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MINI-REVIEW

Microbial biosurfactants production, applications and future potential

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Abstract Microorganisms synthesise a wide range of surface-active compounds (SAC), generally called biosurfactants. These compounds are mainly classified according to their molecular weight, physico-chemical properties and mode of action. The low-molecular-weight SACs or biosurfactants reduce the surface tension at the air/water interfaces and the interfacial tension at oil/water interfaces, whereas the high-molecular-weight SACs, also called bioemulsifiers, are more effective in stabilising oil-in-water emulsions. Biosurfactants are attracting much interest due to their potential advantages over their synthetic counterparts in many fields spanning environmental, food, biomedical, and other industrial applications. Their large-scale application and production, however, are currently limited by the high cost of production and by limited understanding of their interactions with cells and with the abiotic environment. In this paper, we review the current knowledge and the latest advances in biosurfactant applications and the biotechnological strategies being developed for improving production processes and future potential.

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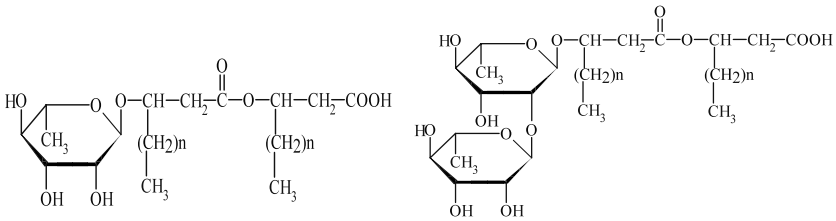
Introduction

Microbial surface-active compounds

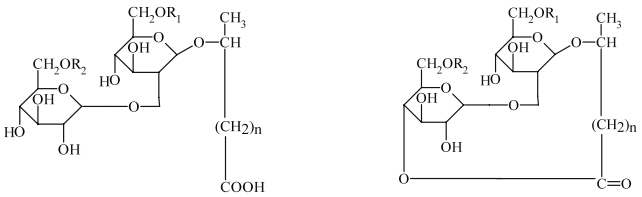
Microbial surface-active compounds are a group of structurally diverse molecules produced by different microorganisms and are mainly classified by their chemical structure and their microbial origin. They are made up of a hydrophilic moiety, comprising an acid, peptide cations, or anions, mono-, di- or polysaccharides and a hydrophobic moiety of unsaturated or saturated hydrocarbon chains or fatty acids. These structures confer a wide range of properties, including the ability to lower surface and interfacial tension of liquids and to form micelles and microemulsions between two different phases. These compounds can be roughly divided into two main classes (Neu 1996): low-molecular-weight compounds called biosurfactants, such as lipopeptides, glycolipids, proteins and high-molecular-weight polymers of polysaccharides, lipopolysaccharides proteins or lipoproteins that are collectively called bioemulsans (Rosenberg and Ron 1997) or bioemulsifiers (Smyth et al. 2010b). The former group includes molecules which can efficiently reduce surface and interfacial tension, while the latter are amphiphilic and polyphilic polymers which are usually more effective in stabilising emulsions of oil-in-water but do not lower the surface tension as much (Smyth et al. 2010a).

The best-studied microbial surfactants are glycolipids. Among these, the best-known compounds are rhamnolipids, trehalolipids, sophorolipids and mannosylerythritol lipids (MELs) (Fig. 1), which contain mono- or disac-



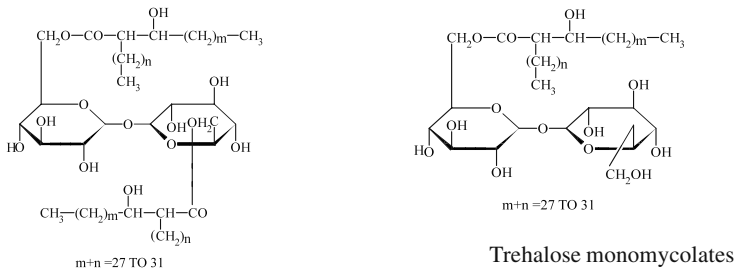
Monorhamnolipids

Dirhamnolipid



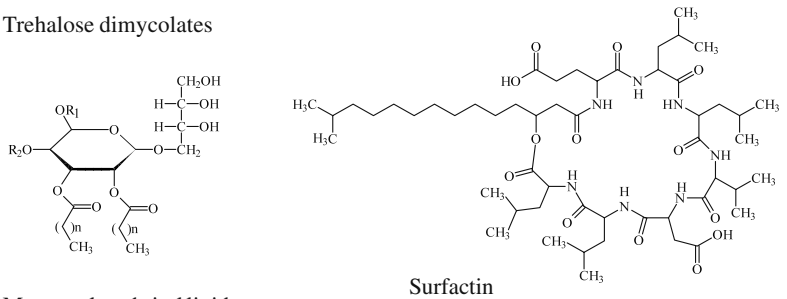
Acidic Sophorolipid

Lactonic Sophorolipid



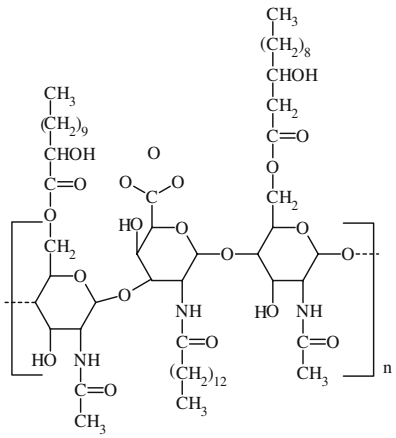
Trehalose dimycolates

Trehalose monomycolates



Mannosylerythritol lipids

Surfactin



Emulsan

Fig. 1 Chemical structure of the most studied microbial surface-active compounds; mono- and dirhamnolipids, acidic and lactonic sophorolipids, monomycolates trehalose lipid and dimycolates trehalose lipids, mannosylerythritol lipids, surfactin and finally emulsan

charides, combined with long-chain aliphatic acids or hydroxyaliphatic acids. Rhamnolipid production by *Pseudomonas* species has been extensively studied, and potential applications have been proposed (Maier and Soberón-Chávez 2000). Rhamnolipids from *Pseudomonas aeruginosa* are currently commercialised by Jeneil Biosurfactant, USA, mainly as a fungicide for agricultural purposes or an additive to enhance bioremediation activities. Trehalolipids are produced by a number of different microorganisms, such as *Mycobacterium*, *Nocardia* and *Corynebacterium*. However, the most extensively studied compounds in this class are trehalose dimycolates produced by *Rhodococcus erythropolis* (Rapp et al. 1979). Sophorolipids, on the other hand, are produced mainly by yeasts, such as *Candida bombicola* (also known as *Torulopsis bombicola), Centrolene petrophilum, Candida apicola* and *Rhodotorula bogoriensis*, while MELs are produced by *Pseudozyma* yeasts, *Pseudozyma aphidis, Pseudozyma antarctica* and *Pseudozyma rugulosa* (Konishi et al. 2007a, b). Cyclic lipopeptides are produced by a number of *Bacillus* species as antibiotic molecules. Among these, the most important compound is surfactin produced by *Bacillus subtilis* because of its very high activity (Desai and Banat 1997; Rosenberg and Ron 1999). A wide variety of microorganisms, including some *Archaea*, produce high-molecular-weight polymers, the most extensively investigated being bioemulsans (Fig. 1) which are synthesised by various species of *Acinetobacter*. The first studied compound was RAG-1 emulsan, an amphiphilic polysaccharide produced by *Acinetobacter calcoaceticus* RAG-1, which is also the only commercially available bioemulsifier at present (Suthar et al. 2008).

Potential applications

Environmental applications

In many cases, environmental contamination caused by industrial activity is due to accidental or deliberate release of organic and/or inorganic compounds into the environment. Such compounds pose problems for remediation, as they become easily bound to soil particles. The application of biosurfactants in the remediation of organic compounds, such as hydrocarbons, aims at increasing their bioavailability (biosurfactant-enhanced bioremediation) or mobilising and removing the contaminants by pseudosolubilisation and emulsification in a washing treatment. The application of biosurfactants in the remediation of inorganic compounds such as heavy metals, on the other hand, is targeted at chelating and removal of such ions during a washing step facilitated by the chemical interactions between the amphiphiles and the metal ions.

Biosurfactant-enhanced bioremediation

Amphiphiles are able to alter the physico-chemical conditions at the interfaces affecting the distribution of the chemicals among the phases (Tiehm 1994). For instance, a hydrocarbon-contaminated soil contains at least six phases: bacteria, soil particles, water, air, immiscible liquid and solid hydrocarbon. The hydrocarbons can be partitioned among different states: solubilised in the water phase, ad/absorbed to soil particle, sorbed to cell surfaces and as a free/insoluble phase. Biosurfactants added to this system can interact with both the abiotic particles and the bacterial cells.

This affects the mechanisms of interaction with environments with regard to the micellarisation and emulsification of organic contaminants, the interaction with sorbed contaminants and the sorption to soil particles which leads to the alteration of cell-envelope composition and hydrophobicity. The interactions between micelles and cells are among the main alterations to the bacterial component (Volkering et al. 1998). These phenomena on the one hand can be exploited to increase the bioavailability of poorly soluble contaminants, thus increasing biodegradation rate, or on the other hand, can result in an inhibition of biodegradation.

In spite of the publication bias which favours an overpublication of successful applications, the main emerging feature of the large body of literature in this area is the contrasting result reported on efficiency. For instance, rhamnolipids can stimulate the degradation of *n*-hexadecane by the producer strain *P. aeruginosa*, but didn't stimulate degradation by *Rhodococcus* strains showing strain specificity. In contrast, biosurfactants from *R. erythropolis* strain 3C-9 significantly increased the degradation rate of *n*-hexadecane by two phylogenetically distant species, *Alcanivorax dieselolei* and *Psychrobacter celer*, in flask tests (Noordman and Janssen 2002; Peng et al. 2007). Therefore, with the current state of knowledge, the modelling of the effect of biosurfactant addition in bioremediation treatment is not predictable, and efficacy has to be evaluated experimentally (Franzetti et al. 2006, 2008b). To gain better insight into this problem, it is useful to review the current knowledge and recent advances regarding these interactions.

For excellent reviews about interactions between surfactants and the environment, see Volkering et al. (1998) and Paria (2008). The interactions between bacteria, contaminants and biosurfactant can be interpreted from a functional perspective, considering that the main natural role attributed to biosurfactants is their involvement in hydrocarbon uptake (Perfumo et al. 2010a). Microbial surfactants can promote the growth of bacteria on hydrocarbons by increasing the surface area between oil and water and through emulsification and increasing pseudosolubility of

hydrocarbons through partitioning into micelles (Miller and Zhang 1997; Volkering et al. 1998).

High-molecular-weight biosurfactants (bioemulsifiers) have great potential for stabilising emulsions between liquid hydrocarbons and water, thus increasing the surface area available for bacterial biodegradation. However, they have been rarely tested as enhancers of hydrocarbon biodegradation in bioremediation systems, and contrasting results are reported in the literature (Barkay et al. 1999; Franzetti et al. 2009a).

For low-molecular-weight biosurfactants, above the Critical Micelle Concentration (CMC), a significant fraction of the hydrophobic contaminant partitions in the surfactant micelle cores. In some cases, this results in a general increase in the bioavailability of contaminants for degrading microorganisms. Successful applications of rhamnolipids and surfactin in enhanced bioremediation have been recently reviewed (Mulligan 2009). In addition, Wang and Mulligan (2009) studied the effect of ammonium ion concentration and pH on the potential application of rhamnolipid and surfactin for enhanced biodegradation of diesel. A lipopeptide and protein–starch–lipid produced by two strains of *P. aeruginosa* significantly enhanced the solubilisation of phenanthrene, pyrene and fluorene, increasing their metabolism and supporting sustained growth (Bordoloi and Konwar 2009). Polycyclic Aromatic Hydrocarbons (PAH) biodegradation was also investigated by Das et al. (2008b); they used *Bacillus circulans* to increase the bioavailability of anthracene. Interestingly, the organism had better growth and biosurfactant production on glycerol containing mineral medium supplemented with anthracene, although it was unable to utilise anthracene as the sole carbon source. These authors were able to demonstrate, however, that anthracene was used as a substrate for the production of the biosurfactant.

The specific modes of hydrocarbon uptake, however, are not fully understood. Recently Cameotra and Singh (2009) elucidated the mechanism of *n*-hexadecane uptake mediated by rhamnolipids in *P. aeruginosa*. The rhamnolipids produced an emulsion with hexadecane, thus facilitating increased contact between the hydrocarbon substrate and the bacteria. It was also observed that uptake of the biosurfactant-coated hydrocarbon droplets occurred, suggesting a mechanism like pinocytosis taking place, a process not previously reported in bacterial hydrocarbon uptake systems.

In contrast, it is well known that the presence of a surfactant can detrimentally affect biodegradation. Micelle cores can trap organic contaminants, creating a barrier between microorganisms and organic molecules, resulting in the potential substrate becoming less rather than more available. For example, Witconol SN70, a non-ionic alcohol ethoxylate surfactant (Colores et al. 2000), reduced the biodegradation rate of hexadecane and phenanthrene, with biodegradation similarly inhibited by Tween 20, sodium dodecyl sulfonate, tetradecyl trimethyl ammonium bromide and Citrikleen at concentrations equal or greater than their CMCs (Billingsley et al. 1999).

Another proposed role of biosurfactants in hydrocarbon uptake is the regulation of cell attachment to hydrophobic and hydrophilic surfaces by exposing different parts of cell-bound biosurfactants, thus changing cell-surface hydrophobicity (Rosenberg et al. 1987; Franzetti et al. 2008a).

This natural role can be exploited by adding (bio) surfactants to increase the hydrophobicity of degrading microorganisms and to allow cells' easier access to hydrophobic substrates (Shreve et al. 1995). The release of LPS by *Pseudomonas* spp. induced by sub-CMC levels of rhamnolipids allowed a more efficient uptake of hexadecane by rendering the cell surface more hydrophobic (Al-Tahhan et al. 2000). Noordman and Janssen (2002) reported that rhamnolipid produced by *P. aeruginosa* UG2 facilitated the hydrocarbon uptake of the producer strain and increased the degradation of hexadecane, while the same product did not stimulate to the same extent the biodegradation of hexadecane by four unrelated species (*Acinetobacter lwoffii* RAG1, *R. erythropolis* ATCC 19558, *R. erythropolis* DSM 43066 and strain BCG112), nor was degradation of hexadecane stimulated by addition of the biosurfactants produced by these species themselves. Zhong et al. (2007) showed that the adsorption of dirhamnolipid biosurfactants on cells of *B. subtilis*, *P. aeruginosa* and *Candida lipolytica* depended on the physiological status of the cells and was specific to the microorganisms. Furthermore, the biosurfactant adsorption affected the cell-surface hydrophobicity depending on the rhamnolipid concentration and the physiological state of the cell. The effect of exogenous rhamnolipids on cell-surface composition of *P. aeruginosa* NBIMCC 1390 was recently studied by Sotirova et al. 2008. They showed that above the CMC, rhamnolipids caused a 22% reduction of total cellular LPS content, while at concentrations below the CMC, they caused changes in the bacterial outer membrane protein composition yet did not affect the LPS component.

Chang et al. (2009) demonstrated that the cell-surface hydrophobicity was enhanced by the accumulation at the cell surface of different fatty acids during growth on hydrocarbon in *R. erythropolis* NTU-1. A significant correlation between the modification of the cell surface by saponins and the degree of hydrocarbon biodegradation was reported by Kaczorek et al. (2008).

Biosurfactant-enhanced soil washing

The application of microbial SACs to remove contaminants from soils is a technology characterised by less

uncertainty than biosurfactant-enhanced bioremediation, since the removal efficiency is mainly driven by the chemico-physical properties of the biosurfactants and not their effect on metabolic activity or changes in cell-surface properties. However, the mechanisms affecting hydrocarbon mobilisation or removal from soils resemble those involved in enhancing bioavailability for bioremediation. The ability to stabilise oil/water emulsions and increase hydrocarbon solubility enhances biodegradation and hydrocarbon removal from soils (Franzetti et al. 2010). These mobilisation and solubilisation effects occur at concentrations above and below the CMC. Mulligan (2009) reviewed the application of biosurfactants in enhanced soil washing for hydrocarbon- and metal-contaminated soils. More recently, Franzetti et al. (2009b) reported efficient removal of crude oil from soil using extracellular bioemulsifier produced by *Gordonia* sp. BS29. Interesting papers reporting enhanced metal removal or mobilisation have been published in the past 2 years. Biosurfactants are efficient in removing bulk arsenic from mine tailings or contaminated soils under alkaline conditions (Wang and Mulligan 2009). Das et al. (2009) showed that cadmium removal from aqueous solution also occurred at concentrations less than the CMC, while at a concentration of five times, the CMC resulted in almost complete removal of 100 ppm of metal ions. Wen et al. (2009) studied rhamnolipid degradation in soils contaminated by Cd and Zn, they suggested that rhamnolipid in the soil remain long enough to enhance metal phytoextraction, yet not long enough to raise concerns regarding metal transport in the long term.

Industrial applications

The main industrial application for biosurfactants is in the field of oil recovery and processing. Since traditional oil recovery technologies can only recover approximately 40–45% of the oil present in the reservoir, some technologies, collectively defined as enhanced oil recovery (EOR), have been developed (Banat 1995; Dastgheib et al. 2008). Among these, microbial EOR (MEOR), which takes advantage of microbial production of surface-active compounds, is considered to have the most cost-effective potential (Sen 2008). When poor oil recovery from oil wells is due to either low permeability of the rocks forming the reservoir or to the high viscosity of the crude oil, the ability of biosurfactants to reduce the oil/water interfacial tension and to form stable emulsions can improve the process efficiency. Three different strategies have been identified for MEOR: biosurfactant production in offsite reactors and subsequent addition to the oil reservoir; biosurfactant production by injected allochthonous microorganisms; injection of nutrients into the reservoir to stimulate biosurfactant production in situ by indigenous bacteria (Singh et al. 2007).

At present, the first strategy is the most exploited. In practice, a major obstacle to the in situ production of biosurfactants is the difficulty of isolating microbial strains adapted to the extreme environment of the reservoirs, which features high pressure, salinity, temperatures up to 85°C and extreme pH values. All the main types of microbial surface-active compounds have been proposed for a MEOR application. Although rhamnolipids have been most frequently used, lipopeptides, such as surfactin, lichenysin and emulsan have also proved very effective in enhancing oil recovery (Sen 2008). Xu et al. (2009) further demonstrated the effectiveness of a polysaccharide produced by *Streptococcus* sp. BT-003 for the same application. The use of biosurfactants in MEOR has been extensively reviewed (Banat et al. 2000; Singh et al. 2007; Sen 2008). Recently, several laboratory experiments were carried out to evaluate the effectiveness of biosurfactant-assisted oil extraction and recovery. Biosurfactants produced by *R. erythropolis* and *Rhodococcus ruber* were used to extract hydrocarbons from oil shale in flask experiments; the maximum recovery was 25% and 26% for the two strains, respectively, with even lower recovery when a high percentage of asphaltenes and resin compounds were present in the oil (Haddadin et al. 2009).

When the main purpose of the laboratory evaluation is a screening to test candidate molecules for full-scale application, most experiments are currently performed in sand-packed column systems to simulate oil reservoir conditions. Pornsunthorntawee et al. (2008) demonstrated that both *B. subtilis* PT2 and *P. aeruginosa* SP4 biosurfactants were more effective than three synthetic surfactants in oil recovery from a sand-packed column, the *B. subtilis* product being the most effective with an oil removal of 61% against 57% for the *P. aeruginosa* surfactants and about 4% when distilled water was used. Some other microbial surface-active compounds were also tested in column systems. For example, a bioemulsifier from *Bacillus licheniformis* K125, which reduced the surface tension to 34 mN/m, gave about 43% additional oil recovery after water extraction (Suthar et al. 2008), while some biosurfactants from different strains of *P. aeruginosa* gave 49–62% oil recovery (Bordoloi and Konwar 2008).

Although offsite biosurfactant production is the most common practice in MEOR, its potential has not been fully realised yet due to its high cost. To reduce costs, Wang et al. (2007) suggested selecting *Pseudomonas* strains, which can efficiently grow on renewable low-cost substrates and genetically engineer them to produce high yields of rhamnolipid. The prospect for such a strategy is probably quite poor since the production of rhamnolipids in *Pseudomonas* regulated through the quorum sensing

system and genetic intervention is difficult. However, an alternative would be an in situ production of biosurfactants, either by injected bacteria or by stimulated autochthonous microorganisms. Therefore some effort has recently been put into isolating new surfactant-producing microbial strains using extreme conditions to reproduce those encountered in oil reservoirs (Agarwal and Sharma 2009), while other research has focused on selective activation of indigenous microorganisms able to enhance oil recovery (Bao et al. 2009). The potential utilisation of selected exogenous microoganisms can be assessed either in the laboratory or directly in the field. The performance of two bacteria, *B. subtilis* and *Leuconostoc mesenteroides*, biosurfactant- and exopolymer-producing strains, respectively, were evaluated by using oil-saturated glass micromodels of a fractured porous medium to determine oil recovery. *B. subtilis* gave better oil recovery due to the reduction of oil viscosity and the interfacial tension (Soudmand-Asli et al. 2007).

While a number of field trials of in situ applications of MEOR are reported in the literature (see Sen 2008 for a review), it has not been completely elucidated whether introduced microorganisms can actually be effective in oil recovery or if they are out-competed by indigenous bacteria. The inability to compare test wells with control wells subjected to similar treatment procedures without introducing live microorganisms or products makes valid conclusions difficult to draw. To provide better insight into the dynamics of the microbial community, Wang et al. (2008) monitored changes in the community using molecular markers by denaturing gradient gel electrophoresis in an oil reservoir during a process of MEOR. They observed that both exogenous and stimulated indigenous bacteria appeared to contribute to the increased oil recovery.

Beside applications in MEOR, microbial surface-active compounds can also be exploited for other applications in the oil industry. For example, the de-emulsifying properties shown by some biosurfactant-producing microorganisms may be used to break emulsions which form at various steps in oil extraction and processing, thus allowing a better recovery of the product. The surface-tension decrease produced by microbial surfactants can also be used to separate oil from tank bottom sludge (Singh et al. 2007; Joseph and Joseph 2009; Perfumo et al. 2010b).

Due to their physico-chemical properties, the use of microbial surface-active compounds has also been proposed for various industrial applications, as additives in foods, cosmetics and detergent formulations (Banat et al. 2000). In the food industry, the most useful property is the ability to form stable emulsions, which improves the texture and creaminess of dairy products. Biosurfactants are also used to retard staling, solubilise flavour oils and improve organoleptic properties in bakery and ice cream formulations and as fat stabilisers during cooking of fats. Although the addition of rhamnolipids has been suggested to improve dough characteristics of bakery products, the use as food ingredients of compounds derived from an opportunistic pathogen such as *P. aeruginosa* is not practically feasible. Instead, it has been suggested to use biosurfactants obtained from yeasts or *Lactobacilli*, which are generally recognised as safe and are already involved in several food-processing technologies (Nitschke and Costa 2007).

Wetting, dispersing and surface-tension reduction properties, as well as low toxicity and high biodegradability, suggested the application of biosurfactants, especially glycolipids, as components of detergent formulations. Low-foaming sophorolipids from *C. bombicola* appear suitable due to their high detergency ability, low cytotoxicity and high biodegradability and general environmentally acceptable properties (Hirata et al. 2009). Also, cyclic lipopeptide biosurfactants from *B. subtilis* improved wash performance by acting additively with other detergent components. Since they have shown better results at low temperature, such formulations are promising from an energy-saving point of view, allowing laundering at lower temperatures (Mukherjee 2007). Several surfactant–enzyme mixtures were tested for rubisco removal from both hydrophobic and hydrophilic surfaces; the most effective formulation was a surfactin–subtilisin A detergent, thus demonstrating that it would be possible to generate fully renewable cleaning formulations with good performance (Onaizi et al. 2009).

Biomedical applications

Biosurfactants, when present in heterogeneous systems, tend to aggregate at the phase boundaries or interfaces. Organic molecules present in aqueous phase are known to be inclined to immobilise at the solid interface is such interfacial systems. They eventually form a conditioning film, which will affect the properties (surface energy and wettability) of the original surface (Neu 1996). In a similar manner to organic-conditioning films, biosurfactants partition at the interfaces and can affect the adhesion properties of microorganisms. Another function valuable for medical application is their ability to disrupt membranes leading to cell lysis through increased membrane permeability leading to metabolite leakage. This occurs due to changes in physical membrane structure or through disrupting protein conformations which alters important membrane functions such as transport and energy generation (Van Hamme et al. 2006; Ortiz et al. 2008, 2009; Sotirova et al. 2008; Sánchez et al. 2009, 2010; Zaragoza et al. 2009).

Antimicrobial activity of biosurfactants

The high demand for new antimicrobial agents following increased resistance shown by pathogenic microorganisms against existing antimicrobial drugs has drawn attention to biosurfactants as antibacterial agents (Běhal 2006). Some biosurfactants have been reported to be suitable alternatives to synthetic medicines and antimicrobial agents and may therefore be used as effective and safe therapeutic agents (Cameotra and Makkar 2004; Singh and Cameotra 2004; Banat et al. 2000).

Lipopeptides form the most widely reported class of biosurfactants with antimicrobial activity. Surfactin, produced by *B. subtilis*, is the best-known lipopeptide (Arima et al. 1968). Other antimicrobial lipopeptides include fengycin, iturin, bacillomycins and mycosubtilins produced by *B. subtilis* (Vater et al. 2002). Lichenysin, pumilacidin and polymyxin B (Naruse et al. 1990; Yakimov et al. 1995; Grangemard et al. 2001; Landman et al. 2008) are other antimicrobial lipopeptides produced by *B. licheniformis*, *Bacillus pumilus* and *Bacillus polymyxa*, respectively. The production of antimicrobial lipopeptides by *Bacillus* probiotic products is one of the main mechanisms by which they inhibit the growth of pathogenic microorganisms in the gastrointestinal tract (Hong et al. 2005). Other reported biosurfactants having antimicrobial activity are daptomycin, a cyclic lipopeptide from *Streptomyces roseosporus* (Baltz et al. 2005), viscosin, a cyclic lipopeptide from *Pseudomonas* (Neu et al. 1990; Saini et al. 2008), rhamnolipids produced by *P. aeruginosa* (Abalos et al. 2001; Benincasa et al. 2004) and sophorolipids produced by *C. bombicola* (Kim et al. 2002; Van Bogaert et al. 2007). Mannosylerythritol lipids (MEL-A and MEL-B) produced by *Candida antarctica* strains have also been reported to exhibit antimicrobial action against Gram-positive bacteria (Kitamoto et al. 1993).

Recently, a lipopeptide biosurfactant produced by a marine organism, *B. circulans*, was found to be active against *Proteus vulgaris*, *Alcaligens faecalis*, methicillin-resistant *Staphylococcus aureus* (MRSA) and other multidrug-resistant pathogenic strains (Das et al. 2008a) while not having any haemolytic activity. A rhamnolipid surfactant produced from soybean oil waste had antimicrobial activity against several bacteria and fungi, namely *Bacillus cereus*, *S. aureus*, *Micrococcus luteus, Mucor miehei* and *Neurospora crassa* (Nitschke et al. 2009b). Flocculosin, a cellobiose lipid produced by the yeast-like fungus *Pseudozyma flocculosa*, was tested against clinical bacterial isolates and the pathogenic yeast *Candida albicans* (Mimee et al. 2009). The glycolipid was particularly effective against *Staphylococcus* species, including MRSA, and its antibacterial activity was not influenced by the presence of common resistance mechanisms (e.g. against methicillin and vancomycin) in tested strains. In addition, flocculosin was able to kill *C. albicans* cells in a very short period of time. Huang et al. (2007) observed that a lipopeptide antimicrobial substance produced by the strain *B. subtilis* fmbj, which is mainly composed of surfactin and fengycin, was able to inactivate endospores of *B. cereus*. Observation by TEM indicated that the lipopeptide could damage the surface structure of the spores.

Antiviral activity of biosurfactants, mainly surfactin and its analogues, has also been described (Naruse et al. 1990). The more effective inactivation of enveloped viruses, such as retroviruses and herpes viruses, compared to non-enveloped viruses, suggests that this inhibitory action may be mainly due to physico-chemical interactions between the virus envelope and the surfactant (Vollenbroich et al. 1997). Antimicrobial lipopeptides produced by *B. subtilis* fmbj inactivated cell-free virus of porcine parvovirus, pseudorabies virus, newcastle disease virus and bursal disease virus, while it effectively inhibited replication and infectivity of the newcastle disease virus and bursal disease virus but had no effect on pseudorabies virus and porcine parvovirus (Huang et al. 2006). Sophorolipids are also claimed to have activity against human immunodeficiency virus (Shah et al. 2005). Similarly, a rhamonolipid and its complex with alginate, both produced by a *Pseudomonas* sp. strain, showed significant antiviral activity against herpes simplex virus types 1 and 2 (Remichkova et al. 2008). The suppressive effect of the compounds on herpes simplex virus replication was dose-dependent and occurred at concentrations lower than the critical micelle concentration.

The antifungal activities of biosurfactants have long been known, although their action against human pathogenic fungi has been rarely described (Tanaka et al. 1997; Chung et al. 2000; Abalos et al. 2001). Recently, a glycolipid isolated from the yeast-like fungus *P. flocculosa*, named flocculosin was shown to display in vitro antifungal activity against several pathogenic yeasts, associated with human mycoses (Mimee et al. 2005). This product positively inhibited all pathogenic strains tested under acidic conditions and showed synergistic activity with amphotericin B, increasing its efficacy while decreasing any toxicity and other side effects.

The antifungal activity against phytopathogenic fungi has been demonstrated for glycolipids, such as cellobiose lipids (Teichmann et al. 2007; Kulakovskaya et al. 2009, 2010) and rhamnolipids (Debode et al. 2007; Varnier et al. 2009), and cyclic lipopeptides (Tran et al. 2007, 2008), including surfactin, iturin and fengycin (Velmurugan et al. 2009; Arguelles-Arias et al. 2009; Chen et al. 2009; Snook et al. 2009; Mohammadipour et al. 2009; Grover et al. 2010).

Biosurfactants as anti-adhesives

Biofilm formation and swarming motility are the key microbial activities in the colonisation of a surface and therefore can increase the chance of nosocomial infections on different medical devices (Khardori and Yassien 1995; Vinh and Embil 2005; McCann et al. 2008; Harriott and Noverr 2009). Current biofilm preventive strategies are essentially aimed at coating medical surfaces with antimicrobial agents (von Eiff et al. 2005; Basak et al. 2009). However, recent studies have suggested that non-antibiotic molecules naturally produced within bacterial communities, including secreted signalling molecules or surface-active biosurfactants, could also interfere with biofilm formation, modulating microbial interaction with interfaces (Neu 1996; Federle and Bassler 2003; Rasmussen and Givskov 2006; Rodrigues et al. 2006a). In addition to their direct action against pathogens, biosurfactants can also alter the physical and chemical condition of the environment where biofilms are developing (Mireles et al. 2001; Merk et al. 2005). Dealing with these biofilms is difficult yet an important goal, since microbes embedded within them are associated with many infections and usually become difficult to treat effectively with traditional antimicrobials (Morikawa 2006).

Recently, the capability of two lipopeptide biosurfactants, produced by *B. subtilis* V9T14 and *B. licheniformis* V19T21, to inhibit biofilm adhesion of pathogenic bacteria to polystyrene was demonstrated using the MBEC device (Rivardo et al. 2009). The two biosurfactants V9T14 and V19T21 showed interesting specific anti-adhesion activity being able to selectively inhibit biofilm formation by two pathogenic strains. In particular, *S. aureus* ATCC 29213 and *Escherichia coli* CFT073 biofilm formation were decreased by 97% and 90%, respectively. V9T14 biosurfactant active on the Gram-negative strain was ineffective against the Gram-positive and the opposite for V19T21 biosurfactant. This activity was observed either by coating the polystyrene surface or by adding the biosurfactant to the inoculum.

Two fractions from each purified biosurfactant, obtained by flash chromatography, fractions (I) (surfactin) and (II) (fengycin), showed that fraction (II) was responsible for the anti-adhesion activity in both strains. Moreover the V9T14 biosurfactant has been shown to increase biofilm eradication efficacy of different antibiotics against a urinary tract-infective *E. coli* strain (Martinotti et al. 2009—deposited patent). More recently, the activity of $AgNO_3$ combined with the lipopeptide biosurfactant V9T14 has been studied against a preformed *E. coli* biofilm on the Calgary Biofilm device (Rivardo et al. 2010). Results indicated that the activity of silver can be synergistically enhanced by the presence of V9T14, allowing a reduction in the quantity of silver used and greater antimicrobial activity. The concentration of silver in the silver–biosurfactant solution was from 129- to 258-fold less than the concentration when silver was used alone. Based on these results, an international patent PCT/IB2009/055334 entitled "Biosurfactant composition produced by a new *B. licheniformis* strain, uses and products thereof" has been deposited in 2009, inventors Martinotti M.G., Rivardo F. Allegrone G., Ceri H., Turner R. Unpublished preliminary results obtained by the same research group showed anti-adhesion effects of two lipopeptides produced by bacterial endophytes, isolated from oleander and rice, on the biofilm of two different pathogenic strains of *C. albicans* (Fig. 2).

Valle et al. (2006) observed that distinct serotypes of group II capsular polysaccharides, produced by the uropathogenic *E. coli* (UPEC strain CFT073) behaved like surface-active polymers that displayed anti-adhesion properties. The treatment of abiotic surfaces with group II capsular polysaccharides significantly inhibit mature biofilm development of a broad range of Gram-positive and Gram-negative bacteria.

Strategies for the prevention of microbial biofilm formation on silicone rubber voice prostheses on vinyl urethral catheters and on other material have also been described (Velraeds et al. 2000; Mireles et al. 2001; Rodrigues et al. 2004, 2006b, c, 2007).

Beside treatment of medical devices, biosurfactants have been used in the pre-treatment of material surfaces found in food-processing environments. *Listeria monocytogenes*, *Salmonella enteritidis* and *Enterobacter sakazakii* are examples of pathogenic bacteria implicated in outbreaks associated with the ingestion of contaminated food. Numerous studies have shown that these bacteria are able to adhere and to form biofilms on food-contact surfaces that are more resistant to sanitation than free-living cells (Kalmokoff et al. 2001; Stepanovic et al. 2004; Kim et al. 2006). The pre-conditioning of surfaces using microbial surface-active compounds could be an interesting strategy to prevent adhesion of food-borne pathogens to solid surfaces.

Meylheuc et al. (2006b) demonstrated that the pre-conditioning of stainless steel and polytetrafluoroethylene surfaces with an anionic biosurfactant produced by *Pseudomonas fluorescens* reduced the number of *L. monocytogenes* LO28-adhering cells and thus resembled the bactericidal activities of the disinfectants sodium hypochlorite (NaOCl) and peracetic acid/hydrogen peroxide (PAH). Similarly, the ability of adsorbed biosurfactants obtained from *Lactobacillus helveticus* and *P. fluorescens* to inhibit the adhesion of four *Listeria* strains to stainless steel was investigated (Meylheuc et al. 2006a). Whichever strain of *L. monocytogenes* used in combination with biosurfactants, the anti-adhesive biological coating developed both reduced

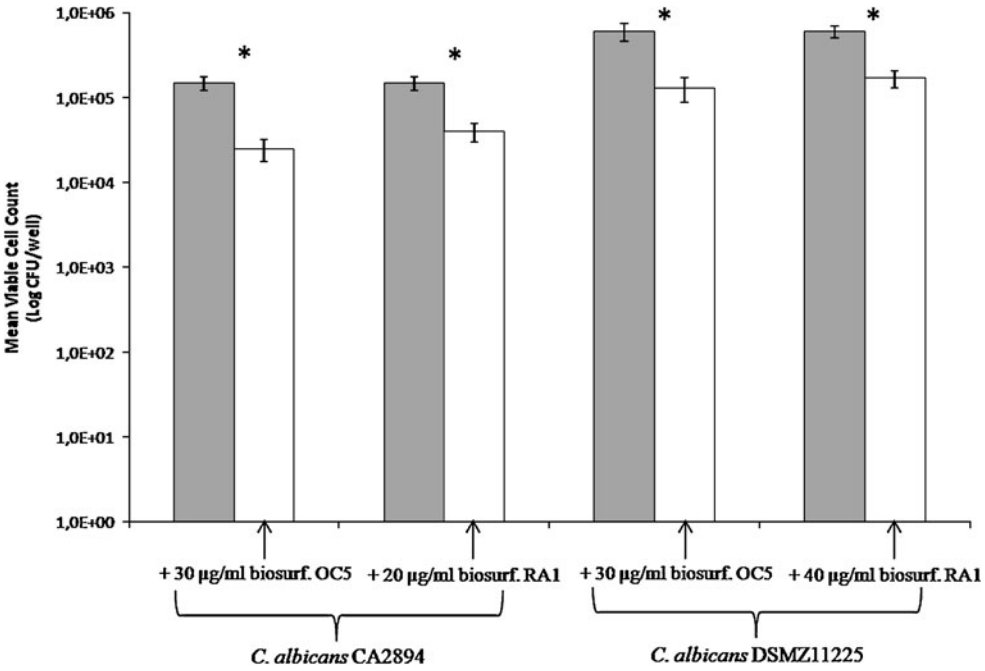


Fig. 2 Anti-adhesion activity of biosurfactants OC5 and RA1 produced by two bacterial endophytes against biofilm produced by *C. albicans* strains. *Shaded columns*, untreated controls; *white columns*, treatments with different biosurfactants concentrations. *Vertical bars* show the standard deviation of the mean based on three independent measurements. *$P<$ 0.05 calculated by *t* test

the total adhering flora and the viable and culturable adherent bacteria on stainless steel surfaces. More recently, another group investigated the effect of rhamnolipids and surfactin biosurfactants on the adhesion of the food pathogens *E. sakazakii, L. monocytogenes* and *S. enteritidis* to polypropylene and stainless steel surfaces (Nitschke et al. 2009a). Preconditioning with surfactin, rather than rhamnolipid, caused a reduction in the number of adhering cells particularly of *L. monocytogenes* and to some extent *E. sakazakii* on stainless steel. Surfactin showed a significant decrease in the adhesion on polypropylene of all strains. The adsorption of surfactin on polystyrene also reduced the adhesion of *S. enteritidis*- and *L. monocytogenes*-growing cells. In addition, surfactin was able to delay bacterial adhesion within short contact periods using non-growing cells or longer contact periods using growing cells.

Probiotics have long been known for their antimicrobial activity and for the capacity to interfere with the adhesion and formation of biofilms of pathogens to epithelial cells of urogenital and intestinal tracts (Reid et al. 1998, 2001), catheter materials (Hawthorn and Reid 1990) and voice prostheses (Rodrigues et al. 2004, 2006b), and the mechanisms of this interference have been demonstrated to include, among others, the release of biosurfactants (Velraeds et al. 1996; Rodrigues et al. 2006d; Gudiña et al. 2010). Probiotics are thus well known to have a positive effect on the maintenance of human health (Reid and Burton 2002; Merk et al. 2005; Gupta and Garg 2009).

Recent work by Walencka et al. (2008) demonstrated that surfactants obtained from three *Lactobacillus acidophilus* strains inhibited *Staphylococcus epidermidis* and *S. aureus* biofilm integrity and formation. Moreover, surfactant addition to preformed mature biofilms accelerated their dispersal and altered the characteristics of the biofilm morphology.

Another interesting application field for probiotics that is gaining more interest is their use in preventing oral infections. The role of probiotics on oral health has been thoroughly investigated (Çaglar et al. 2005; Meurman 2005; Meurman and Stamatova 2007; Hatakka et al. 2007; Kõll et al. 2008). Van Hoogmoed et al. (2004) demonstrated that *Streptococcus mitis* biosurfactant inhibited adhesion of *Streptococcus sobrinus* HG 1025 and *Streptococcus mutans* ATCC 25175 to bare enamel, while *S. mitis* biosurfactant was able to inhibit the adhesion of *S. sobrinus* HG 1025 to salivary pellicles. The authors later reported that these reductions may be attributed to increased electrostatic repulsion between the bacteria and the biosurfactant-coated pellicles (Van Hoogmoed et al. 2006).

Other biomedical and therapeutic applications

Biosurfactants have been shown to have many other roles in biomedical application. Surfactin is one of the most powerful biosurfactants and is known to have anti-inflammatory, antibiotic and anti-tumour functions (Seydlová and Svobodová 2008). Cao et al. (2010) demonstrated that surfactin induces apoptosis in human breast cancer MCF-7 cells through a ROS/JNK-mediated mitochondrial/caspase pathway, whereas Byeon et al. (2008) observed that surfactin was able to down-regulate LPS-induced NO production in RAW264.7 cells and

primary macrophages by inhibiting NF-κB activation. Park and Kim (2009) studied the role of surfactin in the inhibition of the immunostimulatory function of macrophages through blocking the NK-κB, MAPK and Akt pathway. This provided a new insight into the immunopharmacological role of surfactin in autoimmune disease and transplantation. Their work indicated that surfactin has potent immunosuppressive capabilities which suggested important therapeutic implications for transplantation and autoimmune diseases, including allergy, arthritis and diabetes.

Selvam et al. (2009) studied the effect of *B. subtilis* PB6, a natural probiotic, on plasma cytokine levels in inflammatory bowel disease and colon mucosal inflammation. The strain was found to secrete surfactins which are known to inhibit phospholipase A2, involved in the pathophysiology of inflammatory bowel disease. In animal experiments carried out in rat models for trinitrobenzene sulfonic acid-induced colitis, oral administration of PB6 as a probiotic suppressed colitis as measured by mortality rate and changes in colon morphology and weight gain. Plasma levels of pro-inflammatory cytokines were also significantly lowered and the anti-inflammatory cytokine significantly increased after the oral administration of PB6, supporting the concept that PB6 inhibits PLA2 by secreting surfactins. Han et al. 2008 observed that high surfactin micelle concentration affected the aggregation of amyloid β-peptide (Aβ (1-40)) into fibrils, a key pathological process associated with Alzheimer's disease. Another interesting property of surfactin and its synthetic analogues is the ability to alter the nanoscale organisation of supported bilayers and to induce nanoripple structures with intriguing perspectives for biomedical and biotechnological applications (Bouffioux et al. 2007; Brasseur et al. 2007; Francius et al. 2008). Fengycin, another lipopeptide biosurfactant is also able to cause membrane perturbations (Deleu et al. 2008). Recent results by Eeman et al. (2009) emphasised the ability of fengycin to interact with the lipid constituents of the stratum corneum extracellular matrix and with cholesterol.

The biological activities and the numerous potential applications of mannosylerythritol lipids (MELs), one of the most promising glycolipid biosurfactants produced by yeast strains of the genus *Pseudozyma,* have been thoroughly discussed by Kitamoto et al. (2009). Imura et al. (2007) and (2008), Ito et al. (2007) and Konishi et al. (2007a) developed and studied the kinetics of interactions in carbohydrate ligand systems composed of self-assembled monolayers of mannosylerythritol lipid-A (MEL-A) from *P. antarctica* serving as a high-affinity, easy to handle and low-cost ligand system for immunoglobulin G and M and lectins. Igarashi et al. (2006) reported that MEL-A significantly increased the cellular association and the efficiency of gene transfection mediated by cationic liposomes. Their results suggested that MEL-A enhanced the association of lipoplexes with the cells, delivered them widely into the cytoplasm and increased gene expression. Ueno et al. (2007a) observed that MEL-A-containing liposomes exhibited high activity in DNA capsulation and membrane fusion with anionic liposomes, which are important properties for gene transfection. On the other hand, MEL-B- and MEL-C-containing liposomes only increased either the capsulation or the membrane fusion. In another work, Ueno et al. (2007b) suggested that MEL-A was capable of increasing and rapidly promoting the transfection efficiency of target cells by inducing membrane fusion between liposomes and the plasma membrane of these cells.

In another work, a liposome vector containing beta-sitosterol beta-D-glucoside biosurfactant-complexed DNA was successfully used for herpes simplex virus thymidine kinase gene therapy (Maitani et al. 2006). More recently, nano-vectors containing a biosurfactant have been successfully used to increase the efficacy for gene transfection in vitro and in vivo (Nakanishi et al. 2009). On the other hand, Morita et al. (2009), using a three-dimensional cultured human skin model, observed that the viability of the SDS-damaged cells was markedly improved by the addition of MEL-A in a dose-dependent manner. This demonstrated that MEL-A had a ceramide-like moisturising activity toward the skin cells.

Another interesting application for natural surfactant is the possibility to synthesise metal-bound nanoparticles using an environmentally friendly technology benign (Palanisamy and Raichur 2009). The use of gold nanoparticles, in particular, is currently undergoing a dramatic expansion in the field of drug and gene delivery, targeted therapy and imaging (Pissuwan et al. 2009; Boisselier and Astruc 2009). Recently, Reddy et al. (2009) synthesised, for the first time, surfactin-mediated gold nanoparticles, opening the way to a new and fascinating application of biosurfactants in the biomedical field. Most recently Smyth et al. (2010c) reported on the production of selectively deuterated rhamnolipids and sophorolipids using deuterated substrates. The production of such deuterated biosurfactants in particular or other bioactive microbial products in general in which distinct pattern of labelling could be achieved would have great future implications with regards to efficacy and/or persistence or the development of resistance for some bioactives particularly in biomedical related applications.

Production and optimisation

Despite their environmentally favourable characteristics of higher biodegradability, lower toxicity, better foaming

properties compared to their synthetic chemical counterparts while also showing better stability at extreme pH, salinity and temperature, the commercialisation of microbial surfactants has not been fully achieved largely due to production costs. At present, the production costs for most biosurfactants do not compete with those of chemical surfactants. Different strategies have been proposed to make the process more cost effective including: (1) development of more efficient bioprocesses, including optimisation of fermentative conditions and downstream recovery processes, (2) use of cheap and waste substrates (Thavasi et al. 2007, 2008; Raza et al. 2009), (3) development of overproducing strains (Fig. 3). The increasing number of reports of potential antimicrobial and anti-adhesive properties of biosurfactants against pathogenic microorganisms (Rodrigues et al. 2006a) has added to the impetus towards sustainability and reduced carbon foot prints (the greening process) which are helping drive the market towards efficient large-scale production technologies. However, most biosurfactant research related to large-scale economic production trials has been mainly confined to microorganisms, such as *Pseudomonas, Bacillus* and *Candida* (Mukherjee et al. 2006).

Several developments in optimisation of culture conditions and downstream processing have been published recently. The use of agroindustrial by-products has been reported both for yeasts and bacteria (Makkar and Cameotra 2002). Sobrinho et al. (2008) used ground nut oil refinery residues and corn steep liquor as substrates for anionic glycolipid production by *Candida spherical*, while the biosynthesis of glycolipids by *P. aeruginosa* was obtained using cashew apple juice as substrate (Rocha et al. 2007) and vegetable oil refinery wastes (Raza et al. 2007). Very high potential for large-scale industrial application was achieved using the already commercialised Pharmamedia medium for surfactin production by *B. subtilis* MZ-7 (Al-Ajiani et al. 2007). The use of substrates such as soapstick, frying oil and motor oil have all been explored and have had limited success due to the need for more costly or demanding downstream processing. Novel strains able to produce biosurfactants on renewable and low-cost substrates have also been reported during the past few years. Ruggeri et al. (2009) isolated *Rhodococcus* sp. BS32 able to grow on rapeseed oil for the production of extracellular biosurfactants. Glycerol, however, has emerged as one important potential feedstock available in large quantities as a by-product of the biodiesel process (Zheng et al. 2008).

Experimental design techniques have been extensively used to optimise biosurfactant production. The use of surface response methodology effectively enhanced the production of biosurfactant by *Rhodococcus* spp. MTCC 2574 growing on *n*-hexadecane with yields of biosurfactant increasing from 3.2 to 10.9 g/L (Mutalik et al. 2008). Working with *Gordonia* sp. BS29, Franzetti et al. (2009a) increased the production of cell-bound glycolipids by 5-fold using surface response methodology, while the use of an artificial neutral network coupled with a genetic algorithm gave a 3.5-fold enhancement in biosurfactant yield (Pal et al. 2009). The same methodologies were applied by Sivapathasekaran et al. (2010) aimed at optimising biosurfactant production by *B. circulans* MTCC 8281. Kronemberger et al. (2008) reported a significant increase in yield by optimising cultural conditions using statistical tools. They also reported that the yields may be further enhanced by the development of new controlling devices such as oxygen control.

Downstream processing accounts for most of the total cost of a biotechnology product (Mukherjee et al. 2006). The most common isolation techniques for biosurfactants use precipitation, solvent extraction and chromatographic purification. Extraction of low-molecular-weight biosurfactant normally involves an optional precipitation step and the use of different organic solvents according to hydrophobicity and Hydrophilic-Lipophilic Balance (HLB) value of the compounds. Rhamnolipids are usually precipitated by acidification and extracted using ethyl acetate; extraction of sophorolipids is normally carried out

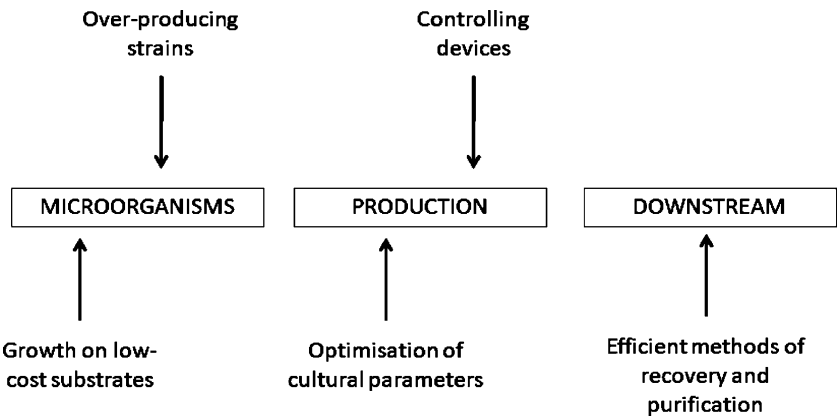


Fig. 3 Different cost-reduction strategies for production of microbial surface-active compounds

with *n*-hexane, while for trehalolipids, the preferred solvent is a mixture of chloroform and methanol. Methanol is also used as a solvent for extraction of lipopeptides after a precipitation by acidification (Smyth et al. 2010a). High-molecular-weight biosurfactants are usually extracted from the culture broth by ammonium sulphate precipitation and purified by dialysis. Other techniques for high-molecular-weight biosurfactant isolation are TCA/acetone precipitation, acid ethanol and chloroform/methanol (Smyth et al. 2010b). These techniques are already well established for lab-scale applications, but their cost does not allow scaling-up for industrial production of biosurfactants. For these reasons, the research effort is now directed towards the development of low-cost extraction and purification procedures, avoiding the use of hazardous and costly organic solvents.

Many advances have been observed in very recent years for recovery and purification of lipopeptides (Smyth et al. 2010a, b). Sivapathasekaran et al. (2009) developed and optimised an efficient method for the separation and purification of fengycin isoforms using high-performance liquid chromatography through manipulating the solvent gradient program and flow rates. Fengycin separation and purification was also obtained directly from the cultivation step without the use of solvent and foam formation by pressing and harvesting the liquid surface layer (Glazyrina et al. 2008). Dimitrov et al. (2008) applied liquid membrane extraction processes for recovery of surfactin, achieving 97% efficiency under optimised conditions. Chen and colleagues, in three different papers (Chen et al. 2008a, b; Chen and Juang 2008), optimised the recovery of surfactin from fermentation broths of *B. subtilis* ATCC 21332 by different methods and achieved improved purity by adsorption or ion exchange after the broth had been treated by a two-stage ultrafiltration process.

With regards to the development of over producer strains, genetic manipulation of selected strains remains limited. Although recombinant strains of *Bacillus* sp. and *Acinetobacter* sp. have been described, most genetic manipulation efforts have been directed towards *P. aeruginosa* due in part to its commercial potential and the more detailed knowledge of its genome. Random mutagenesis using gamma-ray or *N*-methyl-*N'*-nitrosoguanidine increased rhamnolipid production two- to threefold compared to wild strains (Mukherjee et al. 2006).

The ability to produce a hyper-producer strain of *P. aeruginosa*, however, is quite a difficult task due to the complexity of the transcriptional regulatory network of genes involved in rhamnolipid production. This is further complicated by the fact that rhamnolipids are produced as a mixture of congeners. Attempts have been made to limit the products to mono-rhamnolipids only through cloning the *P. aeruginosa rhlABRI* operon into host organisms such as *E. coli* or non-pathogenic *P. putida* (Cabrera-Valladares et al. 2006; Cha et al. 2008). Wang et al. (2007) also reported the use of genetic engineering to obtain an *E. coli* and *P. aeruginosa* that were able to produce rhamnolipids after transposon-mediated chromosome integration of the rhamnosyltrasferase 1 complex. Further yield increase could probably be obtained once the regulation mechanism of biosurfactant production is fully elucidated (Hsueh et al. 2007).

Conclusions and perspectives

In bioremediation, biosurfactant applications are limited even though their high potential has been already demonstrated. This field will probably benefit more from recent and future research on the mechanisms of interactions among hydrocarbons, surfactants and cells than from case-specific studies about applicability of already-known biosurfactant compounds. In the food, biomedical and cosmetics area, in which high-value products are produced, the cost drawback could be less significant. The complex mixture of different components produced by organisms hampers applications, and further research is required to resolve specific issues. This field will have benefits from the very recent attention paid to the isolation and characterisation of biosurfactants produced by extremophiles such as thermophilic and halophilic bacteria (Mnif et al. 2009, Joshi et al. 2008; Kumar et al. 2008) aimed at the isolation of new compounds with novel properties.

The proven antimicrobial, anti-adhesive, immune-modulating properties of biosurfactants and the recent successful applications in gene therapy, immunotherapy and medical insertion safety suggest that it is worth persisting in this field. Advances in the area of biomedical application are probably going to take the lead due to higher potential economic returns. Moreover, due to their self-assembly properties, new and fascinating applications in nanotechnology are predicted for biosurfactants (Palanisamy 2008; Reddy et al. 2009, Kitamoto et al. 2009). In-depth studies of their natural roles in microbial competitive interactions, cell-to-cell communication, pathogenesis, motility and biofilm formation and maintenance could suggest future improved and interesting applications.

The commercial success of microbial surfactants is currently limited by the high cost of production. Optimised growth/production conditions using cheaper renewable substrates and novel and efficient multi-step downstream processing methods could make biosurfactant production more profitable and economically feasible. Furthermore, recombinant and mutant hyper-producer microbial strains, able to grow on a wide range of cheap substrates, could produce biosurfactants in high yield and, potentially, bring the required breakthrough for their economic production.

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