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review

Surfactin – A Review on Biosynthesis, Fermentation, Purification and Applications

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Summary

Surfactin, a bacterial cyclic lipopeptide, is produced by various strains of *Bacillus subtilis* and is primarily recognized as one of the most effective biosurfactants. It has the ability to reduce surface tension of water from 72 to 27 mN/m at a concentration as low as 0.005 %. The structure of surfactin consists of seven amino acids bonded to the carboxyl and hydroxyl groups of a 14-carbon fatty acid. Surfactin possesses a number of biological activities such as the ability to lyse erythrocytes, inhibit clot formation, lyse bacterial spheroplasts and protoplasts, and inhibit cyclic 3',5-monophosphate diesterase. The high cost of production and low yields have limited its use in various commercial applications. Both submerged and solid-state fermentation have been investigated with the mutational approach to improve the productivity. In this review, current state of knowledge on biosynthesis of surfactin, its fermentative production, purification, analytical methods and biomedical applications is presented.

Key words: biosurfactant, surfactin, *Bacillus subtilis*, biosynthesis, fermentation, purification

Introduction

Surfactants are ampiphilic molecules that partition preferentially at the interface between two fluid phases. Formation of a molecular interfacial film lowers the interfacial tension of a solution and is responsible for unique properties of surfactant molecules. The molecular layer in addition to lowering the interfacial tension can have an impact on the interfacial rheological behaviour and mass transfer. Due to these properties, surfactants are gaining importance in various industrial applications such as foam creation and stabilization in food processing, detergents for household cleaning, phase dispersion for cosmetics and textiles, or solubilization of agrochemicals (*1*). They are also applied in oil recovery, crude oil drilling lubricants and bioremediation of water-insoluble pollutants. Biodegradability, low toxicity and solubility of hydrophobic compounds are the characteristics of biosufactants that have attracted industrial attention (*2*).

Surfactin was discovered by Arima *et al.* (*3*) from the culture broth of *Bacillus subtilis* (Fig. 1). It was named thus due to its exceptional surfactant activity (*4*). Natural surfactins are a mixture of isoforms A, B, C and D with various physiological properties obtained from *B. subtilis* BC 1212 (*5*). They contain at least eight depsipeptides with the number of carbon atoms between 13 and 16 as part of the ring system. Surfactin was initially identified as a potent inhibitor of fibrin clot and later found to lyse erythrocytes, protoplasts and spheroplasts. It lowers the surface tension of water from 72 to 27 mN/m and is a very powerful biosurfactant (*6*). However, the high production cost of biosurfactants has been a major concern in commercial applications. Numerous attempts have been made to reduce the cost of the production of biosurfactants. Various strategies have been implemented to achieve improved biosurfactant production such as strain improvement, medium optimization, bioreactor design or using agroindustrial wastes for fermentation to reduce the raw material cost (*7*). The

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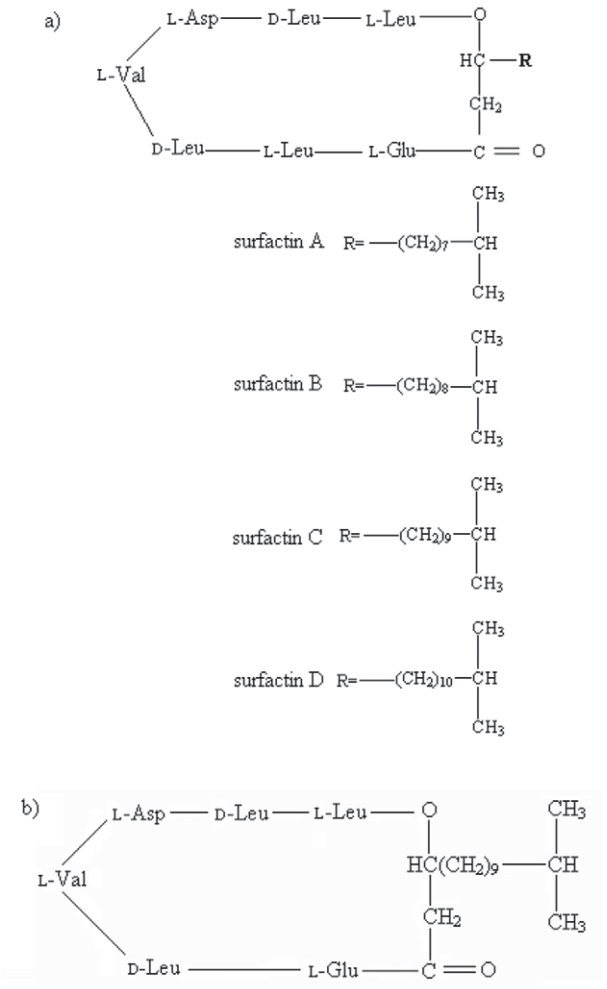

Fig. 1. Structure of: a) surfactin A, B, C, D, and b) lipohexapeptide

surfactant industry exceeds US$ 9 million per year. Although most of them are of petrochemical origin, biosurfactants are gaining importance and need to compete with chemical surfactants with respect to cost, functionality and production capacity to meet the demand. For high-priced products such as cosmetics and medicine, the application of a biosurfactant as a low-volume, high-priced component is acceptable. However, for applications such as oil recovery where high-volume and low-cost surfactants are needed, the high production cost is not justified.

Environmental compatibility is becoming increasingly important in industrial processes with respect to the selection of chemicals. The advantage of biosurfactants over synthetic surfactants has attracted attention for its production on commercial scale. This review provides a general overview with respect to biosynthesis, fermentative production, purification, analytical methods and applications of surfactin.

Biosynthesis

A large number of bioactive oligopeptides are produced by bacteria and fungi through a unique nonribosomal mechanism. Large modular enzymes referred to as nonribosomal peptide synthetases catalyze the biosynthesis of these low molecular mass peptides. All these multimodular enzymatic assemblies carry out acyl chain initiation, elongation and chain termination, catalyzed by the protein molecules. Studies on the biosynthesis of surfactin began with the work of Kluge *et al.* (*8*), who proposed a nonribosomal mechanism catalyzed by multienzymatic thiotemplates constituting the surfactin synthetase. The surfactin synthetase complex consists of four enzymatic subunits. Three of these are enzymes SrfA (E1A, 402 kDa), SrfB (E1B, 401 kDa), SrfC (E2, 144 kDa) and the fourth is SrfD (E3, 40 kDa), which plays an important role in the surfactin initiation reaction (*9*).

Each module of the peptide synthetase consists of different domains and is responsible for the incorporation and modification of one specific amino acid into the peptide and module having co-linear sequence with the sequence of the peptide product. The surfactin synthetase complex is coded by the inducible operon named *srfA* (25 kb), which is also responsible for sporulation and competence development (*10*).

The module comprises three domains: (*i*) from the cognate amino acid and adenosine triphosphate (ATP), the adenylation domain catalyzes the formation of an aminoacyl adenosine and releases pyrophosphate, (*ii*) covalent bonding of the activated amino acid to 4'-phosphopantetheinyl prosthetic group present on the peptidyl carrier protein (PCP) *via* a thioester linkage, and (*iii*) condensation domain catalyzes the direct condensation of the thioesterified intermediate in a growing chain. Chain elongation chemistry in each module is different (*11*). In nonribosomal peptide synthetase (NRPS) assembly lines are positioned both in upstream and downstream direction by C-N amide bond formation. After completion of the synthesis of entire acyl chain, the chain is disconnected from the thioester by a C-terminal thioesterase (TE) domain. The chain-terminating TE domains are 25–30 kDa protein moieties first seen in fatty acid biosynthesis. Some of the TE domains are hydrolases, *e.g.* vancomycin or daunomycin and some carry out regio- and stereo-specific reactions, *e.g.* erythromycin, while intramolecular cyclization is the most striking feature of the TE domains of SrfA-C. The acyl-O-TE intermediate does not undergo hydrolysis but is instead directed to intramolecular capture by a nucleophilic group in the acyl chain. The genetically excised TE domain from the C-terminus of the surfactin synthetase SrfA-C subunit (SrfTE) catalyzes a stereo- and regio-specific macrolactonization by the addition of hydroxyl oxygen of a 3-(*R*)-3-hydroxyacyl heptapeptidyl thioester onto the Leu7 carbonyl to produce the microlactone characteristic of the biosurfactant (*12*).

For catalytic activity, the PCP domain of the peptide synthetases has to be converted from apo- to holo-forms by specific phosphopantetheinyl transferases. These enzymes transfer the 4'-phosphopantetheinyl moiety of coenzyme A (CoA) to the side chain hydroxyl of invariant serine residue in all PCP domains. The production of surfactin from *B. subtilis* depends on the phosphopantetheinyl transferase Sfp, which converts the inactive apo-forms of the seven PCP domains to their active holo-forms. Interaction between CoA and Sfp is somewhat specific as several residues of Sfp that are involved in the interaction with CoA are not conserved in other phosphopantetheinyl transferases (*11*).

4'-Phosphopantethein (Ppant) is an essential prosthetic group of several acyl carrier proteins involved in the secondary metabolism. Ppant carries out two important reactions; first the intermediates remain tethered to the multifunctional enzyme templates through energy-rich linkage, and second, the flexibility and the length of Ppant (about 20 Å) assists the transport of the intermediates to the distinct reaction centres. Ppant moiety is post-translationally transferred onto a serine side chain. This reaction is Mg^{2+}-dependent and catalyzed by 4'--phosphopantethein transferases (PPTases) (*13*,*14*).

PPTases are classified into three groups according to their substrate and sequence homologies: (*i*) acyl carrier proteins (ACPs) of *E. coli*, found in almost all organisms. They carry out the modification of fatty acid ACP, (*ii*) Sfp of *B. subtilis* is the prototype PPTase of the second group, having the length of 240 amino acids mostly found in the gene cluster of nonribosomal peptide synthesis. Sfp exhibits broad substrate specificity, and (*iii*) the third group of PPtases is found as the C-terminal domain of FAS2 (*S. cerevisiae*) (*15*).

Fermentative Production

Commercialization of any biotechnological product depends on its bioprocess economics. Although a large number of biosurfactant producers have been reported in the literature, the product enhancement has been restricted to a few organisms such as *Bacillius*, *Pseudomonas* and *Candida* (*16*). Both submerged (SMF) and solid--state fermentation (SSF) have been tried for surfactin production.

Surfactin production by submerged fermentation

Biosurfactants produced by microorganisms like *Pseudomonas*, *Bacillus*, *Candida* and *Torulopsis* can be categorized into five groups: glycolipids, lipopeptides, phospholipids, fatty acids and polymeric biosurfactants. Surfactin is a lipopeptide produced by *Bacillus*. Several approaches have been tried to improve surfactin yield both at flask level and fermentor level by changing environmental parameters, optimizing medium components or trace elements. Table 1 (*3*,*17*–*22*) summarizes the productivity of surfactin in various media.

Addition of solid carriers

A novel method was reported for the production of surfactin by Yeh *et al.* (*2*). Solid carriers such as activated carbon (AC), agar and expanded clay were added to the fermentation broth that used *B. subtilis* ATCC 21332 for the production of surfactin. The addition of activated carbon (133 g/L) and expanded clay (133 g/L) produced 2150 and 3300 mg/L of surfactin, respectively, while agar (20 %, by volume) produced 140 mg/L of surfactin. AC and expanded clay were beneficial compared to agar, as it dissolved in the medium after prolonged incubation, which is not suitable in practical surfactin fermentation. Expanded clay had a limitation of being very fragile, which generated small fragments after prolonged shaking. This caused difficulties in solid-liquid separation, which ultimately reduced the efficiency of isolation and purification of the surfactant. The most efficient solid carrier was found to be AC due to its good physical strength. The optimized concentration of the same was 25 g/L, above which it decreased the surfactin production. In order to investigate the relationship between the enhanced production of surfactin and the addition of AC carriers, the time course profiles of cell growth and surfactin production were studied with and without AC carriers. Higher cell growth rate with maximum final cell concentration suggested that AC facilitated the growth of cells, and thereby the production of surfactin. This could be due to AC supporting the growth of cells and consequently increasing the surfactin production.

Aqueous two-phase fermentor

Concentration of the product in diluted solutions, low yields and product inhibition are the major obstacles observed in the production of biosurfactant. Drouin and Cooper (*19*) reported about the production of surfactin in aqueous two-phase fermentor. An aqueous two-phase system of polyethylene glycol (PEG-8) and dextran (D-40) was used to partition the surfactant and surfactant-producing organism in a 2-litre cyclone fermentor. The microorganism used was *B. subtilis* ATCC 21332 and the medium contained 5 g/L of glucose, 0.07 M phosphate buffer (pH=6.7) and other salts in minor quantities. Here the partitioning of cells and surfactin was determined by surface tension method. It was observed that the cells

Table 1. Productivity of surfactin in various media

Strain	Medium	Type of fermentation	γ(surfactin)/(mg/L)	Reference
B. subtilis ATCC 21332	semisynthetic	SMF	100	(*3*)
B. subtilis ATCC 21332	synthetic	SMF	250	(*17*)
B. subtilis ATCC 21332	synthetic	SMF	800 (in foam)	(*17*)
B. subtilis ATCC 21332	meat hydrolysate	SMF	160	(*18*)
B. subtilis ATCC 21332	synthetic	SMF, aqueous two phase	350	(*19*)
B. subtilis RB14	semisynthetic	SMF	250	(*20*)
B. subtilis RB14	okara	SSF	200–250 (mg/kg wet mass)	(*20*)
B. subtilis MI113 (pC112)	semisynthetic	SMF	350	(*20*)
B. subtilis MI113 (pC12)	okara	SSF	2000 (mg/kg wet mass)	(*20*)
B. subtilis ATCC 55033	semisynthetic	SMF	3500–4300	(*21*)
Suf-1, a mutant of *B. subtilis* ATCC 21332	synthetic	SMF	550	(*22*)

accumulate in the lower dextran-rich phase and surfactin in the top phase. This was useful when setting up extractive bioconversion fermentation, as the cells were easily recycled to the fermentor. The polymers could be reused after the recovery of surfactin. It was also observed that surfactin caused an end product inhibition and resulted in lower yields. With the aqueous two-phase system, the partitioning between the cells and surfactin lowered the contact of surfactant with the cells, and thus increased the production and minimized the end product inhibition. This method could be applied for continuous fermentation as well.

Airlift reactor

Noah *et al.* (*23*) reported the use of potato process effluent for the continuous production of surfactin by *B. subtilis*. Potato process effluent consists of indigenous bacteria that outcompete the growth of *B. subtilis.* During batch operation in shake flasks, they observed surfactin to remain in the liquid, assisting *B. subtilis* in overtaking the indigenous bacteria. The batch culture was performed in an airlift reactor (3 L) with potato starch, where surfactin was stripped out into the foam and there was better transfer of O_2 into the liquid phase, thus enabling *B. subtilis* to utilize the nutrients more effectively than the indigenous bacteria. The air flow rate was optimized for stripping of surfactin into the foam, and it was found that the air flow rate of 1.5 L/min increased the surfactin concentration in the foam from 1.8 to 3.5 g/L. At higher flow rates, *i.e.* 3 L/min, liquid entrainment occurred and therefore the surfactin concentration in the foam decreased. Continuous run with purified potato starch was studied to find the maximum dilution rate before washout occurred. In continuous operation in the airlift reactor, surfactin was stripped off into the foam; however, the addition of fresh media to the reactor favoured the growth of indigenous bacteria. For the first 25 h, the reactor was operated in batch mode for cell growth and initial surfactin production. The air flow was initiated at 4.5 mL/min, at which stage the contaminants appeared. The flow rate was increased to 11.5 mL/min to wash out the contaminants. Continuous run with potato process effluent resulted in poor quality of foam, and the indigenous bacteria outgrew *B. subtilis* in the reactor after 24 h. The authors proposed a large inoculum volume of *B. subtilis*, pH control and pressurized reactor to minimize the competition by indigenous bacteria.

Noah *et al.* (*24*) reported surfactin production using *B. subtilis* in a chemostat. Runs were performed in New Brunswick BioFlo 3000 (GMI Inc, New Brunswick, Canada) stirred tank reactor. Low solid potato effluents were obtained from two different southeast Idaho processing plants. The run was conducted for 72 h with the air supply of 0.5 vvm. Initial batches were taken at 250 rpm (pH=7.0) and without the baffle where the surfactin concentration reached 0.8 and 0.9 g/L at 30 and 72 h, respectively. Later, the batches were performed at 400 rpm with the baffle that produced 1.1 g/L of surfactin. The increase in oxygen mass transfer resulted in decreased production time from about 48 to 12–17 h (potato effluent from plant 1). Runs with potato effluent from plant 2 resulted in surfactin production of 0.6 g/L in 17–24 h (400 rpm and with the baffle in place).

Effect of nutrients

The importance of glucose and its concentration in surfactin production was studied by Yeh *et al.* (*2*). *B. subtilis* ATCC 21332 was grown in a liquid medium containing 40 g/L of glucose, to which 133 g/L of AC carrier were added. A decline in surfactin concentration was observed after depletion of glucose, as the cells might have been utilizing surfactin as the carbon source for their growth. This indicated that the batch fermentation for surfactin must be terminated at an appropriate time in order to avoid the utilization of surfactin as a carbon source and also hinted that the supply of glucose is critical as it acts as a major carbon source. Higher glucose concentrations (50–60 g/L) led to the accumulation of glucose in the media causing low pH and a resultant decrease in surfactin production. This showed that pH regulation is important in surfactin production. An optimal pH for production was found to be between 6.3–6.7.

Study on diesel biodegradation was performed by Whang *et al.* (*25*). Two biosurfactants, surfactin and rhamnolipid, were evaluated. Surfactin (40 mg/L) was able to enhance biomass production and diesel biodegradation by more than double compared to the batch experiments without surfactin addition.

The nature of the growth-limiting nutrient is also important for the optimized production of many secondary metabolites. Davis *et al.* (*26*) reported the effect of carbon and nitrogen limitations on surfactin production by using a defined medium, under aerobic and oxygen-deficient conditions. The culture used was *B. subtilis* ATCC 21332 in a medium supplemented with glucose, ammonium nitrate and iron sulphate. Surfactin production was the highest in a defined medium with ammonium nitrate as nitrogen source. This was due to the utilization of nitrate, resulting in nitrate-limited growth. Nitrate utilization occurred in two cases, either under anaerobic growth conditions, or when the ammonium was depleted. Low surfactin yields resulted in cultures with no nitrate utilization and such cultures had low carbon content, or contained only ammonium but not nitrate. It was proposed that nitrate acts as a terminal electron acceptor under anaerobic conditions. Nitrate was utilized even though ammonium was present in the culture. During aerobic growth, ammonium ion was utilized preferably over nitrate. The study indicated that by implementing fed batch approach and providing ammonium during growth phase and nitrate in the feed, nitrate utilization can be prolonged and surfactin production increased.

Both Taguchi method and response surface methodology (RSM) have been implemented to optimize culture and environmental parameters, optimization of medium components and trace elements for the fermentative production of surfactin. Sen (*27*) optimized the medium components for surfactin production by RSM with *B. subtilis* DSM 3256. Carbon source (glucose), nitrogen source (ammonium nitrate) and mineral salts, *viz.* ferrous and manganous sulphate were found to be significant parameters. RSM showed glucose and ammonium nitrate to

act as limiting substrates, and any variation affected either the growth or product formation. Although the interaction between these two was significant, the production of surfactin was not increased significantly. The entire biosynthetic pathway and the nature of multi-enzyme systems are not known for surfactin, but the role of iron as a growth stimulator, and that of manganese as a spore inducer and production-enhancing factor have been recognized. The presence of active transport systems has been observed in *B. subtilis* for iron and manganese, both acting as major co-factors for enzymes in the biosynthetic pathway. The yield of 0.76 g/L predicted by the model was achieved in the shake flask experiments, which indicated a close agreement between the predicted model and experimental yields.

Wei and Chu (*28*) reported the addition of iron or manganese salts to improve the yield of surfactin using *B. subtilis* ATCC 21332. Iron (1.7 mM) in the form of ferrous sulphate was added to the medium at different concentrations. It was observed that the time of the addition of iron to the culture did not affect the production of surfactin. Its addition decreased the pH of the broth, and no production was seen below pH=5.0. When the pH was maintained constant, the production of surfactin increased to 3000 mg/L. The experiments proved that iron supplementation and pH maintenance could be used as an efficient way to produce large quantities of surfactin.

Wei and Chu (*29*) confirmed that Mn^{2+} affected nitrogen utilization and K^+ uptake along with other biochemical functions. They studied the effect of manganese on surfactin production using *B. subtilis* ATCC 21332. Manganese added in the form of manganese sulphate at 0.01 mM increased surfactin production from 0.33 to 2.6 g/L.

Wei *et al.* (*30*) optimized the trace element solution for surfactin production with Taguchi method using *Bacillus subtilis* ATCC 21332. Mg^{2+}, K^+, Mn^{2+} and Fe^{2+} were found to be the most significant trace element components for surfactin production. Ca^{2+} was found to be insignificant for cell growth and production. In the absence of Mg^{2+} and K^+, lower surfactin yields were observed, indicating that these two components played a significant role in surfactin production. A medium devoid of Mn^{2+} or Fe^{2+} did not affect surfactin production, although the cell growth was slightly inhibited. In the absence of metal ions, both surfactin production and cell growth decreased, suggesting that one of these ions is essential for the production. Individually, the levels for each trace element component were determined, but lower surfactin production was observed when all the ions were incorporated together in the medium at their optimal levels. This suggested significant interactions between the four metal ions. The statistical data proved that Mg^{2+} and K^+ ions were more critical than the other two metal ions.

It was proposed by Kinsinger *et al.* (*31,32*) that the active site of *B. subtilis* Sfp protein which activates the PCP domains of surfactin synthetase requires Mg^{2+} as the co-factor and thus signifies its role in surfactin production. K^+ was shown to stimulate surfactin secretion. Therefore, Mg^{2+} and K^+ were the two parameters selected for the Taguchi design wherein the concentrations of Mn^{2+} and Fe^{2+} were kept constant. Statistical data gave the optimized concentrations for the four metal ions Mg^{2+} (2.4 mM), K^+ (10 mM), Mn^{2+} (0.008 mM) and Fe^{2+} (7 μM), with surfactin production of 3.34 g/L.

Cooper *et al.* (*17*) reported enhanced productivity of surfactin when various metal cations and hydrocarbon were added to the medium. It was observed that the addition of 2–4 % of hexadecane to the medium inhibited the production of surfactin, although hydrocarbons were reported to increase its production. Attempts were made to improve the yields in the fermentation by adding various salts. $FeSO_4$ and $MnSO_4$ had a significant effect, while $MgSO_4$, $CaCl_2$, Na_2HPO_4, KH_2PO_4, $NaNO_3$ and $ZrOCl_2$ had no effect on either surfactin production or biomass. $ZnSO_4$, $CuSO_4$, $NiSO_4$ and $CoSO_4$ suppressed the growth of *B. subtilis*. Manganese is well known to increase the production of secondary metabolites in *B. subtilis* without showing significant effect on the cell growth. On the other hand, excessive iron is required for cell growth, although it does not increase the yield of surfactin. Poor iron transport system might be one of the reasons for excessive requirement of iron. They also proposed that *B. subtilis* produces a sequestering agent that makes iron unavailable. More detailed study is required with respect to the interaction of surfactin with the metal ions.

Effect of process parameters

Effect of agitation rate on surfactin production was studied by Yeh *et al.* (*2*). The study indicated that the increased dissolved oxygen at higher agitation rates positively impacted surfactin production. However, agitation rates above 250 rpm resulted in foam formation, and decreased the oxygen transfer and surfactin yield.

Yeh *et al.* (*7*) optimized the fermentation parameters for surfactin production in a 5-litre jar fermentor. The effect of different aeration and agitation rates on oxygen transfer and mass transfer rate was studied. The studies carried out in bioreactor supported production of surfactin without the addition of antifoaming agent under extreme foaming conditions. Modified bioreactor design, in which additional foam collector was connected to the gas effluent and a cell recycler, was designed to pump a portion of liquid culture back into the reactor. The foam was collected in acid tank and precipitated at pH=2.0, resulting in primary isolation of surfactin. Studies were carried out using *B. subtilis* ATCC 21332 and AC as a solid carrier in an iron-rich minimal salt (MSI) fermentation medium. A strong relation was observed between oxygen consumption, pH and the production. At an initial glucose concentration of 40 g/L and an agitation and aeration rate of 300 rpm and 1.5 vvm respectively, surfactin production reached maximum level of 6.55 g/L after cultivation for 60 h, when the cell growth ceased. Dissolved oxygen dropped from 70 to 2 % during initial growth and remained at zero during exponential phase, but increased rapidly to 70 % at about 60 h; pH dropped from 6.7 to 6.0 and then increased during the fermentation to reach a value of 6.5.

Oxygen supply and mass transfer efficiency were found to have an important role on the kinetics of surfactin production. Maximum production and productiv-

ity of surfactin were found to increase with increasing the aeration rate from 0.5 to 1.5 vvm and agitation rate from 200 to 300 rpm. Higher agitation (above 350 rpm) and aeration (2.0 vvm) rates resulted in vigorous foaming, thereby decreasing cell recycling and surfactin production. At higher rates of aeration and agitation, rapid foam was produced and caused the culture to overflow. Early foam formation resulted in short fermentation time, decreased biomass and low surfactin production. The best surfactin production was observed at mass transfer coefficient (K_La) of 0.012 s^{-1}.

Sen and Swaminathan (*33*) studied the effect of pH, temperature, rates of agitation and aeration as environmental parameters for the production of surfactin by RSM using *B. subtilis* DSM 3256. The results indicated that surfactin production was maximal at 37.4 °C and pH=6.75, agitation at 140 rpm and aeration at 0.75 vvm. The achieved experimental yield was 1.1 g/L of surfactin.

In another study, Sen and Swaminathan (*34*) optimized the inoculum age and size for the production of surfactin using *B. subtilis* DSM 3256. These two factors directly affected the lag phase, specific growth rate, biomass, sporulation and hence the production. The length of the lag phase was affected by the size of the inoculum and its physiological condition. It was observed that inoculum age was the most important for sporulating bacteria like *B. subtilis* when the inoculum used from the late logarithmic phase contained more spores and resulted in greater lag time in the subsequent step. Two-stage inocula reportedly improved the production of surfactin. The four chosen independent test variables were primary inoculum age, primary inoculum size, secondary inoculum age and secondary inoculum size, and they were critical factors in surfactin production. Thus the optimal levels obtained for the primary inoculum age, primary inoculum size and secondary inoculum age were 55–57 h, 5–6 % (by volume) and 4–6 h, respectively. The optimum value for secondary inoculum size was 9.5 % by volume.

Mutation

Bioindustrial production process is highly dependent on hyperproducing strains. Organisms that produce the final product in high concentrations are preferred and the genetics of the producing organism is important. Apart from natural biosurfactant-producing strains, a few mutants and recombinant strains have been reported in literature (Table 2) (*6,35–38*). One of the recombinant strains of *B. subtilis* ATCC 21332 produced a lipohexapeptide (Fig. 1), which showed less toxicity towards erythrocytes and enhanced lysis of *B. licheniformis* cells, making it useful for therapeutic applications. Thus recombination not only improved the production but also gave better product characteristics. The normal surfactin lyses the erythrocytes because of its membrane-active property, but the recombinant surfactin was produced by generating the modified active peptide synthetase by eliminating a large internal region of this enzyme containing a complete amino acid-incorporating module (*16*).

Liu *et al.* (*39*) generated a surfactin-overproducing mutant from a wild type strain of *B. subtilis* (B.s-E-8) by low energy ion beam implantation. Low energy ion beam implantation is considered as an efficient physical mutagen in plant and microbe breeding. It was reported that N^+ implantation had remarkable effects on inducing mutations in plant seeds. Industrial microbes giving high yields have been obtained by means of ion beam implantation. Low energy ion beam implantation has been widely used in the field of material surface modification since 1970s, and the genetic effect induced by ion implantation in rice has widened the field of ion beam application in life sciences.

Ion beam implantation with the heavy ion beam was carried out at an implantation facility set up by the Institute of Plasma Physics, Chinese Academy of Sciences, Hefei, PR China. Bacteria in the late growth exponential phase from the seed medium were used for ion beam implantation. The survival rate and mutation efficiency were used as the criteria to determine the optimum conditions of ion beam implantation. Dose effect of mutagenesis was determined by carrying out experiments at an energy of 20 keV and different doses of $1.3{\cdot}10^{14}$, $2.08{\cdot}10^{14}$, $2.6{\cdot}10^{15}$, $3.9{\cdot}10^{15}$ and $5.2{\cdot}10^{15}$ N^+/cm^2. In order to determine the effect of energy levels, mutagenesis was also carried out at a dose of $2.6{\cdot}10^{15}$ N^+/cm^2 and different energies of 5, 10, 15, 20, 25 and 30 keV. The optimum conditions for ion beam implantation were a dose of $2.6{\cdot}10^{15}$ N^+/cm^2 with the energy of 20 keV.

According to Song and Yu (*40*), the 'moment etching channels' could be formed as a result of the action of a large number of successively implanted ions, and connected to DNA. Scanning electron microscope clearly showed the damage caused by implanted ions. After ion beam implantation, the mutant B.s-E-8 (obtained from the wild strain of *B. subtilis*) showed differences from the parent strain, including the overproduction of biosurfactant, its metabolite and the intensities of different components. The surface tension of the cell-free Landy

Table 2. Mutant and recombinant strains of *B. subtilis* with enhanced biosurfactant yields and improved product characteristics

Mutant/recombinant strain	Characteristic feature	Yield or improved production property	Reference
B. subtilis ATCC 55033	random mutagenesis with *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine	approx. 4–6 times (2–4 g/L of crude surfactin)	(*35,36*)
Recombinant *B. subtilis* MI113	incorporation of a plasmid containing *Ipa-14* gene	8 times more surfactin production	(*6*)
B. subtilis SD901	random mutagenesis with *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine	4–25 times more surfactin production (8–50 g/L)	(*37*)
Recombinant *B. subtilis* ATCC 21332	contains recombinantly modified peptide synthetase	production of lipohexapeptide with reduced toxicity	(*38*)

medium (LM) was 25.6 mN/m, and it reached 27.1 and 28.5 mN/m when diluted 50- and 100-fold, respectively.

Gong *et al.* (*41*) also reported high-producing surfactin mutant obtained by ion beam implantation, *Bacillus subtilis* E8. The modified bioreactor with a cell/foam recycler was implemented. The concentration of crude surfactin (including recovery from both foam and broth fractionation) increased significantly from 0.75 to 10.26 g/L. The concentration of crude surfactin and biomass reached maximum (12.20 and 6.50 g/L, respectively) after cultivation for 32 h.

There had been a few reports on biosurfactant-overproducing mutants previously. Mulligan *et al.* (*22*) reported a 2-fold higher surfactin production by an ultraviolet mutant of *B. subtilis* than the wild type strain; Lin *et al.* (*42*) reported a 12-fold higher level of surfactin production from a mutant of *Bacillus licheniformis* that was derived from random mutagenesis with *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine, but few reports have focused on the surface activity enhancement. This is even more important than the production enhancement because it will enable the reduction of the cost of biosurfactant and also improve surface activity. It was observed that the intensities of the different components in the mutant strain were vastly different from the parent strain, which might be due to the change in the metabolic pathways induced by the ion implantation. The mode of action of the implanted ions on the biosynthesis of different components, and their contribution to the surface activity of the biosurfactant is still not clear.

Surfactin production by solid-state fermentation (SSF)

One of cost-effective approaches to produce biosurfactants is to use inexpensive raw materials, which accounts for 10–30 % of the production cost. Various agro-industrial wastes for the production of biosurfactants have been reported (*43*), of which the most effective seem to be those from potato processing industry (*44*). Potatoes contain (in %): water 80, carbohydrates 17, protein 2, fat 0.15 and vitamin 0.9, as well as inorganic minerals and trace elements (*45*). Thompson *et al.* (*46*) used high-solids (HS) and low-solids (LS) potato effluents for surfactin production. They used effluents that were diluted in 1:10 ratio, without any supplement or supplemented with trace minerals or corn steep liquor. Growth rate of *B. subtilis* 21332 was higher in all HS- and LS--based media, although the surfactin production was better in LS (0.39 g/L) media compared to HS (0.097 g/L).

Soybean curd residue, called okara in Japan, is a by--product of the tofu manufacturing industry, and is disposed off as waste. One of the disadvantages of its use as a substrate is spoilage by microbial contamination due to its high water content and it being nutrient-rich to support microbial growth. Ohno *et al.* (*6*) checked recombinant *B. subtilis* MI113 and the original strain of *B. subtilis* RB14 for their surfactin-producing capacity both in SSF and SMF. A maximum of 1.8–2.0 g/kg of surfactin wet mass was achieved by *B. subtilis* MI113 at 37 °C and 48 h. High productivity in SSF rather than in SMF is believed to be due to homogenous distribution of the nutrients and oxygen in the liquid medium, which favour cell growth rather than the production of surfactin. Lower growth rate of cell biomass in SSF indicates that nutritional limitations triggered the synthesis of surfactin. Another explanation for increased surfactin production could be overcoming the end product inhibition. One more theory proposed for enhanced production of surfactin in recombinant strain was that the *lpa-14* gene plays an important role in the synthesis of iturin A and surfactin in original *B. subtilis* RB14 strain. The same gene acts as a single controller of surfactin in *B. subtilis* MI113, lacking the function of iturin A gene (*6*).

Ohno *et al.* (*47*) reported the effect of temperature on the dual production of iturin A and surfactin by *B. subtilis* RB14 in SSF using okara as a substrate. Iturin A production decreased with an increase in temperature, while surfactin production increased until 37 °C, after which there was a decline. It was proposed that the common pathways involving *lpa-14* protein were not affected by temperature, but the individual pathways for each product were.

Bacillus polyfermenticus KJS-2 (BP-KJS-2) was used to produce surfactin in SSF using soybeans. It showed antimicrobial activity against bacteria at the concentration of 0.05 mg/mL (*48*).

Purification

The recovery and concentration of biosurfactants account for a major portion of the total production cost. The separation strategies for biosurfactants vary according to the fermentation process and the physicochemical properties of the surfactant in question. The selection of a method for purification and recovery of surfactants depends on the nature of their charge, solubility characteristics, whether the product is intracellular or extracellular, and on the economics of recovery and downstream processing.

Membrane-based techniques

Sen and Swaminathan (*49*) reported about the membrane separation process for the recovery and concentration of surfactin. The filtration characteristics of surfactin when passing through the polymeric membrane were evaluated and the optimum conditions for surfactin recovery were standardized.

pH played an important role in the recovery of surfactin. Effect of initial surfactin concentration on the flux was determined and it was found that the concentration below 0.5 g/L restricted the pre-gel region, above which the flux started declining. A pH of 8.5 gave maximum flux of about 260 L/(m^2/h) at an initial surfactin concentration of 1 g/L. Complete retention of surfactin was possible at an optimum transmembrane pressure of 196.2 kPa and an optimum pH of 8.5. A 166-fold concentration of surfactin was achieved under these conditions. Thus, an understanding of the filtration characteristics of surfactin enables its purification in a single step and thus facilitates the process scale-up.

Chen *et al.* (*50*) reported about the separation and recovery of surfactin from fermentation broth with the culture of *B. subtilis* ATCC 21332 by two-stage ultrafil-

tration (UF) or nanofiltration (NF) processes. The broth was pretreated by acid precipitation and then the precipitate was dissolved in NaOH at pH=11. Experiments were performed at different initial concentrations of surfactin (210–3620 mg/L), of added micelle-destabilizing solvent ethanol (0–44 %, by volume), membrane with molecular mass cut-off (MMCO) of 1–300 kDa, and different transmembrane pressures from $86.184 \cdot 10^3$ to $517.106 \cdot 10^3$ Pa. It was found that in the tested concentration ranges, surfactin micelles could be efficiently destroyed when more than 33 % (by volume) ethanol was added to the broth. The UF membranes with MMCO below 100 kDa were suitable for the retention of surfactin micelles, and the NF membrane with a MMCO lower than 1 kDa was suitable for the retention of surfactin monomers. The separation strategy involving two-stage membrane filtration (UF or NF) processes was proposed. After acid precipitation and centrifugation of the fermentation broth of *B. subtilis* ATCC 21332 culture, the precipitate was dissolved in NaOH solution. A recovery of more than 97 % was obtained, but had a low purity of only 55 %.

A single-stage batch UF experiments showed that the optimal conditions were a transmembrane pressure of $86.184 \cdot 10^3$ Pa, feed pH=7 and stirrer speed of 5 Hz (300 rpm). Of the UF membranes tested, the polyethersulphone (PES) membrane with a MMCO of 100 kDa (PES 100) gave an acceptable rejection of surfactin micelles (88 %), steady flux (*e.g.* 92.4 L/(m^2/h) at a transmembrane pressure of $86.184 \cdot 10^3$ Pa), and the retentate of high purity of about 75 %. Surfactant molecules associate to form micelles or vesicles at concentrations above critical micelle concentrations. This increases the molecular diameter by two to three orders of magnitude as compared to the single unassociated molecule. These surfactin micelles could be destabilized to the form of monomers by adding 33 % (by volume) ethanol, which was allowed to effectively pass through the PES 100 membrane. The use of two-stage UF process for improved purity of surfactin from the treated broth was thus examined. Two-stage UF processes were proposed in two different routes. Surfactin was first retained by PES 100 membrane, and it was further purified by destabilizing the surfactin micelles by adding 33 % (by volume) ethanol. This route gave H-form of surfactin with a purity of 85 % and total recovery yield of 87 % (at a feed surfactin concentration of 2054 mg/L). In another approach, surfactin was first recovered as monomers in the permeate by PES 100 membrane, and smaller molecules such as salts were removed by the second PES 100. This route gave H-form of surfactin with a purity of 83 % and total recovery yield of 72 %. These experiments demonstrated the promising application potential of such two-stage UF processes for surfactin recovery.

In another variation, a two-step membrane filtration process was evaluated using centrifugal and stirred cell devices, while the mechanisms of separation were investigated by particle size and surface charge measurements (*51*). In the first step of ultrafiltration (UF-1), surfactin was retained effectively by membranes above its critical micelle concentration (CMC); subsequently in UF-2, the retentate micelles were disrupted by the addition of 50 % (by volume) methanol solution to allow recovery of surfactin in the permeate. The main protein contaminants were effectively retained by the membrane in UF-2. UF was carried out either using centrifugal devices with 30- and 10-kDa MMCO regenerated cellulose membranes, or a stirred cell device with 10-kDa MMCO PES and regenerated cellulose (RC) membranes. Total rejection of surfactin was consistently observed in UF-1; while in UF-2, PES membranes had the lowest rejection coefficient of 0.08±0.04. UF rejection of micellar surfactin by a semi-porous membrane was achieved in the first step, where surfactin was mainly purified from residual glucose and salts. In the second step, surfactin was purified essentially from proteins. PES membrane was found to be more suitable for the purification of surfactin, especially in the second step of UF. The size and surface charge measurements demonstrated that disruption of surfactin micelles, aggregation of protein contaminants and electrostatic interactions between surfactin molecules and the membrane surface had a major influence on its selective separation. One factor of concern in the second step of UF process was the effect of exposure of UF membranes to a methanolic solution. It was found that the permeability of PES membranes reduced after subsequent exposure to 50 % (by volume) methanol solution. The process could be improved by choosing a membrane that is resistant to organic solvents. PES membranes were found to be affected by concentration polarization, and therefore the need to purify surfactin using a cross-flow filtration unit was suggested. This would undoubtedly be important in the scalability of this separation process.

Chen *et al.* (*52*) also reported on the recovery of surfactin from the fermentation broths with culture of *B. subtilis* ATCC 21332 by cross-flow ultrafiltration process. The broth was pretreated by acid precipitation, and the precipitate was dissolved in NaOH. Two types of membranes, polyethersulphone (PES) and cellulose ester (CE), with MMCO of 100 kDa were implemented for the study. The effect of the presence of micelle-destabilizing solvent ethanol on the performance was studied. Experiments were performed with initial concentrations of surfactin of 1.13–4.57 g/L, transmembrane pressures (TMP) of 20–100 kPa, and cross-flow velocities of 0.16–0.48 m/s. The study indicated that the flux increased with increasing cross-flow velocity, but decreased with an increase in the initial surfactin concentration and TMP. Four cleaning agents and two cleaning methods (flushing and backflushing) were screened to recover the flux. To maximize the reovery of surfactin, flushing with NaOH solution at pH=11 was suggested to clean the fouled PES membrane in the cross-flow UF process. The optimal conditions for surfactin recovery were transmembrane pressure of 20–40 kPa and cross-flow velocity of 0.32 m/s with the feed concentration ranges of surfactin of 1.1–4.5 g/L. The study suggested a flux decline during cross-flow UF with CE 100 and PES 100 membranes. This was attributed to the concentration polarization as well as weak adsorption of small amino acids, and the formation of gel layer on the membrane surface. Although the recovery of surfactin with CE 100 (97 %) was found to be higher than that with PES 100 (88 %), the latter was recommended because of the gradual decline of flux. Destabilization of surfactin micelles by the addition

of ethanol into the feed resulted in the passage of surfactin through the membrane. Recovery of surfactin in the permeate of CE 100 and PES 100 membranes was 64 and 71 %, respectively, and the use of ethanol was not advantageous in the process. Under similar conditions (surfactin concentration of 0.2–3.6 g/L, without ethanol, PES 100 membrane, transmembrane pressure of 60–85 kPa), the cross-flow UF yielded comparable recovery and purity of surfactin in the retentate (83 and 79 %, respectively) in contrast to the dead-end mode (85 and 75 %, respectively). Periodic flushing during cross-flow UF removed most of the reversible components of fouling, leading to satisfactory flux recovery. Flushing had a better performance than backflushing, and NaOH solution at pH=11 was suggested for cleaning.

Chen *et al.* (*53*) reported on the recovery of surfactin by ammonium sulphate salting-out, UF, NF, and their hybrid processes. Surfactin was produced by fermentation using *B. subtilis* ATCC 21332. Different initial concentrations of surfactin (210–3620 mg/L), concentrations of added ammonium sulphate (0–46 %, by mass per volume), micelle-destabilizing solvent ethanol (0–44 %, by volume), and membrane molecular mass cut-off (MMCO, 1–300 kDa) were investigated. When 23 % (by mass per volume) ammonium sulphate and 33 % (by volume) ethanol were added to the broths, the surfactin micelles were efficiently destroyed and other protein macromolecules were removed. On the other hand, the UF membrane with MMCO lower than 100 kDa was found to be suitable for the retention of surfactin micelles, and the NF membrane with a MMCO lower than 1 kDa was found to be suitable for the retention of surfactin monomers. The hybrid process of salting-out and membrane filtration (UF or NF) enhanced the recovery and also improved the purity of surfactin.

Foam fractionation

Another approach for biosurfactant recovery is by foam fractionation. Advantages of this technique are its application to dilute solutions, favourable economics in operation, and application to crude mixtures. Foam production under rapid stirring conditions and aeration to supply the required oxygen causes stripping of the product, essential nutrients and cells, and thus makes the process very difficult. To accommodate the predicted formation of foam, the working capacity of the reactor is reduced, resulting in low productivity and unfavourable process economics. Davis *et al.* (*54*) reported about foam fractionation technique for the recovery of surfactin. Surfactin was recovered from non-integrated semi-batch mode and from integrated mode. In non-integrated semi-batch mode, where foaming occurred after the cell culture stage, the highest surfactin enrichment ratio E_R (concentration of surfactin in the foam/concentration of surfactin remaining in the liquid) occurred (up to 51.6) and the volume of foam collected per unit time was the lowest. Surfactin enrichment was better in the presence of cells than in the absence of cells, because the foamability of the solution was found to increase in their presence. Foam removal was successfully applied to integrated batch mode, where the recovery of surfactin and the production were combined. The efficiency of the system was affected by stirrer speed. At high stirring speeds (204 and 269 rpm) stripping of the majority of the culture was observed due to excess foaming. Surfactin concentration and the mass of surfactin obtained at 166 rpm were 1.2 g/L and 104 mg, while at 146 rpm they were 1.7 g/L and 84 mg, respectively. The study proved that foam recovery method can be applied particularly to integrated production systems.

Extraction

To avoid foaming and formation of stable emulsions in traditional extraction, Chen and Juang (*55*) reported non-dispersive extraction of surfactin from the fermentation broth of *B. subtilis* ATCC 21332 culture with *n*-hexane using microporous polyvinylidene fluoride (PVDF, pore size 0.2 μm) hollow fibre module. The broth was pretreated with acid precipitation followed by dissolution of precipitate in NaOH solution. The treated broth was passed through the lumen side of the module and *n*-hexane flowed across the shell side. Experiments were performed at a fixed pH=8.0 with a flow rate of both phases of 2.5 mL/min, and at different surfactin concentrations (300–3000 mg/L). It was observed that surfactin was adsorbed onto the surface of the fibres, instead of being extracted by *n*-hexane and transported through the pores of the fibres into bulk *n*-hexane phase. This was because surfactin molecule is a cyclic lipopeptide with β-hydroxy fatty acids linked to a heptapeptide and tends to form micelles under the studied conditions. These micelles are difficult to diffuse through the micropores. The purity of surfactin desorbed from the fibres with ethanol was found to be higher than that obtained after solvent extraction with *n*-hexane. An improved purity of surfactin up to 78 % after membrane adsorption was found.

Chen and Juang (*56*) reported about the recovery of surfactin from fermentation broths with the culture of *B. subtilis* ATCC 21332 by physical and chemical extraction. The broths were pretreated by acid precipitation and, if necessary, the precipitate was further dissolved in NaOH solution. The physical solid-liquid and liquid-liquid extractions were performed with different organic solvents (ethyl acetate, *n*-hexane) and at different times of extraction. The extraction was better with ethyl acetate than with *n*-hexane. The extraction could be improved by increasing the extraction time with a given volume of the organic solution. Efficiency was improved using liquid-liquid chemical extraction of surfactin with Aliquat 336 (5–200 mM) in *n*-hexane. The amounts of inorganic salt in the strip solution were also optimized. In chemical extraction, surfactin would readily bind to the quaternary ammonium cations of Aliquat 336. Liquid-liquid extraction with ethyl acetate showed an extraction efficiency of 99 % at surfactin concentration of 2 g/L. In the repeated solid-liquid extraction with ethyl acetate, the maximum extraction was 78 % and the purity was 84 %. The performance in physical solid-liquid and liquid-liquid extraction with *n*-hexane was comparable; for example, repeated extraction with *n*-hexane showed recoveries of 64 and 59 %, respectively, and had a maximum purity of 58 and 63 %, respectively. Surfactin extraction with 5 mM of Aliquat 336 in the pH range of 7–10 in *n*-hexane was able to obtain about 92 % of 3 g/L of surfactin, and adequate addition of sodium chloride or am-

monium sulphate to ethanol/water resulted in 90 and 88 %, respectively, of surfactin recovery from the loaded organic solution. The structure of surfactin was not destroyed after physical extraction with organic solvents.

Adsorption

Liu *et al.* (*57*) reported on the adsorption of surfactin from aqueous solution on AC. The factors evaluated for the process were agitation rate, activated carbon particle size, pH, temperature, initial adsorbate concentration, adsorbent amount and ionic strength of the solution. AC with an approximately spherical geometry was found to be an effective adsorbent for the recovery of surfactin from the model medium. The adsorption was tested by using AC of different particle size (1.40, 0.90, 0.72, 0.56 and 0.45 mm). The smaller the particle size of AC, the faster the rate of adsorption. A pH range of 6.5–8.5 and temperature of 30 °C were found optimal for the adsorption. Temperature had a strong effect on the equilibrium between surfactin in the solution and on the adsorbent surface, and the adsorption rates.

Montastruc *et al.* (*58*) reported on the recovery of surfactin directly from the culture medium using AC as an adsorbent. From the thermodynamic study, the adsorption of pure surfactin on AC was confirmed to be an exothermic process. The study also showed the importance of temperature for process control. Fixed bed column design for surfactin adsorption modelling on a single microporous pellet was demonstrated. The adsorption capacity of surfactin from the culture medium was found to be 26 % lower than that of pure surfactin. Pellet diameter of 0.9 mm was proposed to ensure good recovery conditions.

Chen *et al.* (*59*) reported about purification of surfactin in the fermentation broth of *B. subtilis* ATCC 21332 culture by adsorption or ion exchange after the broth had been treated by a two-stage UF process. The commonly used neutral adsorption resin (macroporous XAD-7) and charged ion-exchange resin (AG1-X4) were applied. Under the tested conditions, the impurities in the treated broth such as macromolecules (proteins, polysaccharides, peptides) or smaller molecules (glycine, serine, threonine, alanine) were adsorbed or exchanged more quickly and preferably within 5 h of operation. XAD-7 resins gave a higher purity of surfactin than AG1-X4 resins. The recovery of surfactin from the remaining solution after adsorption using XAD-7 resin exceeded 95 % at pH=6.5 and the purity increased from 76 % (the feed) to 88 %; however, the purity of surfactin reached up to 80 % only after ion exchange using AG1-X4 resin. The zeta potential and recovery data obtained at pH=7 revealed that surfactin micelles in the treated broth were negatively charged and had less negative charge than the impurities such as macromolecules (proteins, polysaccharides, peptides) and amino acids (glycine, serine, threonine, alanine). The existence of large surfactin micelles made intraparticle diffusion and internal adsorption within the resins difficult. These factors were found to be responsible for the much lower adsorption capacity of surfactin on both resins, particularly on XAD-7 resin. For instance, AG1-X4 resin had adsorption capacity of 1.76 g/g at pH=6.0, whereas XAD-7 resin had 0.41 g/g at pH=6.7. It was thus suggested that surfactin can be further purified by the removal of impurities using adsorption on the resins, more specifically, XAD-7 since it gave higher purity of surfactin than AG1-X4 resin, particularly at pH=6.5.

Liquid membrane extraction

Recovery of biosurfactants from aqueous media was demonstrated using liquid membrane (pertraction) processes by Dimitrov *et al.* (*60*). Transport of pure surfactin in three-liquid-phase system was studied. Surfactin was successfully extracted from slightly acidic media (pH= 5.65–6.05) by batch pertraction in a rotating disc contactor and using *n*-heptane as liquid membrane. Feed solution acidity affected the process efficiency with recovery of 83 % at pH=6.05 and 97 % at pH=5.65 after 4 h of pertraction. An atypical pH effect was observed when the behaviour of surfactin extraction from aqueous media by non-polar solvents (*n*-heptane and *n*-octane) was studied. The authors suggested that the high extraction degrees obtained from both acid and basic media and the clearly reduced degree of extraction from neutral media could be attributed to the different conformations of surfactin in these media. Lower extraction from neutral media could be due to the higher hydrophilicity of the β-sheet micelles formed by surfactin molecules under these conditions. It has been reported that in both acid and basic media, surfactin conformation alters from β-sheets to α-helices. Under these conditions, the non-polar ends of surfactin molecules are more exposed to the contact with organic solvents used, and as a result, give higher extraction. The pertraction process was relatively fast with about 90 % of surfactin removed from feed solution in 30 min. In 2 h, more than 50 % was transferred to the receiving solution phase.

Analytical Methods

The most widely used method for measuring biosurfactants in liquid media consists of measuring surface tension in a series of sample dilutions to estimate the CMC (*61*). It is mostly measured by its dry mass (*62*) or by HPLC (*21*). Polymerase chain reaction (PCR) is used to identify the strains screened for the genetic locus (*sfp*) responsible for surfactin production. Surfactants induce haemolysis at a given concentration due to their ampiphilic nature. Haemolysis, performed on blood agar plates, has been widely used to screen surfactant-producing microorganisms (*63*) and minimal haemolytic concentration (MHC) values have been given for surfactin (*4,64*).

A quantitative assay based on the ability of surfactin to cause haemolysis has been developed. The presence of 145 mL/L of ethanol at 37 °C in the reaction mixture gave an optimal sensitivity. The haemolytic assay gave more reliable results as compared to measurements of dry mass of surfactin because it did not involve separation steps from the supernatant. The assay was developed for measuring biosurfactant concentration by its MHC. A low MHC with respect to biosurfactant concentration is desirable. Red blood cells were obtained from fresh human blood. In the assay protocol, red blood cells were incubated at 30 °C with increasing concentra-

tions of surfactin from *B. subtilis* O9 and checked for MHC. A sigmoid plot was obtained and the corresponding MHC was 0.4 g/L with the limitation that the highest production in the culture was about 0.8 g/L. The MHC was not low enough for the development of a quantitative assay of surfactin based on MHC value titration. The addition of ethanol to the reaction mixture increased the sensitivity of the titration assay. A water-miscible organic solvent such as ethanol dissociated micelles into unassociated molecules and thereby decreased the affinity of the surfactant. This increased the concentration of free monomers in the bulk liquid (*65*), which then interacted with red blood cells and decreased the MHC value and therefore increased the sensitivity of detection of surfactin. Ethanol in excess of 250 mL/L resulted in a high percentage of haemolysis in the absence of surfactin. This unspecific haemolysis could be due to a destabilizing effect of ethanol on red blood cells (*66*). It was proposed that the increase in haemolysis could be attributed to two effects of ethanol, *viz.* (*i*) increased fragility of red blood cells, and (*ii*) increased free monomer concentration in the bulk liquid.

In order to assess the maximal ethanol concentration, the haemolytic assay was performed with increased ethanol concentrations, either without surfactin or with a sample containing 0.033 g/L of surfactin, at 30 and 37 °C. When there was no haemolysis, unspecific haemolysis did not occur up to 167 mL/L of ethanol at 30 °C. At 30 °C haemolysis was not increased by the presence of 0.033 g/L of surfactin in the range of tested ethanol concentrations. When reactions were carried out at 37 °C, an optimal ethanol concentration of 145 mL/L was found, which did not cause unspecific haemolysis and gave 100 % haemolysis with 0.033 g/L of surfactin. One hemolytic unit was defined as the MHC of surfactin measured at 145 mL/L of ethanol at 37 °C, and thereafter the concentration expressed in haemolytic units was calculated as the reciprocal of the maximal dilution of the sample causing 100 % haemolysis. The haemolytic assay values were compared with dry mass of surfactin separated from the supernatant either by solvent extraction or by UF. The advantages of haemolytic assay are rapid assessment of surfactin concentration in the culture supernatants, no requirement of special equipment or expensive reagents, flexibility of using crude supernatant samples with no treatments that might otherwise lead to surfactin losses, and requirement of minimal sample volumes (*67*).

Micelle size of surfactin

Knoblich *et al.* (*68*) studied micelles of surfactin in water by an ice-embedding technique and transmission electron cryo-microscopy, cooling down the specimen to the temperature of liquid helium. The study indicated the relationship between the pH and micelle size. Surfactin micelles were globular, had an ellipsoidal configuration with approximate dimensions of 5–9, 19 and 11 nm for diameter, length and width, respectively, at pH=7. The corresponding values at pH=12 were 8, 9 and 6 nm. At pH=9.5, globular and cylindrical micelles had approximate dimensions of 10–20, 40–160 and 10–14 nm for diameter, length and width, respectively. Characteristic Fourier transform infrared (FTIR) band for a lactone group in the peptide head group of surfactin was observed at pH=7 and 9.5, which disappeared at pH=12. The study indicated that the micelles were formed differently, with the cyclic and linear structure of the head groups at low pH and pH=12, respectively. In the presence of 100 mM NaCl and 20 mM $CaCl_2$ at pH=9.5, the globular and cylindrical micelles transformed their configurations into small spheres.

Hydrolysis of surfactin

Grangemard *et al.* (*69*) studied the hydrolysis of the cyclic lipopeptide surfactin by the V8 endoprotease from *Staphylococcus aureus* at 100 μM. The fragmentation occurred between the two residues, L-Glu1 and L-Leu2, and produced an open-chain lipopeptide. The enzyme action was limited by the aggregation state of the lipopeptide in the solution. One of the advantages of a microbiologically produced surfactant is its biodegradability and consequently a low detrimental environmental impact. This feature leads to a possible degradation of the surfactant by other microorganisms. Therefore, the stability of surfactin was assessed for its susceptibility to proteolytic digestion. Cyclic peptides are known to be almost resistant to common proteases because of their unique structures containing D-amino acid residues. The study indicated that the hydrolysis obtained by V8 protease never exceeded 14 % of the total surfactin. Surfactin resistance is at least to a certain extent provided by YerP (renamed swrC, Kearns *et al*. (*70*)), the first published example of an RND (resistance, nodulation and cell division) family of multidrug efflux pumps in Gram-positive bacteria. Other mechanisms that participate in the efflux of surfactin and the produce self-resistance are possible (*71*).

Potential Applications

Antimycoplasmal activity

Mycoplasmata cause respiratory inflammation, urogenital tract diseases, and also act as co-factors in the pathogenesis of AIDS (*72*). The following are the most important contaminants to tissue culture cells: *Mycoplasma orale* (human species), *M. hyorhinis* (a porcine species), *M. arginini* (a porcine species) and *Acholeplasma laidlawii* (a porcine species). The use of antibiotic therapy to decontaminate mycoplasmata was not effective. Cytotoxic effects of antibiotics on cells and the development of resistant cells were observed. The major reason for antibiotic ineffectiveness was their inability to penetrate cytoplasmic membrane around the mycoplasmata.

Surfactin causes leakage in the plasma membrane at higher concentrations, and finally leads to complete disintegration. It acts on the membrane in the micellar form, inducing an osmotic influx of the medium, thus disrupting the membrane. This method was applied to eliminate mycoplasmata from mammalian cell culture. It was observed that the time-consuming replenishment of the antibiotics during cultivation was not required for cells. Also, reappearance of mycoplasmata after the antibiotic treatment during cultivation is a major disadvantage. Surfactin kills the mycoplasmata by disrupting the membrane and the drug resistance is not possible as in

the case of antibiotics. One disadvantage of this method is that it cannot be used in systems with high protein content due to competitive binding of proteins. Combination of surfactin with enrofloxacin exhibited a synergistic effect and resulted in mycoplasma-killing activity at about two orders of magnitude higher than those of the individual molecules used (*73*).

Effect of surfactin on the cell membranes

Surfactin shows 'detergent-like' action on cell membranes (*74*). The term 'detergent like' was used due to its unspecific mechanisms of membrane permeabilization. Detergents act by inducing curvature in membranes, causing disorder of the acyl chains, a decrease in membrane thickness and lateral packing density with loss of membrane stability. Surfactin tilted the acyl chains, with its peptidic portion inserted into the hydrophobic interface of the membrane. The activity of surfactin was quantified in terms of product partition coefficient and CMC. Surfactant to lipid molar ratio was also an important factor, which was required for the membrane solubilization. Detergent-like action was found to be temperature-dependent with an increased surfactin activity being observed at lower temperature. It was proposed that at lower temperature membranes were more ordered and hence were more sensitive to a disordering agent. The advantage of surfactin was that it exhibited membrane-permeabilizing effect at concentrations much below the CMC values (*75*).

Atomic force microscopy (AFM) combined with surface pressure-area isotherms has been used to probe the interfacial behaviour of phospholipid monolayers following the penetration of surfactin (*76*). It was observed that the increase in the phospholipid chain lengths makes insertion of surfactin into the lipid membrane difficult. Electrostatic repulsions created by the presence of a negative charge on the phospholipid polar head prevented the peptide cycle from coming close to the phospholipid headgroups. The presence of salt decreases the electrostatic repulsion between the adsorbed molecules in favour of hydrophobic interactions. The penetration process is mainly governed by hydrophobic interactions between the fatty acid chain of surfactin and the phospholipid chains in the presence of salt. AFM images obtained for these systems indicated that the presence of a negative net charge in the phospholipid monolayer promotes the immiscibility between the interfacial components.

Surfactin adsorption onto a monolayer-free interface and its penetration into a lipid film is affected by the length of the lipopeptide chain and the nature of the peptide moiety. It was observed that the longer the surfactin acyl chain, the better its insertion into the lipid layer. This indicated that hydrophobic interactions are important for the penetration power of surfactin. The presence of a cyclic polar head (in contrast with a linear one) also favours surfactin penetration into a dipalmitoylphosphatidylcholine (DPPC) monolayer. It was proposed that linear surfactin mainly adopts a random coil conformation in the subphase, favouring a faster diffusion to the interface. At the interface, cyclic molecules with a rigid conformation have a reduced degree of freedom, resulting in rapid interfacial rearrangement. Cyclic surfactins at the interface are most likely to adopt a conformation in which the fatty acid chain is folded on the peptide ring. This folding is governed by the increased intramolecular hydrophobic interactions with lipopeptide chain length (*76–78*).

These observations provide an insight with respect to the interaction of surfactin with the lipid membranes. First, under physiological conditions, surfactin penetrates into cellular membranes independent of their phospholipid nature. The cyclic nature of the peptide moiety and the fatty acid chain length play an important role in the lipopeptide activity. Second, negatively charged phospholipids promote immiscibility of surfactin into the lipid matrix favouring surfactin self-assembly formation, which is the basis of pore-forming activity. Therefore, it was suggested that surfactin, like other antimicrobial peptides, exhibits a target selectivity behaviour based on the composition of the lipid matrix of the target cell. In particular, the lipopeptide could display a high pore-forming activity for membranes with a considerably high amount of anionic lipids such as bacterial membranes, aged blood erythrocytes, and some cancer cells (*78–80*).

The molecular mode of action of the lipopeptide surfactin with zwitterionic and negatively charged model membranes has been investigated with solid-state NMR, light scattering, and electron microscopy (*81*). A mechanism for small vesicle formation by a two-step action was proposed: (*i*) peptide insertion into membranes because of favourable van der Waals forces between the lipophilic part of surfactin and lipid chains, and (*ii*) electrostatic repulsion between the like charges of lipid head groups and the negatively charged surfactin amino acids. The phenomenon has been observed for membrane mixtures containing phosphatidylglycerol or phosphatidylserine.

Heerklotz and Seelig (*82*) reported the leakage and lysis of palmitoyloleoylglycerophosphocholine (POPC) vesicles induced by surfactin using calcein fluorescence dequenching, isothermal titration calorimetry and ^{31}P solid-state NMR. There are different mechanisms by which detergents can permeabilize membranes: (*i*) the bilayer-couple model (*83*) describes the effect of detergents that cannot flip quickly from the outer to the inner monolayer; (*ii*) at higher detergent concentration, detergent-rich clusters in the membrane become abundant, which destabilizes the lamellar structure and results in leaks by covering their edges with a detergent-rich rim; (*iii*) at a characteristic detergent-to-lipid ratio in the membrane, R_b^{sat}, the lipid membrane starts to become solubilized to mixed micelles. Membrane leakage starts at a surfactin-to-lipid ratio in the membrane, $R_b \approx 0.05$, and an aqueous surfactin concentration of $C_S^w \approx 2$ μM.

Anti-adhesive application

Colonization of a surface by bacteria causes nosocomial infections (*84*). Swarming motility and biofilm formation are the two reasons responsible for colonization of bacteria. Biosurfactants possess anti-adhesive properties, which inhibit the adhesion of bacteria onto the surface or infection sites. These infections share common characteristics even though the microbial causes and

host sites vary greatly. Bacteria in biofilms are highly resistant to antibiotics, and so they evade host defenses and withstand antimicrobial chemotherapy. Surfactin inhibits the adhesion of pathogenic organisms to solid surfaces or infection sites. It decreased the amount of biofilm formed by *Salmonella typhimurium*, *Salmonella enterica*, *Escherichia coli* and *Proteus mirabilis* in polyvinyl chloride wells, as well as vinyl urethral catheters. Precoating the catheters by running them through the solution of surfactin before inoculation with media was found to be effective. These results suggested the potential of practical application of surfactin in preventing the colonization by pathogens (*73*).

Antibacterial and anti-inflammatory application

Surfactants play an important role in defense against infection and inflammation in the human body. Pulmonary surfactant is a lipoprotein complex synthesized and secreted by the epithelial cells of lungs into the extracellular space, where it lowers the surface tension at the air/liquid interface of the lung and represents a key factor in host defense. Some biosurfactants may be used as alternatives to synthetic medicines and antimicrobial agents. Currently, resistance of the drugs of last resort is being investigated, including methicillin and vancomycin. These antibiotics are used in the therapy of nosocomial infections caused by enterococci and *Staphylococcus aureus* and also in the therapy of community-acquired methicillin resistant *S. aureus* (caMRSA), which is much more aggressive than its hospital relatives because of the preference it has for the young and healthy. Recently, several studies have revealed the impact of surfactin on silencing the inflammatory effect of the lipopolysaccharide (LPS) interaction with eukaryotic cells. Compounds that inactivate LPS activity have the potential of being new anti-inflammatory agents. It has been reported that surfactin inhibits the LPS-induced expression of inflammatory mediators (IL-1β and iNOS) and reduces the plasma endotoxin, TNF-α and nitric oxide levels in response to septic shock in rats. Also, surfactin was shown to suppress the interaction of lipid A with LPS-binding protein (LBP) that mediates the transport of LPS to its receptors. Surfactin had no influence on the viability of the tested eukaryotic cell lines (*73*).

Surfactin C shows better anti-inflammatory activity as compared to surfactin A, B or D. It inhibits nitric oxide and suppresses the expression of the pro-inflammatory cytokine mRNA, which is stimulated by lipopolysaccharide (*5*) in murine macrophage RAW264.7.

Other applications of surfactin include its antiviral action against semliki forest virus, herpes simplex virus (HSV-1 and HSV-2), suid herpes virus, vesicular stomatitis virus, simian immunodeficiency virus and feline calicivirus. Surfactin also acts as proteolytic agent. The plasminogen-plasmin system is involved in blood clot dissolution. Urokinase-type plasminogen activator (u-PA) activates the plasminogen and u-PA is secreted initially as zymogen prourokinase (pro-u-PA). Surfactin at 3–20 μM increased the prourokinase activation, leading to enhanced fibrinolytic activity.

Surfactin has also been reported to increase the biodegradation of pesticides (*85*). It can be used to wash oil from a sand column, or for removal of metals such as cadmium, copper and zinc from contaminated water by the use of technique called micellar-enhanced UF. It is also known for its selective inhibitory action against cytosolic PLA-2 responsible for inflammatory action.

Concluding Remarks

A host of interesting features of surfactin has led to a wide range of applications such as antibacterial, antiviral agent, anti-adhesive and anti-inflammatory uses. In spite of the immense potential, its use is still limited due to its high production cost and recovery. Novel strategies to improve its yields and recovery are required to lower the production cost. Better understanding of its effect and toxicity towards human cells is needed. Supportive studies are necessary to prove its potential in several biomedical and health related areas. Nevertheless, there appears to be a great potential for its use in the arena of medical science that is waiting to be fully exploited.

Abbreviations

PCP: peptidyl carrier protein
NRPS: nonribosomal peptide synthetase
TE: thioesterase
CoA: coenzyme A
Ppant: 4'-phosphopantethein
PPTases: 4'-phosphopantethein transferases
ACP: acylphosphatase
SMF: submerged fermentation
SSF: solid-state fermentation
AC: activated carbon
RSM: response surface methodology
MSI: iron-rich minimal salt
K_La: mass transfer coefficient
LM: Landy medium
UF: ultrafiltration
NF: nanofiltration
MMCO: membrane molecular mass cut-off
PES: polyethersulphone
CMC: critical micelle concentration
RC: regenerated cellulose
CE: cellulose ester
TMP: transmembrane pressures
PVDF: polyvinylidene fluoride
PCR: polymerase chain reaction
MHC: minimal haemolytic concentration
AFM: atomic force microscopy
DPPC: dipalmitoylphosphatidylcholine
POPC: palmitoyloleoylglycerophosphocholine
LPS: lipopolysaccharide
HSV: herpes simplex virus
u-PA: urokinase-type plasminogen activator

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