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Microbial Production of Surfactants and Their Commercial Potential

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"By which one sees an unperishable entity in all beings
and undivided among the divided then that knowledge is pure.
But if one merely sees the diversity of things with their divisions and limitations, without the truth, then that knowledge is merely an ignorance."

The Bhagavad Gita, chapter XVIII

INTRODUCTION

Surfactants are amphipathic molecules with both hydrophilic and hydrophobic (generally hydrocarbon) moieties that partition preferentially at the interface between fluid phases with different degrees of polarity and hydrogen bonding such as oil/water or air/water interfaces. These properties render surfactants capable of reducing surface and interfacial tension and forming microemulsion where hydrocarbons can solubilize in water or where water can solubilize in hydrocarbons. Such characteristics confer excellent detergency, emulsifying, foaming, and dispersing traits, which makes surfactants some of the most versatile process chemicals (71, 72).

Current worldwide surfactant markets are around $9.4 billion per annum (226), and their demand is expected to increase at a rate of 35% toward the end of the century (71). Almost all surfactants currently in use are chemically derived from petroleum; however, interest in microbial surfactants has been steadily increasing in recent years due to their diversity, environmentally friendly nature, the possibility of their production through fermentation, and their potential applications in the environmental protection, crude oil recovery, health care, and food-processing industries (10, 11, 60, 118, 155, 257).

Biosurfactants are a structurally diverse group of surface-active molecules synthesized by microorganisms. These molecules reduce surface and interfacial tensions in both aqueous solutions and hydrocarbon mixtures, which makes them potential candidates for enhancing oil recovery (219, 227, 234) and

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deemulsification processes (28). Biosurfactants have several advantages over the chemical surfactants, such as lower toxicity; higher biodegradability (266); better environmental compatibility (65); higher foaming (203); high selectivity and specific activity at extreme temperatures, pH, and salinity (126, 257); and the ability to be synthesized from renewable feedstocks. Earlier work on biosurfactants centered mainly on the properties, biosynthesis, and chemistry and has been reviewed by many workers (34, 49, 50, 209a, 244). However, in the last few years, significant work on the fermentative production, genetics, and commercial applications of biosurfactants has been done; this, along with a brief account on the recent developments in microbial screening for biosurfactants, forms the subject matter of the present review.

RECENT ANALYTICAL METHODS

Screening of Potential Biosurfactant-Producing Microorganisms

Recent advances in the field of microbial surfactants are largely attributed to the development of quick, reliable methods for screening biosurfactant-producing microbes and assessing their potential. Van der Vegt et al. (254) developed an axisymmetric drop shape analysis (ADSA) by profile for the assessment of potential biosurfactant-producing bacteria. In this technique, drops of culture broth are placed on a fluoroethylene-propylene surface and the profile of the droplet is determined with a contour monitor. Surface tensions are calculated from the droplet profiles by ADSA. Only biosurfactant-producing bacterial suspensions show reduction in surface tensions. Shulga et al. (231) described a colorimetric estimation of biosurfactants based on the ability of the anionic surfactants to react with the cationic indicator to form a colored complex. Development of other simple methods include the following: (i) a rapid drop-collapsing test (105), in which a drop of a cell suspension is placed on an oil-coated surface, and drops containing biosurfactants collapse whereas non-surfactant-containing drops remain stable; (ii) a direct thin-layer chromatographic technique for rapid characterization of biosurfactant-producing bacterial colonies as described by Matsuyama et al. (143); (iii) colorimetric methods described by Siegmund and Wagner (232) and Hansen et al. (79) for screening of rhamnolipid-producing and hydrocarbon-degrading bacteria, respectively; and (iv) estimation of the emulsification index value (*E*-24) by vigorously shaking culture broth samples with an equal volume of kerosene and measuring the percent emulsification after 24 h by the method of Cooper and Goldenberg (35), which is most suitable for emulsifying biosurfactants.

Estimation of Biosurfactant Activity

Biosurfactant activities can be determined by measuring the changes in surface and interfacial tensions, stabilization or destabilization of emulsions, and hydrophilic-lipophilic balance (HLB). Surface tension at the air/water and oil/water interfaces can easily be measured with a tensiometer. The surface tension of distilled water is 72 mN/m, and addition of surfactant lowers this value to 30 mN/m. When a surfactant is added to air/water or oil/water systems at increasing concentrations, a reduction of surface tension is observed up to a critical level, above which amphiphilic molecules associate readily to form supramolecular structures like micelles, bilayers, and vesicle. This value is known as the critical micelle concentration (CMC). CMC is defined by the solubility of a surfactant within an aqueous phase and is commonly used to measure the efficiency of a surfactant. Microbial culture broth or biosurfactants are diluted severalfold, surface tension is measured for each dilution, and the CMC is calculated from this value. The values of the surface tension, interfacial tension, and CMC of some known biosurfactants are listed in Table 1.

An emulsion is formed when one liquid phase is dispersed as microscopic droplets in another liquid continuous phase. Biosurfactants may stabilize (emulsifiers) or destabilize (deemulsifiers) the emulsion. The emulsification activity is assayed by the ability of the surfactant to generate turbidity due to suspended hydrocarbons such as a hexadecane–2-methylnaphthalene mixture (47, 210) or kerosene (35), etc., in an aqueous assay system. The deemulsification activity is derived by determining the effect of surfactants on a standard emulsion by using a synthetic surfactant (209a, 266).

The HLB value indicates whether a surfactant will promote water-in-oil or oil-in-water emulsion by comparing it with surfactants with known HLB values and properties. The HLB scale can be constructed by assigning a value of 1 for oleic acid and a value of 20 for sodium oleate and using a range of mixtures of these two components in different proportions to obtain the intermediate values. Emulsifiers with HLB values less than 6 favor stabilization of water-in-oil emulsification, whereas emulsifiers with HLB values between 10 and 18 have the opposite effect and favor oil-in-water emulsification.

BIOSURFACTANT CLASSIFICATION AND THEIR MICROBIAL ORIGIN

Unlike chemically synthesized surfactants, which are classified according to the nature of their polar grouping, biosurfactants are categorized mainly by their chemical composition and their microbial origin. In general, their structure includes a hydrophilic moiety consisting of amino acids or peptides anions or cations; mono-, di-, or polysaccharides; and a hydrophobic moiety consisting of unsaturated, saturated, or fatty acids. Accordingly, the major classes of biosurfactants include glycolipids, lipopeptides and lipoproteins, phospholipids and fatty acids, polymeric surfactants, and particulate surfactants.

Although there are a number of reports on the synthesis of biosurfactants by hydrocarbon-degrading microorganisms, some biosurfactants have been reported to be produced on water-soluble compounds such as glucose, sucrose, glycerol, or ethanol (35, 74, 92, 184, 186). The biosurfactant-producing microbes are distributed among a wide variety of genera. The major types of biosurfactants, with their properties and microbial species of origin, are listed in Table 1 and are described briefly in the following section. For more details, readers are referred to Desai and Desai (50), Rosenberg (209a), Kosaric et al. (124), and Banat (11).

Glycolipids

Most known biosurfactants are glycolipids. They are carbohydrates in combination with long-chain aliphatic acids or hydroxyaliphatic acids. Among the glycolipids, the best known are rhamnolipids, trehalolipids, and sophorolipids.

Rhamnolipids. Rhamnolipids, in which one or two molecules of rhamnose are linked to one or two molecules of β-hydroxydecanoic acid, are the best-studied glycolipids. Production of rhamnose-containing glycolipids was first described in *Pseudomonas aeruginosa* by Jarvis and Johnson (108). L-Rhamnosyl-L-rhamnosyl-β-hydroxydecanoyl-β-hydroxydecanoate (Fig. 1A) and L-rhamnosyl-β-hydroxydecanoyl-β-hydroxydecanoate,

TABLE 1. Microbial source and properties of important types of microbial surfactants

Biosurfactant	Organisms	Surface tension (mN/m)	CMC	Interfacial tension (mN/m)	Reference(s)
Glycolipids					
Rhamnolipids	*P. aeruginosa*	29		0.25	74, 208
	Pseudomonas sp.	25–30	0.1–10	1	88, 128, 185
Trehalolipids	*R. erythropolis*	32–36	4	14–17	200
	N. erythropolis	30	20	3.5	140, 142
	Mycobacterium sp.	38	0.3	15	40
Sophorolipids	*T. bombicola*	33		1.8	40, 68
	T. apicola	30		0.9	93, 250
	T. petrophilum				38
Cellobiolipids	*U. zeae*, *U. maydis*				24, 242
Lipopeptides and lipoproteins					
Peptide-lipid	*B. licheniformis*	27	12–20	0.1–0.3	109, 263
Serrawettin	*S. marcescens*	28–33			143
Viscosin	*P. fluorescens*	26.5	150		176
Surfactin	*B. subtilis*	27–32	23–160	1	3, 20
Subtilisin	*B. subtilis*				20
Gramicidins	*B. brevis*				139
Polymyxins	*B. polymyxa*				240
Fatty acids, neutral lipids, and phospholipids					
Fatty acids	*C. lepus*	30	150	2	40, 43
Neutral lipids	*N. erythropolis*	32		3	136
Phospholipids	*T. thiooxidans*				17, 121
Polymeric surfactants					
Emulsan	*A. calcoaceticus*				210, 270
Biodispersan	*A. calcoaceticus*				211, 213
Mannan-lipid-protein	*C. tropicalis*				112
Liposan	*C. lipolytica*				32, 33
Carbohydrate-protein-lipid	*P. fluorescens*	27	10		47, 189
	D. polymorphis				236
Protein PA	*P. aeruginosa*				87, 89
Particulate biosurfactants					
Vesicles and fimbriae	*A. calcoaceticus*				76, 113
Whole cells	Variety of bacteria				58, 209a

referred to as rhamnolipid 1 and 2, respectively, are the principal glycolipids produced by *P. aeruginosa* (57, 88, 102, 103). The formation of rhamnolipid types 3 and 4 containing one β-hydroxydecanoic acid with one and two rhamnose units, respectively (242), methyl ester derivatives of rhamnolipids 1 and 2 (86), and rhamnolipids with alternative fatty acid chains (128, 185, 206) has also been reported. Rhamnolipids from *Pseudomonas* spp. have been demonstrated to lower the interfacial tension against *n*-hexadecane to 1 mN/m and the surface tension to 25 to 30 mN/m (74, 128, 185). They also emulsify

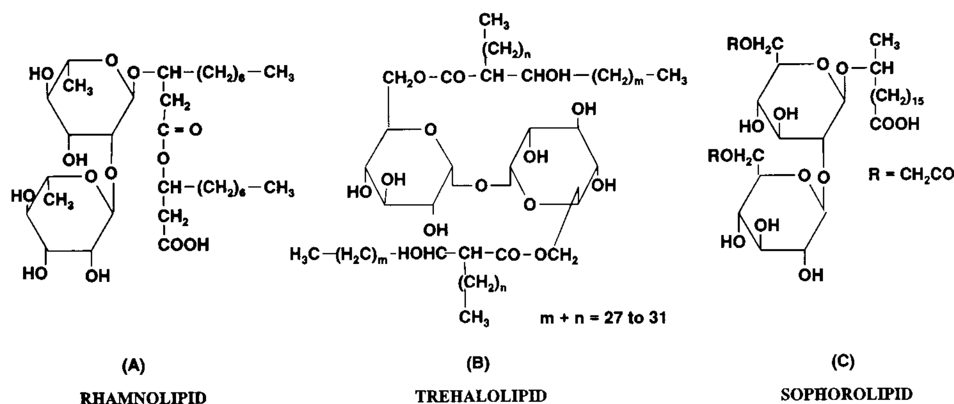

FIG. 1. Structure of some common glycolipid biosurfactants. (A) Rhamnolipid type 1 from *Pseudomonas aeruginosa* in which two rhamnose subunits are linked to two β-hydroxydecanoic acids in a side chain. (B) Trehalose dimycolate from *Rhodococcus erythropolis*, in which disaccharide trehalose is linked to two long-chain α-branched β-hydroxy fatty acids. (C) Sophorolipid from *Torulopsis bombicola* in which dimeric sophorose is linked to a long-chain (C_{18}) hydroxy fatty acid.

alkanes and stimulate the growth of *P. aeruginosa* on hexadecane (88). Itoh and Suzuki (102) isolated two mutants of *P. aeruginosa*, PU-1 and PU-2, which grew poorly on alkanes due to their inability to produce rhamnolipids. These mutants grew normally when the growth medium was supplemented with rhamnolipid.

Trehalolipids. Several structural types of microbial trehalolipid biosurfactants have been reported (128, 133). Disaccharide trehalose linked at C-6 and C-6′ to mycolic acids is associated with most species of *Mycobacterium*, *Nocardia*, and *Corynebacterium*. 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 (5, 40, 128, 244). Trehalose dimycolate produced by *Rhodococcus erythropolis* (Fig. 1B) has been extensively studied (126, 200). *R. erythropolis* also synthesizes a novel anionic trehalose lipid (207). Trehalose lipids from *R. erythropolis* and *Arthrobacter* sp. lowered the surface and interfacial tensions in the culture broth to 25 to 40 and 1 to 5 mN/m, respectively (128, 133, 200).

Sophorolipids. Sophorolipids, which are produced mainly by yeasts such as *Torulopsis bombicola* (39, 68, 99), *T. petrophilum* (38), and *T. apicola* (250), consist of a dimeric carbohydrate sophorose linked to a long-chain hydroxy fatty acid (Fig. 1C). These biosurfactants are a mixture of at least six to nine different hydrophobic sophorosides. Similar mixtures of water-soluble sophorolipids from several yeasts have also been reported (93). Cutler and Light (44) showed that *Candida bogoriensis* produces glycolipids in which sophorose is linked to docosanoic acid diacetate. *T. petrophilum* produced sophorolipids on water-insoluble substrates such as alkanes and vegetable oils (38). These sophorolipids, which were chemically identical to those produced by *T. bombicola*, did not emulsify alkanes or vegetable oils. When *T. petrophilum* was grown on a glucose-yeast extract medium, however, sophorolipids were not produced, but an effective protein-containing alkane emulsifying agent was formed (38). These results appear to contradict the conventional belief that microbial emulsifiers and surfactants are produced to facilitate the uptake of water-insoluble substrates. Although sophorolipids can lower surface and interfacial tension, they are not effective emulsifying agents (39). Both lactonic and acidic sophorolipids lowered the interfacial tension between *n*-hexadecane and water from 40 to 5 mN/m and showed remarkable stability toward pH and temperature changes (38, 128).

Lipopeptides and Lipoproteins

A large number of cyclic lipopetides including decapeptide antibiotics (gramicidins) and lipopeptide antibiotics (polymyxins), produced by *Bacillus brevis* (139) and *B. polymyxa* (240), respectively, possess remarkable surface-active properties. Ornithine-containing lipids from *P. rubescens* (265) and *Thiobacillus thiooxidans* (120), cerilipin, an ornithine- and taurine-containing lipid from *Gluconobacter cerinus* IFO 3267 (246), and lysine-containing lipids from *Agrobacterium tumefaciens* IFO 3058 (245) also exhibit excellent biosurfactant activity. An aminolipid biosurfactant called serratamolide has been isolated from *Serratia marcescens* NS.38 (164). Studies on serratamolide-negative mutants showed that the biosurfactant increased cell hydrophilicity by blocking the hydrophobic sites on the cell surface (14).

The cyclic lipopeptide surfactin (Fig. 2), produced by *B. subtilis* ATCC 21332, is one of the most powerful biosurfactants. It lowers the surface tension from 72 to 27.9 mN/m at concentrations as low as 0.005% (3). *B. licheniformis* produces

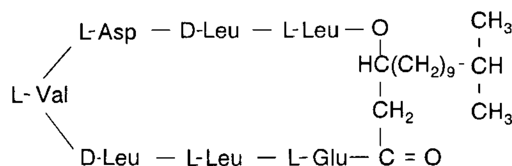

FIG. 2. Structure of cyclic lipopeptide surfactin produced by *Bacillus subtilis.*

several biosurfactants which act synergistically and exhibit excellent temperature, pH, and salt stability (147, 263). The surfactant BL-86, produced by *B. licheniformis* 86, is capable of lowering the surface tension of water to 27 mN/m and the interfacial tension between water and *n*-hexadecane to 0.36 mN/m and promoting excellent dispersion of colloidal β-silicon carbide and aluminum nitride slurries (94, 95). Recent structural analysis revealed that it is a mixture of lipopeptides with major components ranging in size from 979 to 1,091 Da. Each molecule contains seven amino acids and a lipid portion which is composed of 8 to 9 methylene groups and a mixture of linear and branched tails (96). Another important characteristic of this compound is its ability to lyse mammalian erythrocytes and to form spheroplasts (3, 20); this property has been used to detect surfactin production through hemolysis on blood agar.

Recently, Yakimov et al. (263) have shown production of a new lipopetide surfactant, lichenysin A, by *B. licheniformis* BAS-50 containing long-chain β-hydroxy fatty acids. Lichenysin A reduces the surface tension of water from 72 to 28 mN/m with a CMC of as little as 12 μM, comparing favorably with surfactin (24 μM). The detailed characterization of lichenysin A showed that isoleucine was the C-terminal amino acid instead of leucine and an asparagine residue was present instead of aspartic acid as in the surfactin peptide. Addition of branched-chain α-amino acids to the medium caused similar changes in lipophilic moieties of lichenysin-A and lowering of surface tension activity (263a).

Fatty Acids, Phospholipids, and Neutral Lipids

Several bacteria and yeasts produce large quantities of fatty acid and phospholipid surfactants during growth on *n*-alkanes (5, 33, 43, 208). The HLB is directly related to the length of the hydrocarbon chain in their structures. In *Acinetobacter* sp. strain HO1-N phosphatidylethanolamine (Fig. 3), rich vesicles are produced (113), which form optically clear microemulsions of alkanes in water. The quantitative production of phospholipids has also been detected in some *Aspergillus* spp. (113) and *Thiobacillus thiooxidans* (17). *Arthrobacter* strain AK-19 (259) and *P. aeruginosa* 44T1 (208) accumulate up to 40 to 80% (wt/wt) of such lipids when cultivated on hexadecane and olive oil, respectively. Phosphatidylethanolamine produced by *R. erythropolis* grown on *n*-alkane caused a lowering of interfacial

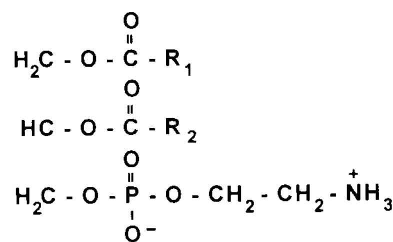

FIG. 3. Structure of phosphatidylethanolamine, a potent biosurfactant produced by *Acinetobacter* sp. R_1 and R_2 are hydrocarbon chains of fatty acids.

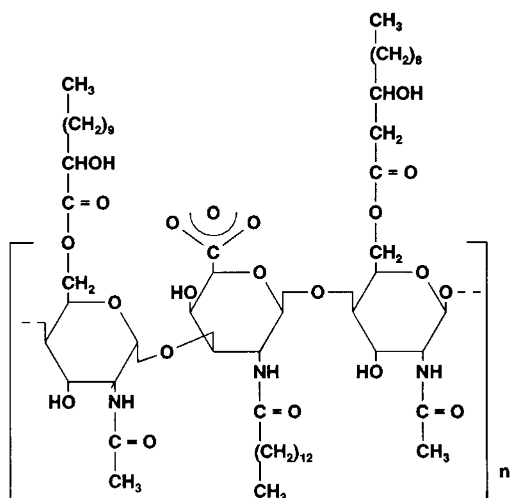

FIG. 4. Structure of emulsan, produced by *Acinetobacter calcoaceticus*, in which fatty acids are linked to a heteropolysaccharide backbone.

tension between water and hexadecane to less than 1 mN/m and a CMC of 30 mg/liter (126).

Polymeric Biosurfactants

The best-studied polymeric biosurfactants are emulsan, liposan, mannoprotein, and other polysaccharide-protein complexes. *Acinetobacter calcoaceticus* RAG-1 produces a potent polyanionic amphipathic heteropolysaccharide bioemulsifier (Fig. 4) called emulsan (210). The heteropolysaccharide backbone contains a repeating trisaccharide of *N*-acetyl-D-galactosamine, *N*-acetylgalactosamine uronic acid, and an unidentified *N*-acetyl amino sugar (271). Fatty acids are covalently linked to the polysaccharide through *o*-ester linkages (18, 225, 271). Emulsan is a very effective emulsifying agent for hydrocarbons in water even at a concentration as low as 0.001 to 0.01%. It is one of the most powerful emulsion stabilizers known today and resists inversion even at a water-to-oil ratio of 1:4 (18, 76, 270). On long standing, this emulsion separates into two layers. The upper cream layer, which is known as emulsanosol, contains 70 to 75% oil (270). Biodispersan is an extracellular, nondialyzable dispersing agent produced by *A. calcoaceticus* A2 (213). It is an anionic heteropolysaccharide, with an average molecular weight of 51,400 and contains four reducing sugars, namely, glucosamine, 6-methylaminohexose, galactosamine uronic acid, and an unidentified amino sugar (211). Recently, Navonvenezia et al. (173) described the isolation of alasan, an anionic alanine-containing heteropolysaccharide-protein biosurfactant from *Acinetobacter radioresistens* KA-53, which was found to be 2.5 to 3 times more active after being heated at 100°C under neutral or alkaline condition.

Liposan is an extracellular water-soluble emulsifier synthesized by *Candida lipolytica* (32, 110) and is composed of 83% carbohydrate and 17% protein (32). The carbohydrate portion is a heteropolysaccharide consisting of glucose, galactose, galactosamine, and galacturonic acid. Palejwala and Desai (184) reported the production by a gram-negative bacterium of a potent bioemulsifier with carbohydrate as a major component. Sar and Rosenberg (218) demonstrated that polysaccharide had no emulsification activity alone but became a potent emulsifier when combined with some proteins released during growth on ethanol.

Cameron et al. (29) recently reported the production of large amounts of mannoprotein by *Saccharomyces cerevisiae*; this protein showed excellent emulsifier activity toward several oils, alkanes, and organic solvents. The purified emulsifier contains 44% mannose and 17% protein. Kappeli et al. (111, 112) have isolated a mannan-fatty acid complex from alkane-grown *Candida tropicalis*; this complex stabilized hexadecane-in-water emulsions. *Schizonella malanogramma* and *Ustilago maydis* produce biosurfactant which has been characterized as erythritol- and mannose-containing lipid (59). Recently, Kitamoto et al. (117) demonstrated the production of two kinds of mannosylerythritol lipids in *Candida antarctica* T-34. Hisatsuka et al. (87, 89) described the isolation from *P. aeruginosa* of a protein-like activator that was involved in emulsification of hydrocarbons. It has a molecular weight of 14,300 and contains 147 amino acids, of which 51 are serine and threonine (89). The production by *P. aeruginosa* P-20 of a peptidoglycolipid bearing 52 amino acids, 11 fatty acids, and a sugar unit has been described previously (123). An emulsifying and solubilizing factor containing protein and carbohydrate from hexadecane-grown *Pseudomonas* spp. has also been reported (17, 153). Desai et al. (47) demonstrated the production of bioemulsifier by *P. fluorescens* during growth on gasoline. This bioemulsifier is composed of 50% carbohydrate, 19.6% protein, and 10% lipid. Trehalose and lipid-*o*-dialkyl monoglycerides were the major components of the carbohydrate and lipid, respectively. Similarly, an extracellular bioemulsifier composed of carbohydrate, protein, and lipids was isolated from *C. tropicalis* (236) and *Phormidium* strain J1 (58).

Particulate Biosurfactants

Extracellular membrane vesicles partition hydrocarbons to form a microemulsion which plays an important role in alkane uptake by microbial cells. Vesicles of *Acinetobacter* sp. strain HO1-N with a diameter of 20 to 50 nm and a buoyant density of 1.158 g/cm^3 are composed of protein, phospholipid, and lipopolysaccharide (113). The membrane vesicles contain about 5 times as much phospholipid and about 350 times as much polysaccharide as does the outer membrane of the same organism.

Surfactant activity in most hydrocarbon-degrading and pathogenic bacteria is attributed to several cell surface components, which include structures such as M protein and lipoteichoic acid in the case of group A streptococci, protein A in *Staphylococcus aureus*, layer A in *Aeromonas salmonicida*, prodigiosin in *Serratia* spp., gramicidins in *Bacillus brevis* spores, and thin fimbriae in *A. calcoaceticus* RAG-1 (49, 58, 128, 209a, 262).

PHYSIOLOGY AND GENETICS

Physiological Role

Biosurfactants are produced by a variety of microbes, secreted either extracellularly or attached to parts of cells, predominantly during growth on water-immiscible substrates (17, 88, 114, 122, 210, 235). Biosurfactant-negative mutants of *P. aeruginosa* KY-4025 (102) and *P. aeruginosa* PG-201 (122) showed poor growth compared to the parent strains on *n*-paraffin and hexadecane, respectively, and addition of rhamnolipid to the medium restored growth on these hydrocarbons. From a physiological point of view, production of such a large amount of polymer will be a waste if it has no function. The genetic system also loses the expression of the gene over a long period by mutation and selection if the gene product has no specific advantage for survival. The function of biosurfactant in

a producing microbial cell is not fully understood. However, there has been speculation about their involvement in emulsification of water-insoluble substrates (64, 81, 178, 267). Direct contact of cells with hydrocarbon droplets and their interaction with emulsified droplets have been described (64, 209a, 235). In addition, biosurfactants have been shown to be involved in cell adherence which imparts greater stability under hostile environmental conditions and virulence (209a, 214), in cell desorption to find new habitats for survival (214), in antagonastic effects toward other microbes in the environment (117, 129, 251), etc.

Biosynthesis

In their amphiphilic structure, the hydrophobic moiety is either a long-chain fatty acid, a hydroxy fatty acid, or α-alkyl-β-hydroxy fatty acid, and the hydrophilic moiety may be a carbohydrate, carboxylic acid, phosphate, amino acid, cyclic peptide, or alcohol. Two primary metabolic pathways, namely, hydrocarbon and carbohydrate, are involved in the synthesis of their hydrophobic and hydrophilic moieties, respectively. The pathways for the synthesis of these two groups of precursors are diverse and utilize specific sets of enzymes. In many cases, the first enzymes for the synthesis of these precursors are regulatory enzymes; therefore, in spite of the diversity, there are some common features of their synthesis and regulation. The detailed biosynthetic pathways for the major hydrophobic and hydrophilic moieties have been extensively investigated and are well documented; however, a brief account by Hommel and Ratledge (91) may be useful. According to Syldatk and Wagner (244), the following possibilities exist for the synthesis of different moieties of biosurfactants and their linkage: (i) the hydrophilic and hydrophobic moieties are synthesized de novo by two independent pathways; (ii) the hydrophilic moiety is synthesized de novo while the synthesis of the hydrophobic moiety is induced by substrate; (iii) the hydrophobic moiety is synthesized de novo, while the synthesis of the hydrophilic moiety is substrate dependent; and (iv) the synthesis of both the hydrophobic and hydrophilic moieties is substrate dependent.

Examples of all the above possibilities have been well documented by Syldatk and Wagner (244) and Desai and Desai (50).

Regulation

In general, three mechanisms, namely, induction, repression, and nitrogen and multivalent ions, operate in the regulation of biosurfactant production.

The induction of sophorolipid synthesis by addition of long-chain fatty acids, hydrocarbons, or glycerides to the growth medium of *Torulopsis magnoliae* (249), of trehalolipid synthesis in *Rhodococcus erythropolis* by addition of hydrocarbons (200, 201), and of glycolipid-EM in *P. aeruginosa* SB-30 by addition of alkanes (30) has been reported. The inducible nature of biosurfactant in *Endomycopsia lipolytica* has also been confirmed (215). Induction also appears to be the general regulatory mechanism used to control the onset synthesis of most lipopeptide biosurfactants (22, 119, 253).

Repression of biosurfactant production by *Acinetobacter calcoaceticus* (68) and *Arthrobacter paraffineus* (54) on hydrocarbon substrates has been observed with organic acids and D-glucose, respectively. Similarly, drastic reduction in the synthesis of rhamnolipids by *P. aeruginosa* (82, 83) and of liposan by *C. lipolytica* (32) upon the addition of D-glucose, acetate, and tricarboxylic acids has been noted. The repression of a protein-like factor responsible for alkane and hexadecane biodegradation in *P. aeruginosa* by glucose, glycerol and palmitic acid has been observed (80, 87, 89). Rapid biosurfactant production was observed in a *Pseudomonas* strain during growth on glucose and oleic acid, when oleic acid was utilized upon the exhaustion of glucose (12).

Nitrogen- or metal ion-dependent regulation also played a prominent role in the synthesis of biosurfactants. The synthesis of rhamnolipid in *P. aeruginosa* upon exhaustion of nitrogen and commencement of the stationary phase of growth has been observed by several investigators (73, 198). Moreover, the addition of a nitrogen source caused an inhibition of rhamnolipid synthesis in resting cells of *Pseudomonas* sp. strain DSM-2874 (242). Mulligan and Gibbs (161) showed a direct relationship between the synthesis of rhamnolipid and glutamine synthetase activity in *P. aeruginosa* RC-II. The concentrations of DL-glutamine, L-glutamic acid, and ammonia in the medium play an important role, and on utilization of glutamate and ammonia, cells become nitrogen limiting, causing derepression of glutamine synthetase. In this event, cell metabolism is switched from nitrogen to glucose, resulting in increased rhamnolipid synthesis, which is again repressed on addition of L-glutamate, DL-glutamine, and ammonia to the medium. Ochsner et al. (182) observed the expression of genes from *P. aeruginosa* for rhamnolipid synthesis in *P. fluorescens* and *P. putida* only under nitrogen-limiting conditions. Glycolipid synthesis by *R. erythropolis* under nitrogen-limiting conditions (207) and emulsan synthesis by *A. calcoaceticus* RAG during amino acid starvation (216) are further examples.

The limitation of multivalent cations also causes overproduction of biosurfactants (73, 104, 243). Guerra-Santos et al. (74) demonstrated that by limiting the concentrations of salts of magnesium, calcium, potassium, sodium, and trace elements, a higher yield of rhamnolipid can be achieved in *P. aeruginosa* DSM 2659. Iron limitation stimulates biosurfactant production in *P. fluorescens* (187, 188) and *P. aeruginosa* (73, 74), whereas addition of iron and manganese salts stimulates biosurfactant production in both *B. subtilis* (41) and *Rhodococcus* sp. (1).

Genetic Characterization

There are numerous reports on the isolation of mutants deficient in biosurfactant production with a concomitant loss in the ability to grow on water-insoluble substrates (14, 15, 30, 65, 217, 224). The recent developments of a thin-layer chromatographic technique (143), drop collapse test (105), hemolytic measurement test (158, 160), technique for rhamnolipid detection on mineral agar (232), and rapid screening method for hydrocarbon-degrading microorganisms (79) have significantly accelerated the ability to rapidly isolate biosurfactant producing organisms. The genetics of biosurfactants have been investigated (48, 50, 65, 205); however, a clear understanding has emerged only through recent studies of *Pseudomonas* and *Bacillus* systems.

Genetics of rhamnolipid synthesis. Recently, Ochsner et al. (179–182) have extensively studied the genetics of rhamnolipid biosynthesis in *P. aeruginosa*. The *rhlABR* gene cluster was found to be responsible for the synthesis of RhlR regulatory protein and a rhamnosyl transferase, both essential for rhamnolipid synthesis. The active rhamnosyl transferase complex is located in the cytoplasmic membrane, with the 32.5-kDa RhlA protein harboring a putative signal sequence, while the 47-kDa RhlB protein is located in the periplasmic region and contains at least two putative membrane-spanning domains (180). The *rhlR* gene encodes a transcriptional activator, the 28-kDa RhlR protein belonging to the LuxR family, which positively regu-

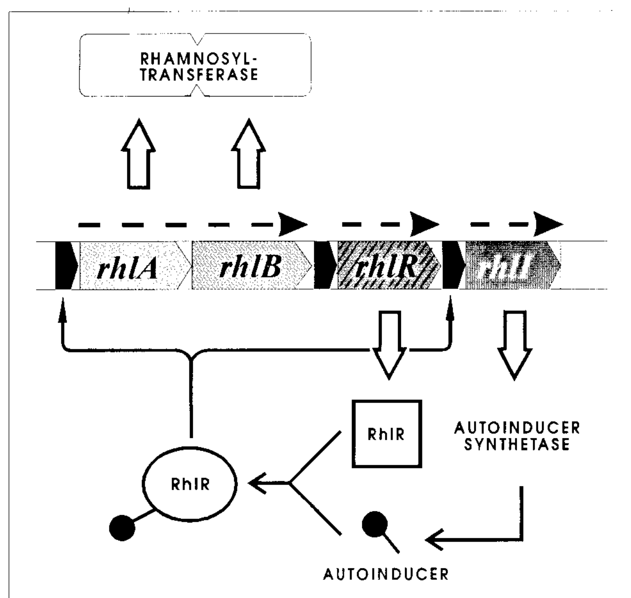

FIG. 5. Genes involved in the synthesis and regulation of rhamnolipid in *Pseudomonas aeruginosa*. The map position at 47 min is shown. The regulatory genes *rhlR* and *rhlI* code for the 28-kDa regulatory protein and the autoinducer synthetase, respectively. The regulatory protein is activated on binding to autoinducer, a product of autoinducer synthetase. The binding of activated regulatory protein to *rhlA* promoter initiates the transcription of the *rhlAB* operon encoding rhamnosyl transferase. The regulatory protein also controls *rhlI* gene expression.

lates rhamnolipid biosynthesis (179). Ochsner and Reiser (181) recently identified another regulatory gene, *rhlI*, located downstream of the *rhlABR* gene cluster. Further, it was shown that the regulation of rhamnolipid production in *P. aeruginosa* is mediated by the *rhlR-rhlI* system involving an autoinducer. The authors presented a model (Fig. 5) for the regulation of rhamnolipid synthesis in which activation of RhlR regulatory protein on binding to its cognate autoinducers (factor 2 and/or VAI), the products of the autoinducer synthetase, and its regulatory role in genetic control of *rhlI* gene are proposed (182). The binding of activated RhlR protein upstream of the *rhlA* promoter enhances the transcription of the *rhlAB* operon encoding rhamnosyl transferase (179). The expression of the *rhlAB* genes in heterologous hosts has also been studied. In *P. fluorescens* and *P. putida* KT-2442, these genes were expressed and produced rhamnolipids. However, in *Escherichia coli*, active rhamnosyl transferase was synthesized but rhamnolipids were not produced (182).

Investigation of the rhamnolipid-nonproducing mutant *P. aeruginosa* 65E12 showed no growth on minimal media containing hexadecane as the carbon source unless surfactants were added exogenously (230). Mutant PG201::rh1R grew very slowly in the absence of exogenously added surfactants. Both mutants are deficient in the positive regulatory gene controlling the activation of rhamnolipid synthesis (230).

Genetics of surfactin synthesis. The studies of the genetics of surfactin production suggest the involvement of three chromosomal genes, *sfp*, *srfA*, and *comA* (166, 167, 170). The *sfp* gene was found in surfactin-producing strains only, and its transformation into a surfactin-nonproducing strain rendered the cells surfactin positive (166). Moreover, mutation in *comA* (earlier designated *srfB*) blocks competence development and renders *sfp*-bearing cells surfactin negative (169). Weinrauch et al. (260) showed that the *comA* product is a response regulator-type protein which can be activated through phosphorylation by the ComP histidine kinase membrane sensor protein. Roggiani and Dubnau (209) purified ComA protein, and after its phosphorylation, it acquired strong binding affinity to the *srfA* promoter. It has been further suggested that two regions upstream of the *srfA* promoter are required for the expression of *srfA*. According to Nakano and Zuber (167), a cooperative interaction of *comA* dimers and binding of such dimers upstream of the *srfA* promoter result in a transcriptional activation of *srfA* genes.

Nakano et al. (166, 170, 172) have isolated null mutants and demonstrated by genetic analysis that the *srfA* locus is a large operon of more than 25 kb of DNA that encodes multifunctional surfactin synthetase (168, 170). Moreover, the *srfA* operon was also shown to be involved in the production of pheromone-like peptide factors which are responsible for the initiation of sporulation in *Bacillus*. The synthesis of these regulatory peptides and surfactin appears to involve the same intermediate (168). Furthermore, the *srfA* promoter region was identified and characterized as inducible by genetic analysis of a *B. subtilis* strain in which the *srfA* promoter was fused with *lacZ* (168, 171).

A closely linked *sfp* locus encodes a 224-amino-acid polypeptide which is responsible for converting intermediates to surfactin (166). This polypeptide also decreases the transcription of the *srfA* operon, indicating that it has a regulatory function in addition to its direct role in surfactin biosynthesis. According to Mulligan et al. (160), the regulatory genes *comA*, *comP*, and *spo0K* are located in the *srfB* region of the chromosome and the gene product of *comA* and *comP* may form a complex which acts as a positive regulator of *srfA* transcription, in response to the levels of L-glutamine and D-glucose in the growth medium.

KINETICS OF FERMENTATIVE PRODUCTION

The kinetics of biosurfactant production exhibit many variations among various systems, and only a few generalizations can be derived. However, for convenience kinetic parameters can be grouped into the following types: (i) growth-associated production, (ii) production under growth-limiting conditions, (iii) production by resting or immobilized cells, and (iv) production with precursor supplementation.

Growth-Associated Production

For growth-associated biosurfactant production, parallel relationships exist between growth, substrate utilization, and biosurfactant production (Fig. 6A). The production of rhamnolipid by some *Pseudomonas* spp. (208, 243, 264), glycoprotein AP-6 by *P. fluorescens* 378 (189), surface-active agent by *B. cereus* IAF 346, and biodispersan by *Bacillus* sp. strain IAF-343 (35) are all examples of growth-associated biosurfactant production. The production of cell-free emulsan by *A. calcoaceticus* RAG-1 has been reported to be a mixed growth-associated and non-growth-associated type (69, 75). Emulsan-like substance accumulates on the cell surfaces during the exponential phase of growth and is released into the medium when protein synthesis decreases (69, 191, 225).

Production under Growth-Limiting Conditions

Production under growth-limiting conditions is characterized by a sharp increase in the biosurfactant level as a result of limitation of one or more medium components (Fig. 6B). A number of investigators have demonstrated an overproduction of biosurfactants by *Pseudomonas* spp. when the culture

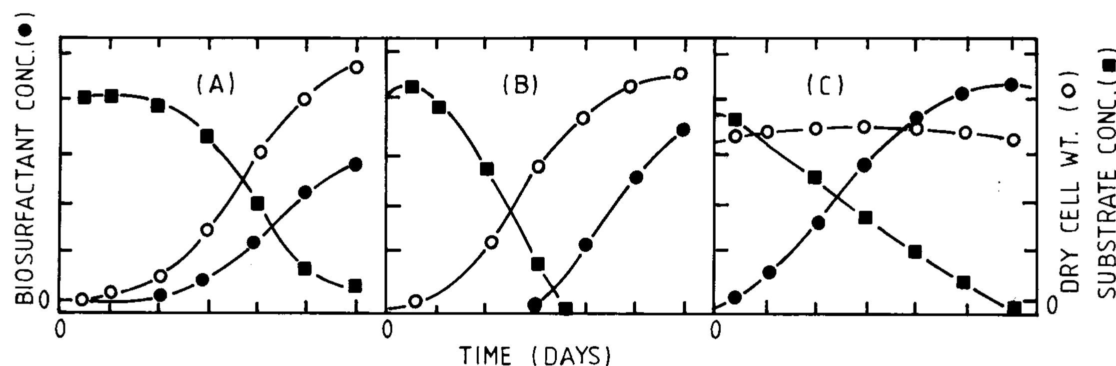

FIG. 6. Schematic illustration showing different types of fermentation kinetics for biosurfactant production. (A) Growth-associated production in which a parallel relationship is obtained between growth, substrate utilization, and biosurfactant production as observed in *Acinetobacter calcoaceticus*. (B) Production under growth-limiting condition in which biosurfactant production is observed only when growth limitation occurs by exhaustion of nutrients such as nitrogen as in the case of *Pseudomonas* spp. (C) Production of biosurfactant by resting or immobilized cells where cells continue to utilize substrate and produce biosurfactant without cell multiplication as observed for some *Torulopsis* spp.

reaches the stationary phase of growth due to the limitation of nitrogen and iron (74, 161, 198, 241). Production of bioemulsifier by *Candida tropicalis* IIP-4 (237), of glycolipid by *Nocardia* sp. strain SFC-D (125), and of water-soluble biosurfactant by *Torulopsis apicola* (93) has also been reported to follow this pattern.

In contrast to the observation in *P. aeruginosa* (159), low phosphate concentration stimulated bioemulsifier production in a gram-negative bacterium during cultivation on ethanol (184). Phosphate, iron, magnesium, and sodium were all important elements for a biosurfactant-producing *Rhodococcus sp*., much more than either potassium or calcium (1). Iron concentration has a dramatic effect on rhamnolipid production by *P. aeruginosa*, resulting in a threefold increase in production when cells were shifted from medium containing 36 μM iron to medium containing 18 μM iron. Interestingly, under these conditions, there was no change in the biomass yield (73, 74).

Production by Resting or Immobilized Cells

Production by resting or immobilized cells is a type of biosurfactant production in which there is no cell multiplication. The cells nevertheless continue to utilize the carbon source for the synthesis of biosurfactants, as illustrated in Fig. 6C. Several examples of biosurfactant production by resting cells are known. They include production of rhamnolipid by *Pseudomonas* spp. (204, 243, 244) and *P. aeruginosa* CFTR-6 (197), sophorolipid production by *Torulopsis bombicola* (99) and *Candida apicola* (90), cellobiolipid production by *Ustilago maydis* (59), trehalose tetraester production by *Rhodococcus erythropolis* (140, 243, 244), and mannosylerythritol lipid production by *Candida antarctica* (115–117). Biosurfactant production by resting cells is important for the reduction of cost of product recovery, as the growth and the product formation phases can be separated.

Production with Precursor Supplementation

Many investigators have reported that the addition of biosurfactant precursors to the growth medium causes both qualitative and quantitative changes in the product. For example, the addition of lipophilic compounds to the culture medium of *T. magnoliae* (249), *T. bombicola* (39, 141), and *T. apicola* IMET 43747 (239) resulted in increased biosurfactant production with yields of about 120 to 150 g · liter^{-1} (132). Similarly, increased production of biosurfactants containing different mono-, di-, or trisaccharides was reported to occur in *Arthrobacter paraffineus* DSM 2567 (133), *Corynebacterium* spp., *Nocardia* spp., and *Brevibacterium* spp. through supplementation with the corresponding sugar in the growth medium (26,104, 241).

FACTORS AFFECTING BIOSURFACTANT PRODUCTION

Carbon Source

Water-soluble carbon sources such as glycerol, glucose, mannitol, and ethanol were all used for rhamnolipid production by *Pseudomonas* spp. Biosurfactant product, however, was inferior to that obtained with water-immiscible substrates such as *n*-alkanes and olive oil (208, 243, 264). Syldatk et al. (242, 243) and Edmonds and Cooney (56) demonstrated that although different carbon sources in the medium affected the composition of biosurfactant production in *Pseudomonas* spp., substrates with different chain lengths exhibited no effect on the chain lengths of fatty acid moieties in glycolipids. On the other hand, Finnerty and Singer (62) and Neidleman and Geigert (174) showed evidence for qualitative variation, reflecting the carbon number of alkane for biosurfactant production in *Acinetobacter* sp. strains H13-A and H01-N, respectively.

When *Arthrobacter paraffineus* ATCC 19558 was grown on D-glucose, supplementation with hexadecane in the medium during the stationary growth phase resulted in a significant increase in biosurfactant yield (54). Duvnjak and Kosaric (55) showed the presence of large amounts of biosurfactant bound to *Corynebacterium lepus* cells when grown on glucose, and addition of hexadecane facilitated the release of surfactant from cells. Others observed little biosurfactant production when cells were growing on a readily available carbon source; only when all the soluble carbon was consumed and when water-immiscible hydrocarbon was available was biosurfactant production triggered (11, 12). Glycolipid production by *T. bombicola* is stimulated by the addition of vegetable oils during growth on 10% D-glucose medium, giving a yield of 80 g · liter^{-1} (4, 37). Davila et al. (46) demonstrated a high yield of sophorose lipids by overcoming product inhibition in *Candida bombicola* CBS 6009 through the addition of ethyl esters of rapeseed oil fatty acids in D-glucose medium. Using *T. apicola* IMET 43747, Stuwer et al. (239) achieved glycolipid yields as high as 90 g · liter^{-1} with a medium containing D-glucose and sunflower oil. In an interesting study, Lee and Kim (132) reported that in batch culture, 37% of the carbon input was

channeled to produce 80 g of sophorolipid per liter by *T. bombicola*. However, in fed-batch cultures, about 60% of the carbon input was incorporated into biosurfactant, increasing the yield to 120 g · liter^{-1}. Mounting evidence leads to the conclusion that the available carbon source, particularly the carbohydrate used, has a great bearing on the type of biosurfactant produced (104, 133, 241).

Nitrogen Source

Medium constituents other than carbon source also affect the production of biosurfactants. Among the inorganic salts tested, ammonium salts and urea were preferred nitrogen sources for biosurfactant production by *Arthrobacter paraffineus* (53), whereas nitrate supported maximum surfactant production in *P. aeruginosa* (73, 137, 208) and *Rhodococcus* spp. (1). Biosurfactant production by *A. paraffineus* is increased by the addition of L-amino acids such as aspartic acid, glutamic acid, asparagine, and glycine to the medium (53). The structure of surfactin is influenced by the L-amino acid concentration in the medium to produce either Val-7 or Leu-7 surfactin (190). Similarly, lichenysin-A production is enhanced two- and fourfold in *B. licheniformis* BAS50 (263a) by addition of L-glutamic acid and L-asparagine, respectively, to the medium.

Robert et al. (208) and Abu-Ruwalda et al. (1) observed nitrate to be the best source of nitrogen for biosurfactant production by *Pseudomonas* strain 44T1 and *Rhodococcus* strain ST-5 growing on olive oil and paraffin, respectively. The production started after 30 h of growth, when the culture reached nitrogen limitation, and continued to increase up to 58 h of fermentation. In *P. aeruginosa*, a simultaneous increase in rhamnolipid production and glutamine synthetase activity was observed when growth slowed as the culture became nitrogen limiting (161). Similarly, nitrogen limitation caused increased biosurfactant production in *P. aeruginosa* (198, 241), *C. tropicalis* IIP-4 (237), and *Nocardia* strain SFC-D (125). Syldatk et al. (242) showed that nitrogen limitation not only causes overproduction of biosurfactant but also changes the composition of the biosurfactant produced. Guerra-Santos et al. (73, 74) showed maximum rhamnolipid production after nitrogen limitation at a C:N ratio of 16:1 to 18:1 and no surfactant production below a C:N ratio of 11:1, where the culture was not nitrogen limited. According to Hommel et al. (93), it is the absolute quantity of nitrogen and not its relative concentration that appears to be important for optimum biomass yield, while the concentration of hydrophobic carbon source determines the conversion of carbon available to the biosurfactant.

Environmental Factors

Environmental factors and growth conditions such as pH, temperature, agitation, and oxygen availability also affect biosurfactant production through their effects on cellular growth or activity. The pH of the medium plays an important role in sophorolipid production by *T. bombicola* (68). Rhamnolipid production in *Pseudomonas* spp. was at its maximum at a pH range from 6 to 6.5 and decreased sharply above pH 7 (73). In contrast, Powalla et al. (194) showed that penta- and disaccharide lipid production in *N. corynbacteroides* is unaffected in the pH range of 6.5 to 8. In addition, surface tension and CMCs of a biosurfactant product remained stable over a wide range of pH values, whereas emulsification had a narrower pH range (1, 2).

In *A. paraffineus* (52) and *Pseudomonas* sp. strain DSM-2874 (243), temperature causes alteration in the composition of the biosurfactant produced. A thermophilic *Bacillus* sp. grew and produced biosurfactant at temperatures above 40°C (9). Heat treatment of some biosurfactants caused no appreciable change in biosurfactant properties such as the lowering of surface tension and interfacial tension and the emulsification efficiency, all of which remained stable after autoclaving at 120°C for 15 min (2).

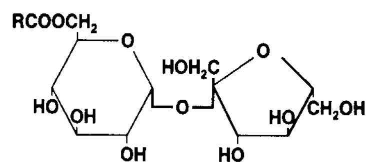

FIG. 7. Structure of sucrose monoester.

An increase in agitation speed results in the reduction of biosurfactant yield due to the effect of shear in *Nocardia erythropolis* (140–142). While studying the mechanism of biosurfactant production in *A. calcoaceticus* RAG-1, Wang and Wang (258) revealed that the cell-bound polymer/dry-cell ratio decreases as the shear stress increases. On the other hand, in yeast, biosurfactant production increases when the agitation and aeration rates are increased (238). Sheppard and Cooper (228) have recently concluded that oxygen transfer is one of the key parameters for the process optimization and scale-up of surfactin production in *B. subtilis.*

Salt concentration also affected biosurfactant production depending on its effect on cellular activity. Some biosurfactant products, however, were not affected by salt concentrations up to 10% (wt/vol), although slight reductions in the CMCs were detected (2).

BIOSURFACTANT PRODUCTION BY BIOTRANSFORMATION

Considerable attention has been directed toward the production of biosurfactants through the biotransformation route in the last few years, largely due to a close structural similarity between sucrose esters, a group of commercial surfactants (Fig. 7), and glycolipid biosurfactants. The main thrust has been to use microbial fermentation to obtain different hydrophobic and hydrophilic moieties of biosurfactants, which could be then joined by enzymatic treatment to produce commercial surfactants. Such enzyme systems are highly specific, and the reactions can be carried out easily at normal temperature and pressure.

The simplest transformation reported is through using selected yeast strains to upgrade oil quality by desaturating or saturating the component fatty acids (78, 154, 202). The potential of microbial transformation in the production of specific fatty acids includes 70% conversion of *n*-alkane to α,ω-dioic acids by mutants of *Candida tropicalis* (85) and to arachidonic and eicosapentaenoic acids by soil isolates of *Mortierella elongata* IS-4 and *M. alpina* (229) and conversion of oleic acid to recinoleic acid by soil bacterium BMD-120 (265). Another important conversion is that of soybean lecithin to a new biosurfactant by phospholipase D obtained from *Streptococcus chromofuscus* (265).

Although several enzymes are involved in the biotransformation of lipids, lipases and phospholipases are the most important commercially as they perform nonspecific or specific transesterification reactions (127, 131, 138). Phospholipids were successfully modified by *Candida cylindracea* and *Rhizo-*

pus delemar lipase-catalyzed transesterification to produce polyunsaturated phospholipids from soy phospholipids and sardine oil (165, 248). Seino (223), in collaboration with Daiichi Ltd., has achieved production of D-isomers of sucrose, glucose, fructose and sorbitol esters with oleic, linoleic, and stearic acids by using lipase obtained from *Candida cylindracea* with a conversion efficiency up to 68%. Janssen et al. (107) used a lipase from *Candida rugosa* in a continuous two-phase membrane reactor to esterify decanoic acid with D-sorbitol. Conversion at a rate of 6.8 mmol $\cdot$ g^{-1} $\cdot$ h^{-1} for 24 days without any significant reduction in the production rate was achieved. Recently, Forsell et al. (63) reported a continuous enzymatic transesterification of rapeseed oil and lauric acid in a fixed-bed bioreactor. Sarney and Vulfson (220) and Siemann and Wagner (233) have recently presented detailed accounts of the prospects and limitations of enzymatic synthesis of surfactants.

RECOVERY OF BIOSURFACTANTS

Downstream processing in many biotechnological processes is responsible for up to 60% of the total production cost. Due to economic considerations, most biosurfactant would have to involve either whole-cell spent culture broths or other crude preparations. In addition, biosurfactant activity may be affected by other materials present in these preparations. Biosurfactant recovery depends mainly on its ionic charge, water solubility, and location (intracellular, extracellular or cell bound).

The most commonly used biosurfactant recovery techniques are listed in Table 2. The most widely used techniques are extractions with chloroform-methanol, dichloromethane-methanol, butanol, ethyl acetate, pentane, hexane, acetic acid, ether, etc. Trehalose lipids of *Mycobacterium* spp. (5, 128, 244) and *Arthrobacter paraffineus* (104, 133, 241), trehalose corynomycolates and tetraesters of *Rhodococcus erythropolis* (126, 136, 200, 207), mono-, di-, and pentasaccharide lipids of *A. paraffineus* (133, 241) and *Nocardia corynebacterioids* (194), cellobiolipids of *Ustilago* spp. (24, 238), sophorolipids of several yeast species (38, 39, 44, 68, 207), liposan from *C. lipolytica* (33), and rhamnolipids of *Pseudomonas* spp. (47, 88, 264) are some of the well-known examples of biosurfactant recovery by solvent extraction.

Glycolipids produced by *T. bombicola* (39, 68, 99), *T. petrophilum* (37), and *T. apicola* (93, 250) are extracted by chilled ethyl acetate after adsorption on charcoal. Biosurfactant from *P. aeruginosa* has also been recovered in a similar way, except that extraction was carried out in acetone (175). Both the glycolipid produced by *Ustilago zeae* and the mannosylerythritol lipid produced by *Candida* spp. (82, 238) are sedimented as heavy oils upon centrifugation and then extracted in either ethanol or methanol.

Glycolipids from *P. aeruginosa* and *U. zeae* have also been recovered by acid precipitation at low temperature (24, 238). Other glycolipids from a mixed microbial population (177) and rhamnolipids from both *P. aeruginosa* (185) and *C. lipolytica* (32, 33) have been recovered by acidification followed by extraction in chloroform-methanol mixtures. Recently, Cameron et al. (29) extracted a cell-bound bioemulsifier from *S. cerevisiae* at 121°C in a buffer containing potassium metabisulfite followed by precipitation in an ethanol-acetic acid mixture.

Ammonium sulfate precipitation has also been successfully used in isolating emulsan (69, 210, 225) and biodispersan (213) from *Acinetobacter calcoaceticus* A2 as well as a bioemulsifier from an unidentified gram-negative bacterium (184). Surfactin and surfactin-like biosurfactants produced by *B. subtilis* (3) and *B. licheniformis* (109), respectively, have been recovered by acid precipitation, whereas other biosurfactants from *Pseudomonas* spp. (31, 70, 196), *Endomycopsis lipolytica* (215), and *C. tropicalis* and *Debaryomyces polymorphus* (236) were best recovered by acetone precipitation.

In a recent development, continuous removal of biosurfactant during fermentation by different techniques has increased the cell density in the reactor and eliminated product inhibition, resulting in a severalfold net increase in biosurfactant yield (41, 145, 176). In addition, substantial reductions in the cost of product recovery and effluent treatment were achieved (145). One of the successful techniques involved a continuous in situ removal of surfactin from fermentation broth by foam fractionation. In this technique, foam was collected and acidified to pH 2 with concentrated HCl and the precipitated surfactin was extracted in dichloromethane (41, 160). Neu and Poralla (176), in contrast, recovered a *Bacillus* biosurfactant by blowing the foam out of the fermentor to be collected, centrifuged, and extracted by acetone precipitation. A tangential-flow filtration system has been described by Mattei and co-investigators (144, 145) for continuous recovery of

TABLE 2. Downstream processes for recovery of important biosurfactants

Process	Reference(s)
Batch mode	
Ammonium sulfate precipitation	
Emulsan	210, 271
Biodispersan	213
Bioemulsifier	184
Acetone precipitation	
Bioemulsifier	31, 236
Acid precipitation	
Surfactin	3, 109
Solvent extraction	
Trehalolipids	36, 133, 241
Sophorolipids	207
Liposan	33
Crystallization	
Cellobiolipids	238, 250
Glycolipids	177
Continuous mode	
Centrifugation	
Glycolipids	82, 117
Adsorption	
Rhamnolipids	264
Lipopetide	143, 204
Glycolipids	39, 68, 175
Foam separation and precipitation	
Surfactin	41, 160, 176
Tangential flow filtration	
Mixed biosurfactant	27, 145
Diafiltration and precipitation	
Glycolipids	27, 31
Ultrafiltration	
Glycolipids	162

biosurfactant. In this system, biosurfactants were recovered from the filtrate while cells and residual hydrocarbons were recycled and up to 3 g of biosurfactant per liter (60% purity) was recovered. It is now established that the cross-flow filtration techniques for continuous recovery are more cost effective than centrifugation for cell separation.

Other continuous-recovery techniques, involving adsorption on Amberlite XAD-2 followed by purification and freeze-drying, have also been reported to give 60% recovery with 90% purity (204). The ability of biosurfactants to aggregate has also been used to retain them on high-molecular-weight-cutoff membranes. For example, Amicon XM-50 membrane (a 5,000-molecular-weight-cutoff membrane) has been successfully used for 98% recovery of 97% pure surfactin and rhamnolipid, while YM-10 membrane (a 10,000-molecular-weight-cutoff membrane) gave 92% rhamnolipid recovery (21). Thus, this technique can be successfully used by selecting the membranes with molecular weight cutoff for most surfactants present in the fermentation broth at a level higher than the CMC.

POTENTIAL COMMERCIAL APPLICATIONS

The surfactant industry has been increasing at a high rate, and the total quantity of surfactants produced during 1989 in the United States and globally has been estimated to be around 7.6×10^9 and 15.5×10^9 lb, respectively (70). Virtually all surfactants are chemically synthesized. Nevertheless, in recent years, much attention has been directed toward biosurfactants due to their broad-range functional properties and the diverse synthetic capabilities of microbes. The structural analysis of biosurfactants has also opened possibilities for their chemical synthesis. Most important is their environmental acceptability, because they are readily biodegradable and have lower toxicity than synthetic surfactants.

A number of industrial applications of biosurfactants have been envisaged (48, 60, 257). One of the potential uses is in the oil industry (10, 30, 219, 227) with minimum purity specification so that whole-cell broth could be used. Compared with chemical surfactants, they are very selective, required in small quantities, effective under broad ranges of oil and reservoir conditions (191, 199, 209a), and environmentally friendly in protection of coastal areas from additional damage inflicted by synthetic chemicals (193). About a 30% increase in total oil recovery from underground sandstone by using trehalolipids from *Nocardia rhodochrous* has been documented (201).

Multi-biotech, a subsidiary of Geodyne Technology, has commercialized biosurfactants for enhanced oil recovery applications. *B. licheniformis* JF-2, an isolate from oilfield injection water which, in addition to producing most effective biosurfactants (CMC, 10 $\mu g \cdot liter^{-1}$; interfacial tension of saline against decane lowered to 10^{-3} dynes $\cdot$ cm^{-1}), has other properties such as being anaerobic, halotolerant, and thermotolerant, makes biosurfactants that are potentially useful for in situ microbially enhanced oil recovery (106, 109, 135). Hayes et al. (84) have demonstrated that when Boscan, Venezuelan heavy crude oil, was treated with emulsan, oil viscosity was reduced from 200,000 to 100 cP. Thus, it was feasible to pump heavy oil 26,000 miles in a commercial pipeline after this treatment although conventional chemical surfactant treatment failed. Banat et al. (12) have demonstrated the use of a biosurfactant for desludging of a crude oil storage tank for Kuwait Oil Co. and achieved 90% recovery of the oil trapped in the sludge by using a proprietary biosurfactant-producing strain which belonged to an American company, Petrogen Inc. (19).

Microbial remediation of hydrocarbon and crude oil-contaminated soils is an emerging technology involving the application of biosurfactants (10, 11, 16, 20, 66, 81, 104). Biodegradation of hydrocarbons by native microbial populations is the primary mechanism by which hydrocarbon contaminants are removed from the environment (7, 8). The effectiveness of enhancing hydrocarbon degradation through addition of microbial inocula prepared from nonindigenous populations (bioaugmentation) has been ambiguous (6). However, the addition of biosurfactant stimulated the indigenous bacterial population to degrade hydrocarbons at rates higher than those which could be achieved through addition of nutrients alone. Rhamnolipid from *P. aeruginosa* has removed substantial quantities of oil from contaminated Alaskan gravel from the *Exxon Valdez* oil spill (81). Recently, in a large-scale experiment, the effectiveness of in situ bioremediation on the *Exxon Valdez* oil spill has been demonstrated by Bragg et al. (25). In another experiment, Van Dyke et al. (255) demonstrated a 25 to 70% and 40 to 80% increase in the recovery of hydrocarbons from contaminated sandy-loam and silt-loam soil, respectively, by rhamnolipid from *P. aeruginosa*. Similarly, 56% of the aliphatic and 73% of the aromatic hydrocarbons were recovered from hydrocarbon-contaminated sandy-loam soil by treatment with *P. aeruginosa* biosurfactant (221). Glycolipid biosurfactants enhanced the hydrocarbon removal (from 80 to 90–95%), and also increased hydrocarbon mineralization by twofold and shortened the adaptation time of microbial populations to a few hours (155, 156, 178).

A stimulatory effect of different rhamnolipids on the degradation of hexadecane and octadecane by seven *Pseudomonas* strains has recently been demonstrated (268, 269). In some cases, however, the desired effect was not obtained when oxygen was limiting; hence, pretreatment with hydrogen peroxide is a common practice in bioremediation technology which depends mainly on aerobic microorganisms (13, 155). The efficiency of biosurfactants for the remediation of metal (152), phenanthrene (195), and polychlorinated biphenyl (256) contamination in soil has recently been shown. According to Zhang and Miller (269) the three-way interaction between biosurfactant, substrate, and cells is very critical to achieve an enhanced biodegradation rate.

The ability of biosurfactants to emulsify hydrocarbon-water mixtures has been very well documented (64, 81, 178, 267). This property has been demonstrated to increase hydrocarbon degradation significantly and is thus potentially useful for oil spill management (1, 2, 7–10, 21). While screening oil-degrading marine microorganisms from the North Sea, Schulz et al. (222) isolated biosurfactant-producing *Alcaligenes* sp. strain MM-1, *Arthrobacter* sp. strain EK1, and *Arthrobacter* strain S-II. Dixon (51) recently observed the development of blue-green mats of oil-degrading organotrophic bacteria attached to mucilage produced by cyanobacteria during the deliberate discharge of oil in the Persian Gulf during the Iraqi war and during the *Braer* tanker leakage off the Shetland Isles. Dave et al. (45) recently demonstrated that the use of a mixture of hydrocarbon-degrading microbes for bioaugmentation of soil contaminated with slop oil from a petrochemical industry resulted in the bioreclamation of soil, while Ghosh et al. (66) demonstrated an enhancement in bioremediation of polyaromatic hydrocarbons and soil contaminated with polychlorinated biphenyls. Deziel et al. (50a) have recently shown biosurfactant production by polycyclic aromatic hydrocarbon-metabolizing *Pseudomonas aeruginosa* 19SJ. Biosurfactants are also useful in bioremediation of sites contaminated with toxic heavy metals like uranium, cadmium, and lead, and this aspect has been very well reviewed recently by Miller (152). These observations suggest the usefulness of natural microbial con-

sortia and their products in solving environmental related problems.

Biosurfactants also have several promising applications in the food industry as food additives. Lecithin and its derivatives, fatty acid esters containing glycerol, sorbitan, or ethylene glycol, and ethyoxylated derivatives of monoglycerides including a recently synthesized oligopeptide (23) are currently in use as emulsifiers in the food industries worldwide. A novel bioemulsifier from *Candida utilis* has shown potential use in salad dressing (227a). Biosurfactants are potential candidates in the search for functionally different products, as they meet the requirements of functional food additives.

Biosurfactants are also very attractive in the health care and cosmetic industries (118). A product containing 1 mol of sophorolipid and 12 mol of propylene glycol has excellent skin compatibility and is used commercially as a skin moisturizer (265). Production of sophorolipids by microbial fermentation with yields up to 90 to 150 g $\cdot$ liter^{-1} has been documented (4, 100, 132, 239). A large number of compounds for cosmetic applications are prepared by enzymatic conversion of hydrophobic molecules by various lipases and whole cells (247). The cosmetic industry demands surfactants with a minimum shelf life of 3 years. Therefore, saturated acyl groups are preferred over the unsaturated compounds. Sophorolipid is commercially used by Kao Co. Ltd. as a humectant for cosmetic makeup brands such as Sofina. This company has developed a fermentation process for sophorolipid production, and after a two-step esterification process, the product finds application in lipstick and as moisturizer for skin and hair products (97, 98). Monoglyceride, one of the widely used surfactants in the cosmetic industry, has been reported to be produced from glycerol-tallow (1.5:2) with a 90% yield by using *P. fluorescens* lipase treatment (148, 149).

Antibiotic effects and inhibition of growth of human immunodeficiency virus in leukocytes by biosurfactants have been cited in the literature (117, 129, 175). A deficiency of pulmonary surfactant, a phospholipid protein complex, is responsible for the failure of respiration in prematurely born infants. The isolation of genes for protein molecules of this surfactant and cloning in bacteria have made possible its fermentative production for medical application (261). The succinoyl-trehalose lipid of *Rhodococcus erythropolis* has been reported to inhibit herpes simplex virus and influenza virus with a lethal dose of 10 to 30 μg $\cdot$ ml^{-1} (251, 252). Haferburg et al. (77) successfully used a 1% emulsion of rhamnolipids for the treatment of leaves of *Nicotiana glutinosa* infected with tobacco mosaic virus and for the control of potato virus-x disease.

A cost-effective application of surfactin in mechanical dewatering of peat at concentrations as low as 13 g $\cdot$ ton^{-1} has been demonstrated (37, 42). Other potential areas of application are in the pulp and paper (212), coal, textile (94, 95), and uranium ore-processing (147) industries. A heteropolysaccharide containing D-mannuronic acid and D-guluronic acid from *Macrocystis pyrifera* (kelp) (186a) and *Azotobacter vinelandii* and polyglutamic acid from *B. licheniformis* (186b) have been used as effective dispersants in the ceramic processing industry.

Glycolipids with an HLB value close to 10 and CMCs in the range of 5 to 150 mg $\cdot$ liter^{-1} are capable of reducing the surface tension of water to 30 mN $\cdot$ m^{-1}. Enzymatically produced sugar esters at 0.01% reduce interfacial tension between xylenes and water to 1 mN $\cdot$ m^{-1}. These values are favorable compared to those for the chemically synthesized surfactants, which have a higher CMC and less efficiency.

CONCLUDING REMARKS

Surfactants have long been among the most versatile of process chemicals. Their market is extremely competitive, and manufacturers will have to expand their arsenal to develop products for the 1990s and beyond. In this regard, biosurfactants are promising candidates.

Many biosurfactants and their production procedures have been patented, but so far only a few have been commercialized. For enhanced oil recovery application, chemical surfactants cost in the range of $4 to 6 per kg (209b), whereas lignin-based sulfonates are 40% cheaper; at these prices, they are economically viable only at a sale price of oil of $35 and 25 per barrel, respectively. The cost of biosurfactant production is about 3 to 10 times higher than that of the chemical counterparts (157). From the above it is evident that the current major target of biosurfactant applications is the oil sector; however at current costs, these industries cannot adopt biosurfactants for microbially enhanced oil recovery applications. Thus, future biosurfactant applications will be governed mainly by the overall economic gain of production versus application.

The fermentation process holds the key to improving the overall process economics in biosurfactant production. It has been estimated that raw materials account for about 10 to 30% of the overall cost of biosurfactant production (157). Generally, biosurfactants are produced during growth on hydrocarbons which are usually expensive and therefore increase the overall process cost. However, other cheaper, water-soluble substrates such as glucose (55, 74, 92, 239) and ethanol (160, 161, 184, 213, 271) are sometimes used. In the search for cheaper raw materials for biosurfactant production, industrial effluents have recently shown good promise. Striking recent developments in this area include rhamnolipid production from olive oil mill effluent by *Pseudomonas* spp. (150, 208), the use of agroindustrial by-products (151), potato-processing industrial residues (192), the soybean curd residue okara (183), chicken fat residues (30), and wastewater pressate from fuel-grade peat processing (163).

A number of attempts have been made to increase biosurfactant productivity by manipulating physiological conditions and medium composition. There have also been attempts to improve yields by genetic manipulation. Mutants capable of producing severalfold higher biosurfactant concentrations than those of the parent microbial strains have been isolated (114, 160, 183, 224). Alteration in substrate requirements of *P. aeruginosa* strains by insertion of a *lac* plasmid from *E. coli* has made it possible to produce rhamnolipid from whey, a waste product from the dairy industry (121). Thus, one can combine the biosurfactant-producing ability of one microbe with the ability of others to utilize waste substrates.

Recent developments in the area of optimization of fermentation conditions have resulted in a significant increase in production yields, making them more commercially attractive. These developments include a practical approach using nonaseptic biosurfactant production by a mixed microbial culture (67); use of a fed-batch technique in which the yield of sophorolipids of *T. bombicola* increased from 0.37 g $\cdot$ g of substrate^{-1} in batch culture to 0.6 g $\cdot$ g of substrate^{-1} (132); development of a continuous process for lipopeptide surfactant production from *B. licheniformis* JF-2, which usually disappears from the fermentation broth shortly after production, by operating in a narrow range of dilution rates of about 0.12/h and low dissolved oxygen concentration of about 30% saturation, resulting in an increase in the yield from 5.3 to 11.2 mg $\cdot$ liter^{-1} (134, 135); and the self-cycling technique developed by McCaffrey and Cooper (146) for sophorolipid produc-

tion from *C. bombicola*. Recently, Ohno et al. (183) reported surfactin production by solid-state fermentation on the soybean curd residue okara, which normally is incinerated as an industrial waste, by using *B. subtilis* MI113 with recombinant plasmid pC112 containing the *Ipa-14* gene related to surfactin production from *B. subtilis* RB14. The production is devoid of product inhibition and foaming, resulting in a yield as high as 2 g (wet weight) $\cdot$ kg^{-1}, about 10 times higher than that reported in the parent strain RB-14 under submerged culture conditions (3, 160).

Downstream recovery processes can greatly increase the cost of biosurfactants. To combat this, significant developments have taken place which include the continuous recovery of surfactants by foam fractionation (41, 160, 176), tangential flow filtration (144), adsorption on Amberlite XAD-2 (204), and membrane ultrafiltration (27). Fiechter (61) has developed an integrated fermentation system for *P. fluorescens* involving on-line high-pressure liquid chromatography and membrane filtration for cell retention, which resulted in biosurfactant productivity of 7.7 mg $\cdot$ liter^{-1} $\cdot$ h^{-1}. Drouin and Cooper (52) developed an aqueous two-phase fermentation system involving polyethylene glycol and dextran in which *B. subtilis* partitioned to the bottom phase while biosurfactant partitioned to the top phase. These techniques not only replaced expensive extraction procedures but also resulted in improved recovery and increased overall output by relieving the product inhibition.

In recent years, the development of rapid and simple screening methods for biosurfactant-producing microorganisms has had a remarkable impact on biochemical and genetic information related to the degradation of hydrophobic compounds. Genes responsible for the production of biosurfactants have also been isolated, characterized (181), and cloned in heterogeneous hosts (80, 182). They have also resulted in a severalfold increase in the yield of biosurfactants (182) and a change in the raw-material requirement for production (121, 182, 183). It is therefore conceivable that superactive microbial strains can be constructed through genetic engineering to produce potent biosurfactants in quantity with a variety of wastewaters as substrate.

The cosmetics, food, and pharmaceutical industries are low-volume, high-value categories which could absorb the higher cost of biosurfactants (118). Surfactin is currently sold at $9.6 mg^{-1} by Calbiochem for research purposes to inhibit protein denaturation and blood coagulation. The enzymatic synthesis of tailor-made surfactants and sugar esters by lipase, in addition to enzymatic catalysis in a nonaqueous medium (130), offers a new dimension in biosurfactant production. This class of biosurfactants is desired in the cosmetics and health care industries, where mildness and performance of surfactants are important.

Because of the economic considerations in the oil industry, most biosurfactant applications would involve either whole-cell cultures or crude preparations. Interestingly, biosurfactants have about a 10- to 40-fold-lower CMC than do chemical surfactants. Thus, this factor narrows the actual gap in cost compared with their chemical counterparts. Further reductions in the cost of biosurfactants which will make them economically attractive will depend largely on the development of cheaper processes, the use of low-cost raw materials, and increased product yields through superactive mutants and genetically engineered bacteria.

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