipids, hydroxylated and crossed-linked and fatty acids, polymeric surfactants and particulate surfactants.[4,5]

Glycolipids

Glycolipids are carbohydrates like mono-, di-, tri- and tetrasaccharides that include glucose, mannose, galactose, glucuronic acid, rhamnose and galactose sulphate combined with long chain aliphatic acids or hydroxy aliphatic acids. The best examples of glycolipids include trehalose lipids,

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rhamnolipids, sophorolipids, diglycosyl diglycerides and mannosylerythritol lipids. Other types of glycolipids have been reported in the literature such as glycoglycerolipid,[6] sugar-based bioemulsifiers,[7,8] mannosylerythritol lipid A and many different hexose lipids.[9]

Trehalose Lipids

Several structural types of microbial trehalose lipid biosurfactants have been reported. Disaccharide trehalose linked at C-6 and C-6′ to mycolic acids is associated with most species of *Mycobacterium*, *Nocardia* and *Corynebacterium*.[4,10] Mycolic acids are long-chain, α-branched -β- hydroxy fatty acids. Trehalolipids from different organisms differ in the size and structure of mycolic acid, the number of carbon atoms and the degree of unsaturation.[4,11] In 2002, Philp and his colleagues[12] reported the production of trehalose lipids from alkanotrophic *Rhodococcus ruber* on gaseous alkanes propane and butane.

Rhamnolipids

Certain species of *Pseudomonas* are characterized to produce large amounts biosurfactant containing one or two molecules of rhamnose linked to one or two molecules of β-hydroxydecanoic acid.[13-16] In 1965, Edward and Hayashi[17] have reported formation of glycolipid, type R-1 containing two rhamnose and two β hydroxydecanoic units by *Pseudomonas aeruginosa*. A second kind of rhamnolipid (R-2) containing one rhamnose unit was reported by Itoh et al.[18] Gas-chromatographic analysis of hydroxyl fatty acids rhamnolipid produced by *P. aeruginosa* DAUPE 614 showed that positions of the fatty acids in the lipid moiety were variable.[16]

Sophorolipids

Sophorolipids consist of a dimeric carbohydrate sophorose attached with a long chain hydroxy fatty acid and are mainly produced by yeasts such as *Torulopsis bombicola, T. apicola*[19] and *Wickerhamiella domericqiae*.[20] Sophorolipids have the capacity to lower the surface tension of water from 72.8 mN/m to 40 to 30 mN/m, with a critical micelle concentration of 40 to 100 mg/l.[21] It has been shown that *T. petrophilum* produces sophorolipids on water insoluble substrates such as alkanes and vegetable oil.[22] Moreover, it has been reported that critical micelle concentration (CMC) and the solubilization ratio of the sophorolipids biosurfactant were found to be in a good range compared with synthetic surfactants.[23]

Mannosylerythritol Lipids

This glycolipid biosurfactant consists of a sugar called mannosylerythritol and are synthesized by yeast like *Candida antarctica*[24,25] and *Candida* sp. SY 16.[26] The fatty acid component of biosurfactant was determined to be hexanoic, dodecanoic, tetradecanoic or tetradecenoic acids.[26] Mannosylerythritol lipids synthesized by *Candida* sp. SY 16[26] lowered the surface tension of water to 29 dyne/cm at critical micelle concentration of 10 mg/l and the minimum interfacial tension was 0.1 dyne/cm against kerosene.[26] Fukuoka et al[27] have characterized the surface active properties of a new glycolipid biosurfactant, mono acylated mannosylerythritol lipid produced by *Psdozyma antarctica* and *P. rugulosa.*

Lipopeptides

Surfactin

Surfactin, a cyclic lipopeptide is one of the most effective biosurfactants known so far, which was first reported in *B. subtilis* ATCC-21332.[28] Because of its exceptional surfactant activity it is named as surfactin.[29] Surfactin can lower the surface tension from 72 to 27.9 mN/m[30] and have a critical micelle concentration of 0.017 g/l.[31] The surfactin groups of compounds are shown to be a cyclic lipoheptapeptides which contain a β-hydroxy fatty acid in its side chain.[32] Recent studies indicate that surfactin shows potent antiviral, antimycoplasma, antitumoral, anticoagulant activities as well as inhibitors of enzymes.[30,33] Although, such properties of surfactins qualify them for potential applications in medicine or biotechnology, they have not been exploited extensively till date.

Iturin

Iturin A, the first compound discovered of the iturin group and its best known member, was isolated from a *Bacillus subtilis* strain taken from the soil in Ituri (Zaire) and its structure was elucidated.[34] The subsequent isolation from other strains of *Bacillus subtilis* of five other lipopeptides such as iturin A_L, mycosubtilin, bacillomycin L, D, F and L_C (or bacillopeptin), all having a common pattern of chemical constitution, led to the adoption of the generic name of "iturins" for this group of lipopeptides.[35] The iturin group of compounds are cyclic lipoheptapeptides which contain a β- amino fatty acid in its side chain. Lipopeptids belonging to the iturin family are potent antifungal agents which can also be used as biopesticides for plant protection.[32,36,37]

Fengycin

Fengycin is a lipodecapeptide containing β- hydroxy fatty acid in its side chain and comprises of C_{15} to C_{17} variants which have a characteristic Ala-Val dimorphy at position 6 in the peptide ring.[32,38] Wang et al[39] have demonstrated the identification of fengycin homologues produced by *B. subtilis* by using electrospray ionization mass spectrometry (ESI-MS) technique.

Lichenysin

Lichenysin, produced by *Bacillus licheniformis* exhibits similar structure and physiochemical properties to that of surfactin.[40] *B. licheniformis* also produce several other surface active agents which act synergistically and exhibit excellent temperature, pH and salt stability.[40] Lichenysin A produced by *Bacillus licheniformis* strain BAS50, is characterized to contain a long chain beta-hydroxy fatty acid molecule.[41] Lichenysin is reported to be stable over a wide range of pH, temperature and NaCl concentration and promotes dispersion of colloidal 3- silicon carbide and aluminum nitride slurries much more efficiently than chemical agents.[42] It has also been reported that lichenysin is a more efficient cation chelator compared with surfactin.[43]

Fatty Acid Biosurfactant

Certain hydrocarbon degrading microbes produce extracellular free fatty acids when grown on alkanes and exhibit good surfactant activity. The fatty acid biosurfactants are saturated fatty acids in the range of C_{12} to C_{14} and complex fatty acids containing hydroxyl groups and alkyl branches.[44,45] It was shown that *Arthobacter* strain AK-19[46] and *P. aeruginosa* 44T1[47] accumulated up to 40-80% (w/w) of such lipids when cultivated on hexadecane and olive oil respectively.

Polymeric Biosurfactants

Polymeric biosurfactants are high molecular weight biopolymers, which exhibit properties like high viscosity, tensile strength and resistance to shear. The following are the examples of different classes of polymeric biosurfactants.

Emulsan

Acinetobacter calcoaceticus RAG-1 produces a potent extracellular polymeric bioemulsifier called emulsan[48] which is characterized as a polyanionic amphipathic heteropolysaccharide. The heteropolysaccharide backbone consists of repeating units of trisaccharide of N- acetyl-D-galactosamine, N-acetylgalactosamine uronic acid and an unidentified N- acetylamino sugar.[49] Removal of the protein fraction yields a product, apoemulsan, which exhibits much lower emulsifying activity on hydrophobic substrates such as n-hexadecane. One of the key proteins associated with the emulsan complex is a cell surface esterase.[50]

Biodispersan

A. calcoaceticus A-2 produces an extracellular, nondialyzable surface-active dispersing substance called biodispersan.[51] The surface active component of biodispersan is an anionic heteropolysaccharide, with an average molecular weight of 51,400 and contains four reducing sugars namely glucosamine, 6- methylaminohexose, galactosamine uronic acids and an unidentified amino sugar.[51] Elkeles and his colleagues[52] have suggested that mutants of strain *A. calcoaceticus* A-2 that were defective in protein secretion are potentially useful for the production of biodispersan.

Alasan

Alasan is an anionic alanine- containing heteropolysaccharide protein biosurfactant produced by *A. radioresistens* KA-53.[53] Alasan produced by *A. radioresistens* KA-53 was reported to solubilise and degrade polyaromatic hydrocarbons.[54] The surface active component of alasan is a 35.77 kD protein called as AlnA. This surface-active protein AlnA have a high amino acid sequence homology to *Escherichia coli* outer membrane protein A (OmpA), but however OmpA does not possess any emulsifying activity.[55]

Three of alasan proteins were purified from *A. radioresistens* KA-53 are having molecular masses of 16, 31 and 45 kD and it was demonstrated that the 45-kD protein had the highest specific emulsifying activity, 11% higher than the intact alasan complex.[56] The 16- and 31-kD proteins gave relatively low emulsifying activities, but they were significantly higher than that of apo-alasan.[56]

Liposan

C. lipolytica produce an extracellular water soluble emulsifier called Liposan which is composed of 83% (w/v) carbohydrate and 17% (w/v) protein.[57] The carbohydrate portion is a heteropolysaccharide consisting of glucose, galactose, galactosamine and galacturonic acid.[57]

Emulsifying Biopolymer from Fungus

The production of large amounts of mannoprotein by *Saccharomyces cerevisiae* exhibiting excellent emulsifier activity toward several oils, alkanes and organic solvents had been reported.[58] The purified emulsifier contains 44% mannose and 17% protein. A manose- fatty acid complex from alkane grown *C. tropicalis* was isolated.[59] This complex stabilizes hexadecane-in-water emulsion.

Emulsifying Protein

An emulsifying peptidoglycolipid containing 52 amino acids, 11 fatty acids and a sugar unit produced by *P. aeruginosa* P-20 has been reported by Koronelli et al.[60] Also, a bioemulsifier, composed of 50% carbohydrate, 19.6% protein and 10% lipid produced by *P. fluorescens* was reported.[61]

Particulate Biosurfactant

Some examples of particulate biosurfactant are extracellular membranes vesicles of microbial cells, which help in emulsification of hydrocarbon. Accumulation of extracellular membrane vesicles having 20-50 mm diameter and a bouyant density of 1.158 g/cm^2 has been reported in *Acinetobacter* sp. HO1-N cells.[62] The purified vesicles are composed of protein, phospholipid and lipopolysaccharide.

Potential Applications of Biosurfactant

Biosurfactants are becoming important biotechnology products for industrial and medical applications due to their specific modes of action, low toxicity, relative ease of preparation and widespread applicability.[63-65] Biosurfactants also exhibit natural physiological roles in increasing bioavailability of hydrophobic molecules and can complex with heavy metals, promoting improved degradation of chemical contaminants.[66] They can be used as emulsifiers, de-emulsifiers, wetting and foaming agents, functional food ingredients and as detergents in petroleum, petrochemicals, environmental management, agrochemicals, foods and beverages, cosmetics and pharmaceuticals, commercial laundry detergents and in the mining and metallurgical industries.[67-71]

Role of Microbial Surfactants in Bioremediation of Oil Pollutants

Oil-contamination of soil is a common problem and its physical treatment methods or remediation techniques can be difficult or economically not feasible. One of the most economically feasible methods includes in situ bioremediation by the use of microorganisms which is the partial simplification or complete destruction of the molecular structure of environmental pollutants.[64,70-72] Permeability of the microbial cell membrane might be adversely affected by the use of synthetic surfactant, which would interfere with the capacity of a microorganisms to biodegrade.[73] Microbial surfactants are generally much less toxic than chemical surfactants, but are as effective and more readily biodegradable. Using microorganisms that produce their own biosurfactants capable of degrading pollutants can further lower treatment costs.

Numerous attempts have been made to successfully remediate the oil contaminated soil by using microbial inoculation and by biosurfactant treatment. The rhamnolipid biosurfactant produced by *P. aeruginosa* stimulates the uptake of hydrophobic compounds finally leading to its degradation.[74] Similarly, Das and Mukherjee[71] have demonstrated the crude petroleum-oil biodegradation efficiency of biosurfactant producing *B. subtilis* DM-04 and *P. aeruginosa* M and NM strains isolated from the petroleum oil contaminated soil from North-East India. Study has shown that all the three bacteria are efficient biosurfactant producers in petroleum oil-contaminated soil which offers the advantage of a continuous supply of natural, nontoxic and biodegradable biosurfactants by bacteria at low cost for solubilizing the hydrophobic oil hydrocarbons prior to biodegradation. In an another study, it was shown that the biosurfactant secreted by the *B. subtilis* and *P. aeruginosa* strains enhanced the apparent solubility of pyrene (a toxic polyaromatic hydrocarbon) by factors 5 to 7, and also influenced the bacterial cell surface hydrophobicity resulting in higher uptake and utilization of pyrene by bacteria.[70]

Application of Biosurfactant in Petroleum Industry

Biosurfactant in Oil Clean Up of Storage Tanks

Due to excellent emulsifying properties of biosurfactants, they are used as detergents in cleaning up hydrocarbon/crude oil storage tank. Banat et al[75] reported the ability of biosurfactants produced by a bacterial strain (Pet 1006) for cleaning up oil storage tanks and to recover hydrocarbons from emulsified sludge. In a test for cleaning up of oil storage tank, about 91% crude oil could be recovered from the total sludge. Such clean up process is highly desirable as it is economically rewarding and environmentally friendly.[76]

Microbial Surfactants in Microbial Enhanced Oil Recovery (MEOR)

Approximately 30% of the oil present in a reservoir can be recovered using current enhanced oil recovery (EOR) technology.[77] The low permeability or the high viscosity of the oil, as well as, high interfacial tensions between the water and oil may also result in high capillary forces retaining the oil in the reservoir rock leading to poor recovery of oil.[64,78] Due to failure of primary and secondary recovery techniques to recover the oil from reservoirs, interests have evolved in tertiary recovery techniques (MEOR) by utilizing microorganisms and/or their biosurfactant.[79]

There are several strategies involving the use of biosurfactant in MEOR. The first strategy involves injection of biosurfactant-producing microorganisms into a reservoir through the well, with subsequent in-situ propagation of microbes through the reservoir rock.[80] The second strategy involves the injection of selected nutrients into a reservoir, to favor and encourage the growth of indigenous biosurfactant-producing microorganisms. The third mechanism involves the production of biosurfactants in bioreactors ex situ and subsequent injection into the reservoir.

Laboratory studies on MEOR usually utilize core samples and columns containing the desired substrate. Fermentative culture broth containing biosurfactant from *Rhodococcus* ST-5[81] and the thermophilic *Bacillus* AB-2[82] could release 80% and 95% oil from sand-pack columns respectively. Studies from our lab have shown that biosurfactant from the *B. subtilis* strains can release appreciable amount of crude kerosene oil from sand pack column reinforcing it's potential application in MEOR.[83]

Field studies involving MEOR increases the production of oil by 250% using *Clostridium acetobutylicum*.[84] MEOR investigations in carbonate reservoirs showed an increase of 60-120% in oil production in Hungary.[85] Recently it has been demonstrated that biosurfactant produced by *Bacillus* strains inside a limestone petroleum reservoir may be promising candidates for MEOR.[86]

Use of Biosurfactants in Food Industries

Biosurfactants have several applications in food industries such as to control the agglomeration of fat globules, stabilize aerated systems, improve texture and shelf-life of starch-containing products, modify rheological properties of wheat dough and improve consistency and texture of fat-based products.[63,65,87] In the food industry, biosurfactants are used as emulsifiers in the processing of raw materials whereas in bakery and meat products they influence the rheological characteristics of flour or the emulsification of partially broken fat tissue.[88] An improvement of dough stability, texture, volume and conservation of bakery products was obtained by the addition of rhamnolipid surfactants.[89] Recently, a bioemulsifier isolated from a marine strain of *Enterobacter cloaceae* was used as a potential viscosity enhancement agent of interest in food industry especially due to the good viscosity observed at acidic pH allowing its use in food products containing edible acids like citric acid or ascorbic acid.[90]

Use of Biosurfactants in Agricultural Sectors

Surface active compounds like polymeric fatty acids, or short- chained alkyl sulfonates are used in agricultural sector for hydrophilization of heavy soil. Good wettability and equal distribution are the preconditions for loosening the soil. Hydrate formation between emulsifiers and water helps in soil improvement.[87]

Biodegradation of the chlorinated pesticide α- and β-endosulfan by using the biosurfactant from *B. subtilis* MTCC2423 was reported by Banat et al.[91] The use of biosurfactant leads to around 40% biodegradation of the said pesticides.[91] This furnishes another example of the role of microbial surfactant in environment protection.

The rhamnolipid biosurfactant, mostly produced by the genus *Pseudomonas* is known to possess potent antimicrobial activity.[92] For example, Zonix^{TM} biogungicide, which is a trade product of mixture of two rhamnolipid biosurfactants (known as technical grade active ingredient-TGAI) has been claimed as biofungicide to prevent and control pathogenic fungi on horticultural and agricultural crops. Further, no adverse effects on humans or the environment are anticipated from aggregate exposure to rhamnolipid biosurfactants. Fengycins are also reported to possess antifungal activity and, therefore may be employed in biocontrol of plant diseases.[93,94]

Application of Biosurfactant as a Substitute of Synthetic Chemical Surfactant in Commercial Laundry Detergents

Almost all surfactants, an important component used in modern day commercial laundry detergents, are chemically synthesized and exert toxicity to fresh water living organisms. Furthermore, these components often produce undesirable effects. Therefore, growing public disquiet about the environmental hazards and risks associated with chemical surfactants has stimulated the search for ecofriendly, natural substitutes of chemical surfactants in laundry detergents.

A recent study from our laboratory has shown that cyclic lipopeptide (CLP) biosurfactants produced by *B. subtilis* strains were stable over a pH range of 7.0-12.0 and heating them at 80°C for 60 min did not result in any loss of their surface-active property.[69] Crude CLP biosurfactants showed good emulsion formation capability with vegetable oils and demonstrated excellent compatibility and stability with commercial laundry detergents favoring their inclusion in laundry detergents formulations.[69]

Biosurfactant as Biopesticide

Conventional arthropod control strategy involves application of broad-spectrum chemicals and pesticides, which often produce undesirable effects. Further, emergence of pesticide resistant insect populations as well as rising prices of new chemical pesticides have stimulated the search for new eco-friendly vector control tools. Eventually, biocontrol of insect pests and vectors is becoming one of the most promising alternatives to chemical pesticides. Studies have shown that the lipopeptide biosurfactant produced by *B. subtilis* exhibit insecticide activity against fruit fly *Drosophila melanogaster*.[95]

Since mosquitoes continue to pose a serious public health problem throughout the world, therefore, the mosquito larvicidal potency of cyclic lipopeptides (CLPs) secreted by two *B. subtilis* strains were determined in our laboratory.[68] LC_{50} of the crude CLPs secreted by *B. subtilis* DM-03 and DM-04 strains against 3rd instar larvae of *Culex quinquefasciatus* was 120.0 ± 5.0 mg/l and 300.0 ± 8.0 mg/l respectively post 24 h of treatment. Physico-chemical factors such as pH of water, incubation temperature, heating and exposure to sunlight did not influence the larvicidal potency of these CLPs.[68] Further, *B. subtilis* CLPs were insensitive to UV or sunlight exposure demonstrating the greater UV radiation stability of *B. subtilis* lipopeptides as compared to bactoculicide (Bti) and *B. sphaericus* insecticidal toxins. Moreover, the crude CLPs secreted by *B. subtilis* strain can withstand many environmental stresses like extreme pH, sunlight/UV radiation etc. and they did not impart toxicity to the tested aquatic vertebrate *Labeo rohita* up to a concentration that induced mortality to the mosquito larvae.[68] These properties can be exploited for the formulation of a safer, novel biopesticide for effective control of mosquito larvae.

Use of Biosurfactants in Pharmaceutical Sectors and Molecular Biology Research

Rhamnolipids produced by *P. aeruginosa*,[67] lipopeptides produced by *B. subtilis*[30,96] and *B. licheniformis*[97] and mannosylerythritol lipids from *C. antarctica*[25] have been reported to have antimicrobial activities. Rhamnolipid biosurfactant produced by *P. aeruginosa* was recently reported to have potential algicidal activity against some harmful algae.[98] Surfactin was reported to have properties like hemolysis and inhibiting fibrin clot formation that indicates its potential use in the pharmaceutical sector.[33] Iturin produced by *B. subtilis* was reported to have antifungal properties.[33,99] Pumilacidin, a surfactin analog was reported to have inhibitory effect against herpes simplex virus 1 (HSV-1), H^+, K^+- Atpase and gastic ulcer in vivo. Itokawa et al[100] reported the potential application of surfactin against human immunodeficiency virus 1(HIV-1) showing this class of biosurfactant is a deserving candidate for the development of rational anti-HIV drug. Takizawa et al[101] reported significant stimulation of the proliferation of bone marrow cells from BALB/c female mice by lipopeptide biosurfactant produced by *S. amethystogenes*. The reports on antibiotic effects and inhibition of HIV virus growth in white blood corpuscles have opened up new arena in the potential application of these microbial surface active compounds in pharmaceutical sector.[33,100]

Gene transfection is a fundamental technology for molecular and cell biology and also clinical gene therapy. Recently it was found that a biosurfactant, monnosylerythritol lipid (MEL)-A, dramatically increased the efficiency in transfection of plasmid DNA mediated by cationic liposomes.[102]

Conclusion

During the recent years there is an increasing environmental awareness and therefore, it might be reasonable to assume that microbial surfactants have a promising role to play in the years to come. Considering the importance of biosurfactants, there is an urgent need to gain a greater understanding of the physiology, genetics and biochemistry of biosurfactant-producing strains and to improve the process technology to reduce production costs for commercial level production of biosurfactant. Therefore, an extensive cooperation among different science disciplines is needed in order to fully characterize the biochemical properties of biosurfactant and exploration of their potential applications in different industrial sectors.

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