# PRODUCTION OF BIOSURFACTANTS

**F. Siñeriz**
*Institute of Microbiology, University of Tucuman- PROIMI, Argentina*

**R. K. Hommel**
*CellTechnologie Leipzig Germany*

**H–P. Kleber**
*Institute of Biochemistry, University of Leipzig, Germany*

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**Contents**

**Summary**

Several prokaryotes and eukaryotes produce surface-active molecules, which are generically known as biosurfactants. These molecules present two distinct moieties: a hydrophilic and a hydrophobic one, and it is this combination that allows the microorganism to interact with hydrophobic substrates or surfaces. Biosurfactants reduce the interfacial tension and/or produce stable emulsions with insoluble compounds like hydrocarbons. The types of molecules are variable and can be classified according to their structure as:

- glycolipids (a carbohydrate moiety linked, generally to fatty acids of different type);
- proteolipids (the hydrophilic moiety consists of amino acids); and
- polymeric surfactants (high molecular weight structures).

Depending on the carbohydrate moiety, glycolipids can further be subdivided into: sophorolipids (derived from sophorose, a disaccharide), rhamnolipids (derived from rhamnose), and trehaloselipids (derived from trehalose).

Biosynthetic and regulatory pathways are best known in the cases of *Pseudomonas fluorescens*., producer of rhamnolipids and of *Bacillus subtilis*, producer of surfactin or subtilisin. In other cases, the depth of knowledge is still variable and very much strain dependent, so ageneral approach to production is unavailable.

Some production processes are well developed and at pre-commercial or commercial scale. For example: rhamnolipid production, which can also provide L-rhamnose, an intermediary in the food additive industry, and Emulsan (a polymeric bioemulsifier), are available commercially.

Due to their biodegradability they can safely be used in the environment without the risks observed of some of their chemically synthesized counterparts. Biosurfactants can be used for oil recovery, treatment of oil spillage and bioremediation, in foods, cosmetics and pharmaceuticals.

## 1. Introduction

Surfactants are surface-active, amphipathic molecules. They comprise both hydrophilic and hydrophobic moieties. Usually the hydrophobic domain is a hydrocarbon, whereas the hydrophilic group can be non-ionic, positively or negatively charged, or amphoteric. Surfactants have special properties due to the presence of a hydrophilic and hydrophobic group within the same molecule.

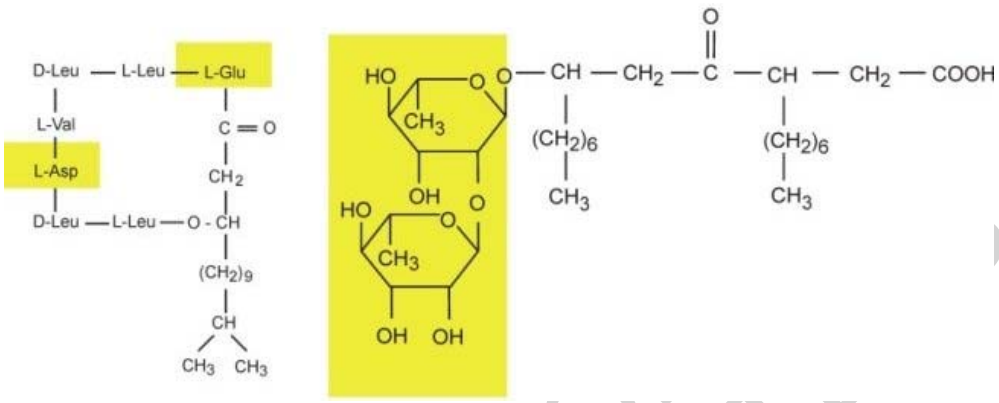


Figure 1. Surfactin, a lipopeptidic surfactant (left) and rhamnolipid, aglycolipid surfactant (right). The hydrophilic parts of the molecules are shaded.

They reduce the surface tension of aqueous media (air-water), and the interfacial tension of liquid-liquid (e.g. oil-water) or liquid-solid (e.g. wetting phenomena) systems. They also effect changes in the foaming properties of aqueous mixtures. Because of these properties, surfactants find application in a variety of industrial processes, such as bioremediation of oil-polluted soil and water (see *Bioremediation in the Marine Environment*), enhanced oil recovery, use in the detergent industry, and formation of stable oil-in-water emulsions for the food (see *Food Processing and Preservation Technology*) and cosmetic industries. The economic importance of surfactants is reflected by increasing worldwide consumption. The market value for surfactants reached US$ 39 billion in 1990. It is estimated that the worldwide demand for surfactants will have increased by 35% by 2000. However, nearly all the surface-active compounds currently in use are chemically synthesized. Some of these synthetic surfactants are partly toxic and not so readily biodegradable (see *Biodegradation of Xenobiotics*). Under this aspect, biological sources of surfactants or surfactant precursors are of interest.

Biosurfactants are defined as surface-active compounds produced by living cells, usually by microorganisms. Very often the growth of microorganisms on hydrocarbons is accompanied by the emulsification of the hydrocarbon in the medium, and in most cases this has been attributed to the production of surface-active compounds. Frequently, biosurfactants have several advantages over synthetic surfactants such as: higher biodegradability; lower toxicity; good biocompatibility with eucaryotic organisms; effectiveness at a wide range of temperatures, pH values, and salinities; synthesis under user-friendly conditions (e.g. low temperatures and pressures). An additional advantage is the chemical diversity of biosurfactants, offering a wider

selection of surface-active agents with properties closely tailored to specific applications.

This diversity in the chemical structure, composition, and physical properties of biosurfactants can be increased by genetic, biological, or chemical manipulations. However, biosurfactants have not yet been employed extensively in industry due to technical and/or economic reasons. To reduce the costs of biosurfactant production, it is necessary to select microorganisms capable of high-yield production and to optimize large-scale fermentation (see *Process Optimization Strategies for Biotechnology Products*) and recovery conditions.

Most biosurfactants are exolipids, although in some cases the surfactant is cell bound. The excretion can be detected in certain phases of the growth cycle (the late exponential or stationary phases) (see *Microbial Cell Culture*,) and probably depends on the metabolic state of the cell, which is also reflected by the morphology of the cell-boundary. In addition, it is frequently possible to change the fermentation conditions to alter the relative distribution of the cell bound (non-ionic) and cell free (anionic) forms.

This specificity seems to be a general feature of such molecules, related to whether the cells need to emulsify the immiscible carbon source with the extracellular anionic surfactant or adhere to the hydrocarbon directly via the non-ionic cell-bound surfactants. Some amphipathic molecules, usually of high molecular weight, can display emulsifying properties (bioemulsifiers), and are sometimes included in the general term biosurfactant, although occasionally they do not significantly reduce surface tension.

In addition to production by growing cells, resting cell systems have been described which are able to produce biosurfactants. These systems may be modified to use inexpensive, renewable carbon sources. The economic, ecological, and technological potential of biosurfactants is enormous but remains largely untapped today.

## 2. Evaluation

Surfactants are characterized functionally mainly by their ability to change surface-surface interactions. Quantitative parameters are the surface tension, the critical micelle concentration, and the hydrophilic and lipophilic balance.

Surface tension is an important parameter since, due to their amphiphilic character, surfactants partition preferentially at the interface between fluid phases of different degrees of polarity and hydrogen bonding, such as oil-water, air-water or solid-water interfaces. The formation of such an ordered molecular film at the interface lowers the interfacial energy and tension, and is responsible for the unique properties of surfactant molecules.

Measurements of surface tension at oil-water and air-water interfaces are easily performed with a tensiometer. However, it is difficult to quantify the surface tension at a solid-water interface. The surface tension of distilled water is 72mN $m^{-1}$, and addition of a good surfactant lowers this value to approximately 30mN $m^{-1}$. Similarly, the hexadecane-water interfacial tension is reduced from 40mN $m^{-1}$ to approximately 1mN

$m^{-1}$. The interfacial properties of surfactants depend on the ionic composition of the aqueous phase.

The critical micelle concentration (CMC) is one of the most widely used parameters for evaluating surfactant activity. At concentrations above the CMC, amphiphilic molecules associate readily to form supramolecular structures like micelles, bilayers, and vesicles. The forces that maintain these structures include hydrophobic, van der Waal's, electrostatic, and hydrogen bonding interactions.

The hydrophilic and lipophilic balance (HLB) is a commonly used parameter to correlate structure, interfacial functionality, and the CMC. The HLB value indicates whether a surfactant will promote water-in-oil, or oil-in-water emulsions. A new surfactant is compared with the surfactants of known HLB values.

The HLB scale can be constructed by assigning a value of 1 to oleic acid and a value of 20 to sodium oleate; intermediate values are derived by mixing oleic acid and oleate in varying proportions. Generally, surfactants with HLB values of less than 6 are more soluble in the oil phase and favour stabilization of water-in-oil emulsion. Surfactants with HLB values between 10 and 18 have the opposite effect and favor oil-in-water emulsification.

## 3. Structural Types and Producers

Biosurfactants may be classified according to their chemical structure, function, and their microbial origin. The hydrophobic moiety of biosurfactants consists of the hydrocarbon chain of a saturated, unsaturated, hydroxylated, or branched fatty acid. The hydrophilic moiety may be as simple as the carboxylategroup of fatty acids or amino acids or the phosphate-containing portions of phospholipids; or as complex as the mono-, di-, and polysaccharides of glycolipids, and the polar side chains and peptide backbone of lipopeptide biosurfactants. Biosurfactants from *Corynebacterium*, *Mycobacterium* and related microorganisms also contain alpha-branched beta-hydroxymycolic acids with very long chains, containing approximately 30 to 90 carbon atoms.

The hydrophilic part of biosurfactants is responsible for their degree of solubility in water. The lipophilic part is responsible for capillary activity. The two parts are joined by (a) ester linkage (including lactones) with organic and inorganic acids; (b) amide linkage (single and peptide); or (c) glycosidic linkage (sugar-sugar and sugar-hydroxy fatty acids). The ionization of functional groups plays an important role in surface activity, especially if simple carboxylic acids act at the water-oil interface. A reasonable classification of biosurfactants can be based on molecular weight or charge.

In Table 1 some representative values of the most promising and extensively studied biosurfactants are listed. The most widespread microbial surfactants areglycolipids, but lipopeptide biosurfactants are structurally more heterogeneous. Care should be taken in comparing the interfacial properties presented in Table 1 because the experimental conditions used in different reports vary significantly. Some biosurfactants also exhibit good thermal and chemical stability.

| Biosurfactant | Producing organism | C-source | Localization[1)] | Charge | Surface tension (mN $m^{-1}$) | CMC (mg $l^{-1}$) | Interfacial Tension (mN $m^{-1}$) |
|---|---|---|---|---|---|---|---|
| Cellobioselipids | *Ustilago* sp. | Vegetable oil | c.f. | anionic | 30 | 20 | < 1 |
| Corynomycolates | *Arthrobacter* sp. | different sugars | c.b. | non-ionic | 33–46 | 1–19 | 1–19 |
| Mannosylerythriol lipids | *Candida* sp. | glucose, soybean oil | c.f. | non-ionic | 28 | | < 1 |
| Rhamnoselipids | *Pseudomonas* sp. | n-alkanes, glycerol | c.f. | anionic | 25–31 | 10–200 | 4–<1 |
| Sophoroselipids | *Torulopsis* sp. | glucose, vegetable oil | c.f. | non-ionic /anionic[2)] | 25–35 | 60–82 | 1–9 |
| Trehalose coryno-mycolates | *Rhodococcus erythropolis* | n-alkanes, carbohydrates | | | | | |
| mono | | | c.b. | non-ionic | 32 | 3 | 16 |
| di | | | c.b. | non-ionic | 36 | 4 | 17 |
| tri | | | c.b. | non-ionic | 26 | 15 | < 1 |
| Lipopeptides | Bacillus licheniformis | glucose | c.f. | non-ionic | 27 | 0.02–10 | < 1 |
| Surfactatin | Bacillus subtilis | glucose | c.f. | non-ionic | 27 | 5 | 1 |

[1)] c.b. = cell-bound; c.f. = cell-free (extracellular)
[2)] in dependence on culture conditions

Table 1. Some properties of selected biosurfactants from a range of microorganisms.

The biosurfactants discussed in this article differ from high molecular weight biological molecules, which are sometimes classified as bioemulsifiers. Bioemulsifier are also synthesized by micoorganisms and include lipoproteins, lipopolysaccharide-protein complexes, or polysaccharide-protein-fatty acid complexes. These compounds do not reduce interfacial tension but stabilize oil-in-water emulsions. The term bioemulsifier is often used in an application-oriented manner. The most representative bioemulsifier is emulsan, currently the only such product on the market.

Many microorganisms, such as bacteria, yeasts, and filamentous fungi, are able to produce surface-active agents during their growth. These surfactant producing microorganisms may be divided into three groups with respect to alkane utilization and the synthesis of extracellular lipids:

- Microorganisms which produce biosurfactants exclusively during growth on alkanes (e.g. some strains of *Arthrobacter* sp., *Corynebacterium* sp. and *Nocardia* sp.)
- Microorganisms that produce biosurfactants on both alkanes and water-soluble compounds. An extraordinary number of producers fall into this category with the best-known example being *Pseudomonas aeruginosa* producing rhamnolipids.
- Microorganisms that exclusively produce biosurfactants duringgrowth on water-soluble compounds. Surfactin-producing *Bacillus subtilis* is one example. Some species of the yeast *Rhodotorula* produce a mixture of mannitol- and pentitolesters of beta-D-hydroxypalmitic acid and beta-D-hydroxystearic acid duringgrowth on a complex medium withglucose as carbon source. Theglycolipid is partly acetylated.

## 4. Biosynthesis and Regulation

Biosurfactants display a range of different amphiphilic structures (see Section 3). In biosurfactants of all species, hydrophobic and hydrophilic moieties are present within the molecule, which means that at least two different synthetic pathways must be considered: one leading to the hydrophilic and one to the hydrophobic moiety. The hydrophobic fatty acid components—which may be a long-chain fatty acid, a hydroxy fatty acid, or alpha-alkyl-beta-hydroxy fatty acid are synthesized by rather common pathways of lipid metabolism. The hydrophilic moieties, however, exhibit a greater degree of structural complexity. This explains the wide variety of biosynthetic pathways involved in their synthesis.

For the biosynthesis of such amphiphilic molecules four principle possibilities are well accepted:

1. hydrophilic and hydrophobic moieties are synthesized *de novo* via independent pathways;
2. the hydrophilic moiety is synthesized *de novo* and the substrate induces the hydrophobic moiety;
3. the synthesis of the hydrophilic moiety is substrate dependent while the hydrophobic moiety is synthesized *de novo;*

4. the synthesis of both residues depends on the carbon substrate used.

The elucidation of these mechanisms for a particular system is important for design of growth media and growth conditions for large-scale production, as well as for the induction of biosynthetic pathways by addition of precursor molecules (see Section 6). The carbon source influences the biosurfactant synthesis either by induction or repression.

Some bacterial systems are relatively well understood in regard to the biosynthetic mechanisms involved. Two of them will be discussed in brief: The first is the formation of rhamnolipid by *P. aeruginosa* and the second is the formation of surfactin by *Bacillus* sp.

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**Biographical Sketches**

**Faustino A. Siñeriz** graduated at the University of Buenos Aires in 1965 and received his Ph.D. in 1973 at the same University. From 1974 to 1977 he did post-doctoral studies at Queen Elizabeth College, University of London, with Professor John Pirt and at the New York State Health Department in Albany. He was an Alexander von Humboldt fellow at the University of Konstanz, Germany, in 1984–1985. He held several positions at the University of Buenos Aires, University of Cordoba, and University of Tucumán, where he is now Professor of Microbiology. In 1978 he entered a Research career in CONICET and since 1986 has been Director of PROIMI, a research institute from CONICET specializing in fermentations and microbial biotechnology. His research interests include microbial physiology applied to biotechnological processes, continuous culture, bioremediation, and wastewater treatment. He has participated as author or coauthor in more than 80 scientific publications in international journals. He is a fellow of the American Academy of Microbiology since 1998.

**Rolf K. Hommel** received his diploma degree in biochemistry in 1976 and his Ph.D. in 1983 both from the University of Leipzig. From 1982 to 1987 he held different positions in industrial biotechnological research centers. Main topics of his work were bacterial degradation of pollutants like hydrocarbons and microbial formation of phytohormones by fungi. From 1987 to 1992 he was a postdoctoral fellow at the University of Leipzig, where his research efforts focused on microbial physiology and biosurfactant production by yeasts and bacteria. Special emphasis was directed to fermentation technology, enzymology, biotransformation, and *in vivo* NMR studies. Since 1994 he is head of microbiology at IfN gmbH and at the CellTechnologie, Leipzig. There he has been involved in the development and optimization of several remediation processes using microbial consortia in oil- and polycyclic aromatic hydrocarbon-contaminated soils and water. He is author and coauthor of several reviews and papers on biosurfactants, bacterial enzymes of alkane metabolism, etc.

**Hans-Peter Kleber** finished the study of medicine in 1963 and received in the same year his Ph.D. (Dr. med.) from the University of Leipzig, Germany. Following this he worked for one year as an assistant physician at various clinics of the Medical Faculty in Leipzig. From 1964–1974 he was an Assistant Researcher and Senior Research Worker at the Institute of Physiological Chemistry and the Department of Biochemistry, respectively, of the University Leipzig. After his "Habilitation" (1972) he became a biochemistry lecturer at the University of Leipzig in 1974. Since 1978 he has been professor for biochemistry and enzymology, at the University of Leipzig. Main topics of his research work are the bacterial degradation of hydrocarbons including bacterial cytochrome P-450 and biosurfactant production by bacteria and yeasts, and the metabolism of quaternary ammonium compounds. He is author and coauthor of several text books, lexicons, reviews, and papers on biosurfactants.