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Recovery and purification of the lipopeptide biosurfactant of *Bacillus subtilis* by ultrafiltration

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Surfactin, a lipopeptide biosurfactant produced as micelles by *Bacillus subtilis*, was recovered from the fermentation broth by ultrafiltration with a 30 kDa MWCO membrane. The retained surfactin micelles were then ruptured and collected in the permeate by adding methanol to 50% (v/v). The final yield of surfactin was 95%.

Introduction

The recovery and purification of biosurfactants from complex fermentation broth is a major problem in the commercialization of biosurfactants. The most widely used approaches for the recovery and purification of biosurfactants involve precipitation at extreme pHs and extraction with organic solvents. For example, the current practice for the recovery of surfactin, a lipopeptide biosurfactant produced by *Bacillus subtilis*, involves precipitation at pH 2.0 followed by organic extraction, adsorption chromatography, or thin layer chromatography (Arima *et al.*, 1968), which usually lead to the generation and release of hazardous wastes. Therefore, it is necessary to develop a more economic and environmentally-friendly approach for the recovery and purification of biosurfactants.

At concentrations above the critical micelle concentration (CMC), surfactant molecules associate to form supramolecular structures, such as micelles or vesicles, with nominal molecular diameters up to two to three orders of magnitude larger than that of the single unassociated molecules. It is, therefore, possible to retain surfactant molecules in the form of micelles in the retentate by ultrafiltration. This approach has been successfully employed for the recovery of surfactin, a lipopeptide surfactant produced by *Bacillus subtilis*, from complex fermentation medium (Mulligan and Gibbs, 1990). In this study, a modified ultrafiltration process for the recovery and purification of surfactin from fermentation medium will be discussed.

Materials and methods

Microorganism and growth conditions

Bacillus subtilis ATCC 21332 was grown in 2 liters of mineral salt medium containing 4% (w/v) glucose (Cooper *et al.*, 1981) at 30ºC in a 3-liter fermenter for 48 h. Cells were removed from the culture by centrifugation at 12,000 g for 10 minutes.

Ultrafiltration

Cell-free culture was concentrated by an Amicon magnetically stirred ultrafiltration cell (Beverly, MA, USA) with cellulose membranes of varying molecular weight cut-offs (MWCO) at pressure in the range of 6.9×10^4 and 2.1×10^5 Pa. Hollow fiber ultrafiltration cartridges (9 × 337 mm, A/G Technology, Needham, MA, USA) were used for continuous operation at a pressure of 1.7×10^5 Pa.

HPLC

The concentration of surfactin in the cell-free culture was determined by HPLC with with a Techsphere 5 μm ODS C18 reverse phase column. For each assay 100 μl cell-free culture was injected and eluted with a linear gradient of 70–79% methanol in 10 mM KH_2PO_4 buffer at pH 6.0 at 0.5 ml/min within 60 min. The absorbancy of the eluent was monitored at 210 nm. Surfactin purchased from Sigma was used as standard.

Results and discussion

Identification of surfactin

HPLC of the cell-free *B. subtilis* culture and the permeate through a ultrafiltration membrane with a MWCO of 3,000 daltons are shown in Fig. 1A and 1B, respectively. Compared with the chromatogram of surfactin standard from Sigma (data not shown), the peaks eluted between 31 and 45 minutes in Fig. 1A were identified as those of surfactin. The presence of multiple peaks on the chromatograms for standard surfactin and fermentation broth resulted from the existence of several surfactin structures produced by

B. subtilis. Like most secondary metabolites, surfactin consists of a family of lipopeptides with similar chemical structures. So far at least nine different surfactin structures have been identified (Horowitz and Griffin, 1991; Peypoux and Michel, 1992; Lin *et al.*, 1994; Wu and Lin, 1996). The absence of peaks eluted during the same period in Fig. 1B further confirmed that those peaks represented surfactin, because only a small number of unassociated surfactin molecules could penetrate the membrane with a MWCO of 3,000 daltons. The surfactin concentration in the cell-free broth was approximately 250 mg/l by HPLC analysis.

Recovery of surfactin

Surfactin was concentrated from 120 ml to 6 ml by ultrafiltration with membranes of MWCOs ranging from 1,000 to 100,000 daltons. In ultrafiltration systems with high MWCO membranes, small molecules in the fermentation medium, such as salts, amino acids, organic acids and alcohols, and other small metabolites are allowed to pass the membrane freely and are thus released into the permeate, while macromolecules, such as extracellular proteins, with nominal molecular diameters higher than MWCO of the membarne are concentrated in the retentate. At concentrations above its CMC, the excess surfactin molecules associated into micelles leaving only a small number of surfactin molecules, at concentration close to its CMC, unassociated. While the unassociated surfactin molecules can easily penetrate ultrafiltration membrane and be collected in the permeate, surfactin micelles are restricted from permeation and are concentrated in the retentate. The concentrations of surfactin in the retentate and the permeate were determined by HPLC. The retention factors of surfactin by ultrafiltration, defined as [surfactin in the retentate]/[total surfactin], with different MWCO membranes is shown in Fig. 2. Percentages of surfactin retained in the form of associated supramolecules with 10,000 and 30,000 daltons MWCO membranes were about 98.8 and 97.9%, respectively. The percentages of surfactin retained decreased significantly to 86 and 53% when 50,000 and 100,000 daltons MWCO membranes were used.

The results indicated that a majority of the surfactin micelles behaved as macromolecules with molecular weights in the range of 30,000 to 50,000 daltons, which could be effectively retained with membranes with a

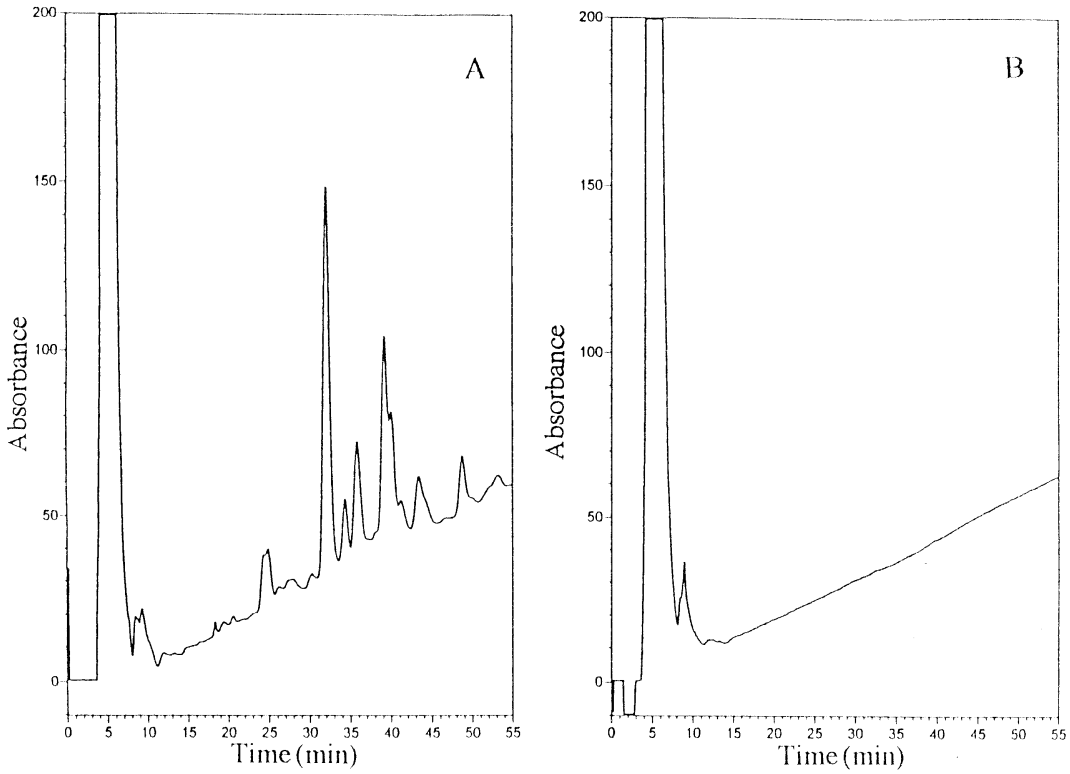

Figure 1 HPLC of cell-free *B. subtilis* fermentation broth (A) and permeate through a 3,000 MWCO ultrafiltration membrane (B).

MWCO of up to at least 30,000 daltons. Although the loss of surfactin was minimal with 3,000 daltons MWCO membrane, the filtration rate was relatively low. Taking filtration rate and retention factor into consideration, ultrafiltration membranes with MWCO of 10,000 and 30,000 daltons were used for further investigation.

Dissociation of surfactin micelles

The employment of ultrafiltration for the recovery of surfactin from complex fermentation broth has been previously reported (Mulligan and Gibbs, 1990). However, in addition to surfactin micelles, other macromolecules such as extracellular proteins and polysaccharides can also be simultaneously concentrated by ultrafiltration. It is, therefore, desirable to develop a process that can easily separate surfactin from these contaminating macromolecules.

This can be accomplished by dissociating surfactin micelles into unassociated molecules, which can then be easily forced through ultrafiltration membranes. It is well known that organic solvents such as alcohol and acetone can destabilize surfactant micelles. The effect of methanol concentration on the stability of surfactin micelles was studied, Fig. 3. The concentration of surfactin in the permeate in the form of unassociated molecules was again monitored by HPLC. The percentage of micelles ruptured was defined as [surfactin in the permeate]/[initial surfactin in the retentate]. The increase in methanol concentration resulted in the decrease in micelle stability, indicated by the increase in surfactin concentration in the permeate. At a concentration of 50%, methanol was effective in breaking down almost all micelles, i.e. the complete leakage of surfactin molecules into the permeate. High molecular weight components were retained in the retentate because their structures were not significantly affected by the presence of methanol.

The surfactin preparation collected in the permeate was further concentrated by drying under vacuum. TLC analysis on silica gel 60 in the solvent chloroform: methanol: water (65:25:4; by vol.) indicated that the purity of the surfactin preparation obtained by the proposed process was at least as high as that of the standard surfactin obtained by conventional process. The final yield of surfactin was 95%.

Conclusions

The recovery and purification of biosurfactants from the complex fermentation broth have been one of the major hurdles to the successful commercialization of biosurfactants. The current practices for biosurfactant recovery and purification usually leads to the generation of large volume of hazardous wastes. It is, therefore, necessary to develop alternative approaches

Surfactant micelles can be effectively concentrated by ultrafiltration. A modified ultrafiltraiion process, characterized by the subsequent elution of surfactin from the filtration system by the addition of methanol

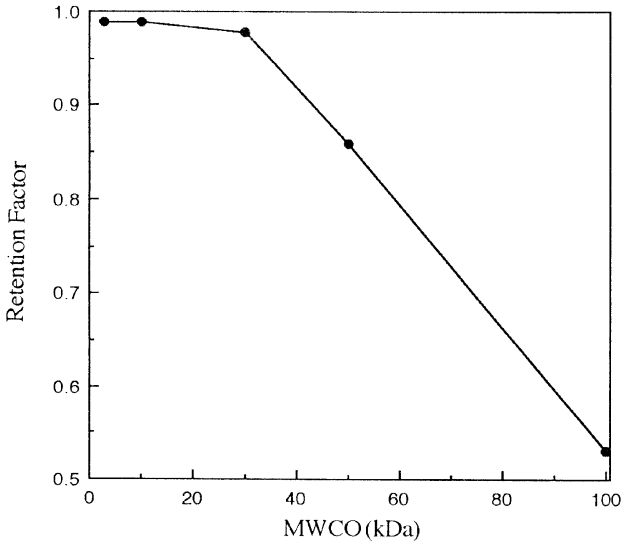

Figure 2 Retention factors of surfactin by ultrafiltration with different MWCO membranes.

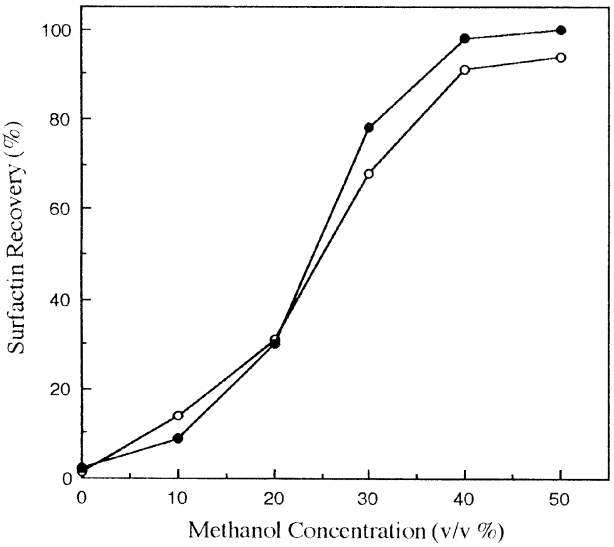

Figure 3 The effect of methanol concentration on the stability of surfactin micelles, indicated by the percentage of surfactin recovery in the permeate with ultrafiltration membranes with MWCO of 10,000 (○) and 30,000 (●) daltons.

following preliminary concentration, allows the recovery and purification of surfactin from fermentation broth in a single ultrafiltration unit. Furthermore, arrays of hollow-fiber cartridges can be developed for continuous recovery and purification of surfactin from fermentation broth.

Compared to conventional concentration processes, ultrafiltration processes have the advantages of minimal use and release of hazardous compounds and ease of scaling up. Furthermore, the elution of surfactin from the filtration system can not only effectively separate surfactin from other contaminating macromolecules but also minimize surfactin loss due to adhesion to ultrafiltration membranes, which can be very significant when more hydrophobic membranes are employed. This process can be further modified and employed for the recovery and purification of most surfactants from aqueous solutions at concentrations above the critical micelle concentration. Operational systems involving the employment of arrays of hollow fiber ultrafiltration cartridges can also be developed for continuous operation.

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