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General approach for the development of high-performance liquid chromatography methods for biosurfactant analysis and purification

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Abstract

A general approach for the development of HPLC methods for biosurfactant analysis and purification was proposed. By comparing the chromatograms of the cell-free fermentation broth, the ultrafiltration filtrate, and the ultrafiltration filtrate of a methanol–surfactant mixture, the peaks corresponding to biosurfactants can be identified without any prior structural information of the biosurfactants. It can be assumed that the peaks observed only on the chromatogram of the filtrate of methanol–surfactant mixture but not on the chromatogram of the filtrate are biosurfactant peaks. This approach can be applied for the development of a HPLC assay for any biosurfactants as long as the concentration of biosurfactants in the fermentation broth is higher than the critical micelle concentration. The HPLC methods thus developed can also be adapted for the preparation of homogeneous biosurfactant samples useful for chemical analysis for the elucidation of chemical structure of biosurfactants and for the determination of the physical properties of biosurfactants. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: Ultrafiltration; Surfactants; Lipopeptides; Surfactin

1. Introduction

Surfactants are amphiphilic molecules, consisting of hydrophilic and hydrophobic domains, which tend to partition preferentially at the interface between fluids of different degrees of polarity and hydrogen bonding. The formation of an ordered molecular layer at the interface lowers the interfacial tension and attributes to the unique surface properties of surfactants. Due to the unique interfacial behavior, surfactants find applications in various industrial processes involving emulsification, foaming, detergency, wetting and phase dispersion or solubilization.

Many biological molecules exhibiting particularly high surface activity are classified as biosurfactants. Microbial biosurfactants included a wide variety of chemical structures, such as glycolipids, lipopeptides, polysaccharide–protein complexes, phospholipids, fatty acids and neutral lipids [1–7]. In terms of physicochemical properties such as surface activity as well as pH and heat stability, many biosurfactants are comparable to synthetic surfactants [6]. Biosurfactants possess some advantages, such as low critical micelle concentration (CMC) and high biodegradability, over synthetic surfactants and, therefore, are particularly well suited for environ-

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mental applications such as bioremediation and the dispersion of oil spills [8–12].

Due to some technical and/or economic reasons, biosurfactants have not been employed extensively in industry. Like most microbial metabolites, biosurfactants exist in fermentation broth of complex composition at relatively low concentrations, which often makes the costs associated with the isolation and purification of biosurfactants prohibitively high. To make the large-scale production of biosurfactants possible, it is generally necessary to undergo the time-consuming and labor-intensive strain improvement programs and the systematic medium optimization studies. The success of strain improvement programs and medium optimization studies generally relies on the availability of efficient and specific analysis techniques for biosurfactants. Unfortunately, for most biosurfactants reported so far the techniques frequently employed for the detection of biosurfactants have been surface/interfacial tension measurements [13–17], which do not meet the desired criteria. The results of tension measurements for the quantification of biosurfactants are impractical and can be misleading in some instances. The correlation between surface/interfacial tension reduction and surfactant concentration holds for surfactant concentrations below the CMC, at which the surface/interfacial tensions reach a minimum. However, at concentrations above the CMC, the reduction in surface/interfacial tensions becomes negligible due to the association of excess surfactant molecules into supramolecular structures such as micelles, making the estimation of surfactant concentration impossible without serial dilutions. Although the results of tension measurements of the serially diluted solutions can provide a rough estimation about how much higher the surfactant concentration is than the CMC, exact biosurfactant concentrations can not be quantified without information about the value of the CMC. For example, it has been reported that a *Rhodococcus aurantiacus* strain produced a glycolipid biosurfactant at a concentration as high as 400×CMC [16]. However, the exact concentration of the glycolipid biosurfactant in the fermentation broth was still unknown because the CMC of the glycolipid biosurfactant has not been determined.

The employment of surface/interfacial tension measurement for medium optimization studies is also hindered by the fact that tensions of surfactant solutions are also strongly affected by many parameters such as pH and ionic strength frequently investigated in medium optimization studies. Therefore, even at concentrations below its CMC, the extent of surface tension reduction does not always correspond to the level of biosurfactant in the fermentation broth.

Another technique frequently employed for the characterization and quantification of biosurfactants has been thin-layer chromatography (TLC) [18,19]. Although TLC analysis can provide qualitative and quantitative information about biosurfactants, time-consuming pre-purification procedures, such as precipitation and organic extraction, are generally required.

Compared to tension measurements and TLC analysis, high-performance liquid chromatography (HPLC) represents an effective alternative for biosurfactant analysis with the desired sensitivity and selectivity. HPLC methods for quantitative analysis and/or for the purification of some lipopeptide biosurfactants have been reported [20–25]. However, the development of these HPLC methods generally required relatively pure biosurfactant samples, which cannot be obtained without tedious isolation and purification operations including HPLC. It is, therefore, necessary to explore a general approach for the development of efficient HPLC methods for biosurfactant analysis and purification.

In this study, a general approach incorporating ultrafiltration analysis was proposed for the development of HPLC analysis for biosurfactants without any prior structural or physicochemical information about the biosurfactants. The development of HPLC analysis for surfactin, a lipopeptide biosurfactant produced by *Bacillus subtilis*, was reported to demonstrate the feasibility of this approach. Nevertheless, the proposed approach can be used for the development of HPLC analysis for practically any microbial surfactants. The techniques used are also useful for the preparation of homogeneous biosurfactant samples required for determining the CMC and for performing chemical analysis, such as Fourier transformation infrared (FT-IR) analysis and nuclear magnetic resonance (NMR) spectroscopy, for the elucidation of chemical structures of biosurfactants.

2. Experimental

2.1. Microorganism and growth conditions

Bacillus subtilis ATCC 21332 (American Type Culture Collection, Rockville, MD, USA) was grown in a mineral salt medium supplemented with 4% glucose (Sigma, St. Louis, MO, USA) [26] at 30°C for 48 h. Cells were removed from the fermentation broth by centrifugation at 12 000 *g* for 10 min.

2.2. Ultrafiltration

Solutions of sodium dodecyl sulfate (SDS, Sigma), 2% (w/v), hexadecyltrimethylammonium bromide (CTAB, Sigma), 0.5% (w/v), lysozyme (Sigma), 0.1 mg/ml, in 10 m*M* potassium phosphate, pH 6.0 and cell-free broth of *B. subtilis* were concentrated from 10 ml to 2 ml by ultrafiltration with an Amicon magnetically stirred ultrafiltration cell (Beverly, MA, USA) assembled with ultrafiltration membranes of molecular mass cut offs (MWCOs) ranging from 500 to 100 000 at operation pressure in the ranges of $7 \cdot 10^4$ to $2 \cdot 10^5$ Pa. Feeds and filtrates from all ultrafiltration runs were collected for HPLC analysis. In some experiments, methanol was added into the concentrate obtained by ultrafiltration to a final concentration ranging from 10 to 60% (v/v) before further concentrations were conducted.

2.3. HPLC analysis

All analytical experiments were performed by reversed-phase HPLC with a Jasco HPLC system (Tokyo, Japan) equipped with a C_{18} column (Bondclone, 5 μm, 300×3.9 mm, Phenomenex, Torrance, CA, USA). The mobile phase used for the analysis of SDS was a mixture of methanol–10 m*M* potassium phosphate buffer at pH 6.0 (75:25, v/v) at a flow-rate of 1 ml/min; the mobile phase used for the analysis of CTAB was a mixture of acetonitrile–water both containing 0.1% trifluoroacetic acid (TFA) (70:30, v/v) at a flow-rate of 1 ml/min. The elution for SDS and CTAB analysis was monitored with a refractive index (RI) detector. The solvent system used for the analysis of lysozyme were mobile phase A (0.1% TFA in acetonitrile) and mobile phase B (0.1% TFA in water). The elution was conducted with a linear gradient from 40 to 45% A within 15 min at a flow-rate of 1 ml/min and monitored with an UV detector at 280 nm. Cell-free fermentation broth of *B. subtilis* and surfactin solution were analyzed with a mobile phase consisting of a mixture of methanol, mobile phase A, and 10 m*M* potassium phosphate buffer at pH 6.0, mobile phase B. The elution was conducted with a linear gradient from 60 to 75% A within 35 min at a flow-rate of 1 ml/min at 50°C and monitored with UV at 210 nm. All HPLC experiments were repeated at least twice. For each assay, a sample of 10 μl was injected.

3. Results and discussion

Surface active molecules at concentrations above its CMC tend to aggregate spontaneously into supramolecular micelles. The nominal molecular diameters of these surfactant micelles can be up to two-orders of magnitudes higher than that of the unassociated molecules. It is therefore possible to concentrate biosurfactants from fermentation broth by ultrafiltration with high MWCO membranes [27,28]. To demonstrate the feasibility of ultrafiltration for the recovery of biosurfactant from fermentation broth, the recovery of SDS, an anionic surfactant, and CTAB, a cationic surfactant, from aqueous solutions by ultrafiltration with various MWCO membranes were conducted. The HPLC chromatograms of the SDS solution and the filtrate collected from an ultrafiltration run with a MWCO 3000 membrane are shown in Fig. 1a and b, respectively. The peak corresponding to SDS, eluted at 6.3 min, was only observed on chromatogram of the SDS solution, Fig. 1a, but not on the chromatogram of the filtrate, indicating that most of the SDS molecules associated into micelles with high nominal molecular diameters and thus were effectively concentrated in the retentate. Only trace amount of unassociated SDS molecules were detected in the filtrate. Similar results were also observed for CTAB. The HPLC chromatograms of the CTAB solution and the filtrate collected from ultrafiltration run with the MWCO 3000 membrane are shown in Fig. 2a and b, respectively. The peak corresponding to CTAB, eluted at 10.1 min, was only observed on chromatogram of the CTAB solution, Fig. 2a, but not on the chromato-

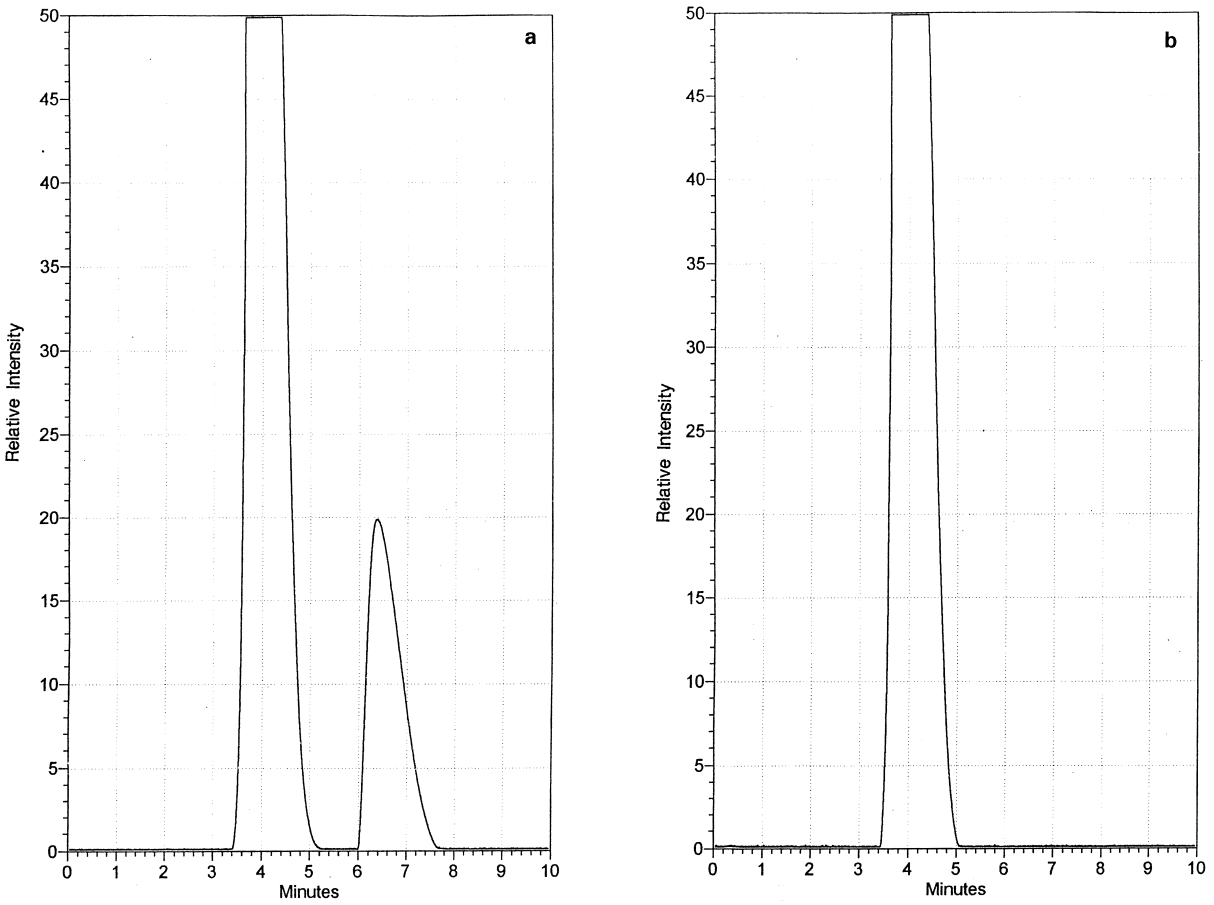

Fig. 1. Chromatograms of a 2% SDS solution (a) and of the filtrate collected from the concentration of SDS solution by ultrafiltration with a MWCO 3000 membrane (b). Ten μl of SDS solution was injected for each assay. The peak eluted at 6.3 min was identified as SDS. The peak eluted between 3 and 5 min was buffer front.

gram of the filtrate. The losses of unassociated SDS and CTAB into the filtrate, defined as [surfactant concentration in the filtrate]/[surfactant concentration in the feed], during ultrafiltration with various MWCO membranes are shown in Fig. 3. The loss of SDS during concentration by ultrafiltration with a membrane of MWCO as high as 10 000 was only 8.19%, although the molecular mass of SDS is only 288.4. The losses of SDS into the filtrates increased significantly to 14.13% with a MWCO 30 000 membrane and to 73.92% with a MWCO 50 000 membrane. These results indicates that the nominal molecular masses of most SDS micelles are between 30 000 and 50 000, about two-orders of magnitude higher than that of the unassociated molecules. The losses of CTAB, a cationic surfactant with a molecular mass of 364.5, were significantly lower than that of SDS, presumably due to the electrostatic repulsion between the charges on surfactant molecules and the charges on membrane surface. No significantly losses of CTAB were observed with membranes of MWCO below 30 000. The losses of CTAB into the filtrate with MWCO 50 000 and 100 000 membranes were 11.4 and 54.35%, respectively.

Based on these observations, it is possible to identify peaks corresponding to biosurfactants without any prior structural or physicochemical information about the biosurfactant by comparing the chromatograms of the fermentation broth and of the filtrate from ultrafiltration experiments. However, it

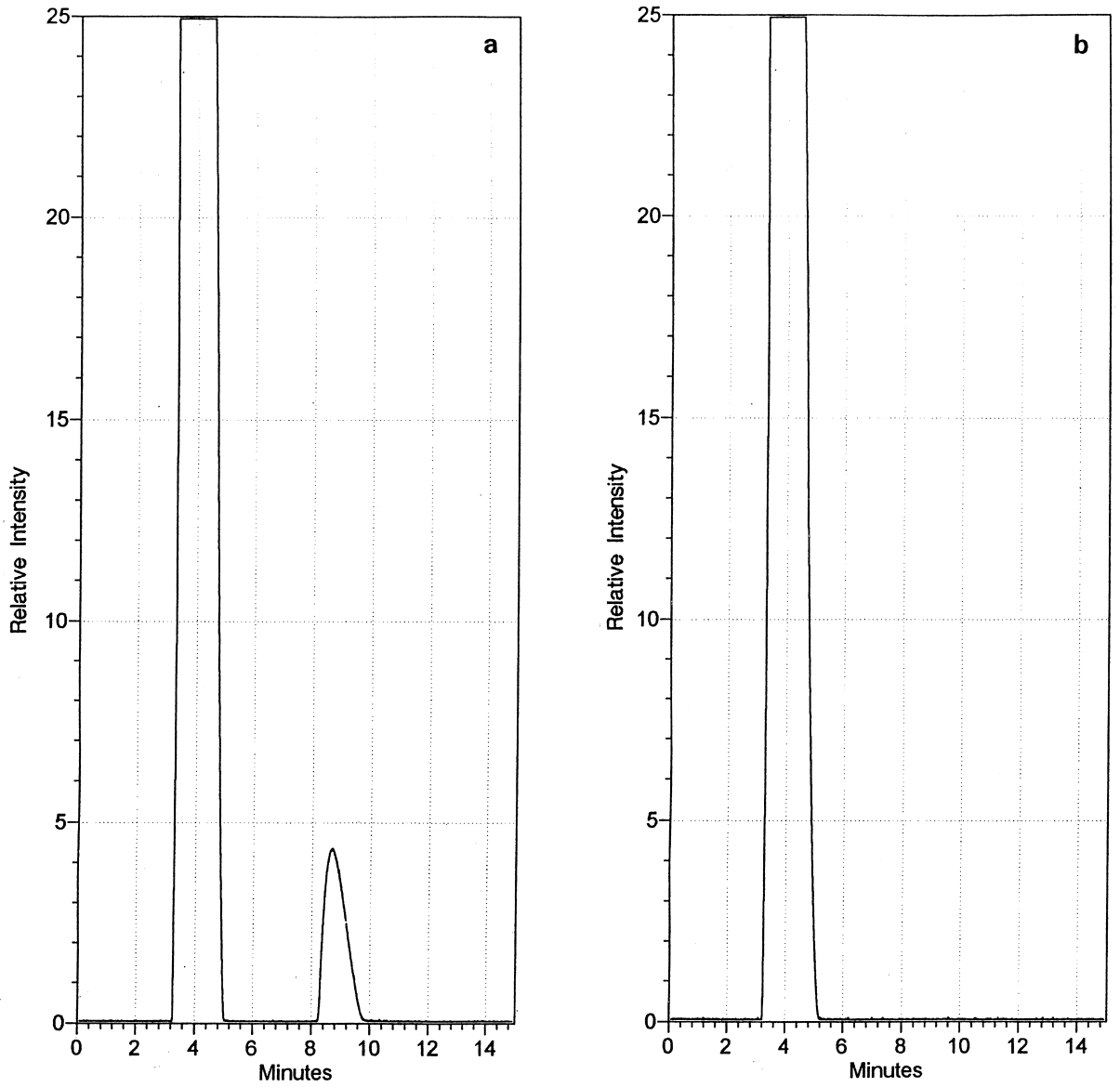

Fig. 2. Chromatograms of a 0.5% CTAB solution (a) and of the filtrate collected from the concentration of CTAB solution by ultrafiltration with a MWCO 3000 membrane (b). Ten μl of CTAB solution was injected for each assay. The peak eluted at 9.1 min was identified as CTAB. The peak eluted between 3 and 5 min was buffer front.

should be pointed out that some of the peaks observed only on the chromatogram of the fermentation broth but not on that of the filtrate may correspond to biological macromolecules, such as extracellular proteins or polysaccharides, other than biosurfactants. It is therefore necessary to develop a technique capable of differentiating peaks of biosurfactants and other macromolecular contaminants.

Alcohols and acetone are capable of dissociating surfactant micelles into free molecules. The dissociated surfactant molecules with molecular diameters well below the MWCO of the ultrafiltration membrane employed for surfactant concentration are free to permeate the membrane and can therefore be

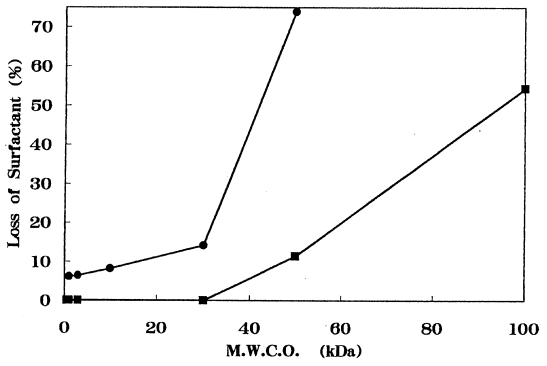

Fig. 3. Losses of unassociated SDS (●) and CTAB (■) molecules into the filtrates during ultrafiltration with membranes of MWCO ranging from 500 to 100 000. The degree of surfactant loss during ultrafiltration was defined as [surfactant concentration in the filtrate]/[surfactant concentration in the feed]×100%.

collected in the filtrate. The chromatograms of SDS solution containing 50% methanol and of the ultrafiltration filtrate of the solution are shown in Fig. 4a and b. The peak corresponding to SDS was observed on both chromatograms, indicating that most SDS micelles were dissociated into free molecules by 50% methanol and thus were collected in the filtrate. Similar phenomena were also observed for CTAB, Fig. 5. An extra peak eluted around 4.5 min corresponding to methanol was observed in Fig. 5. The presence of methanol in the samples also led to the small shift in CTAB retention time. The effectiveness of methanol in dissociating SDS and CTAB micelles was shown in Fig. 6. The degree of micelle dissociation was defined as [surfactant concentration in the filtrate]/[surfactant concentration in the surfac-

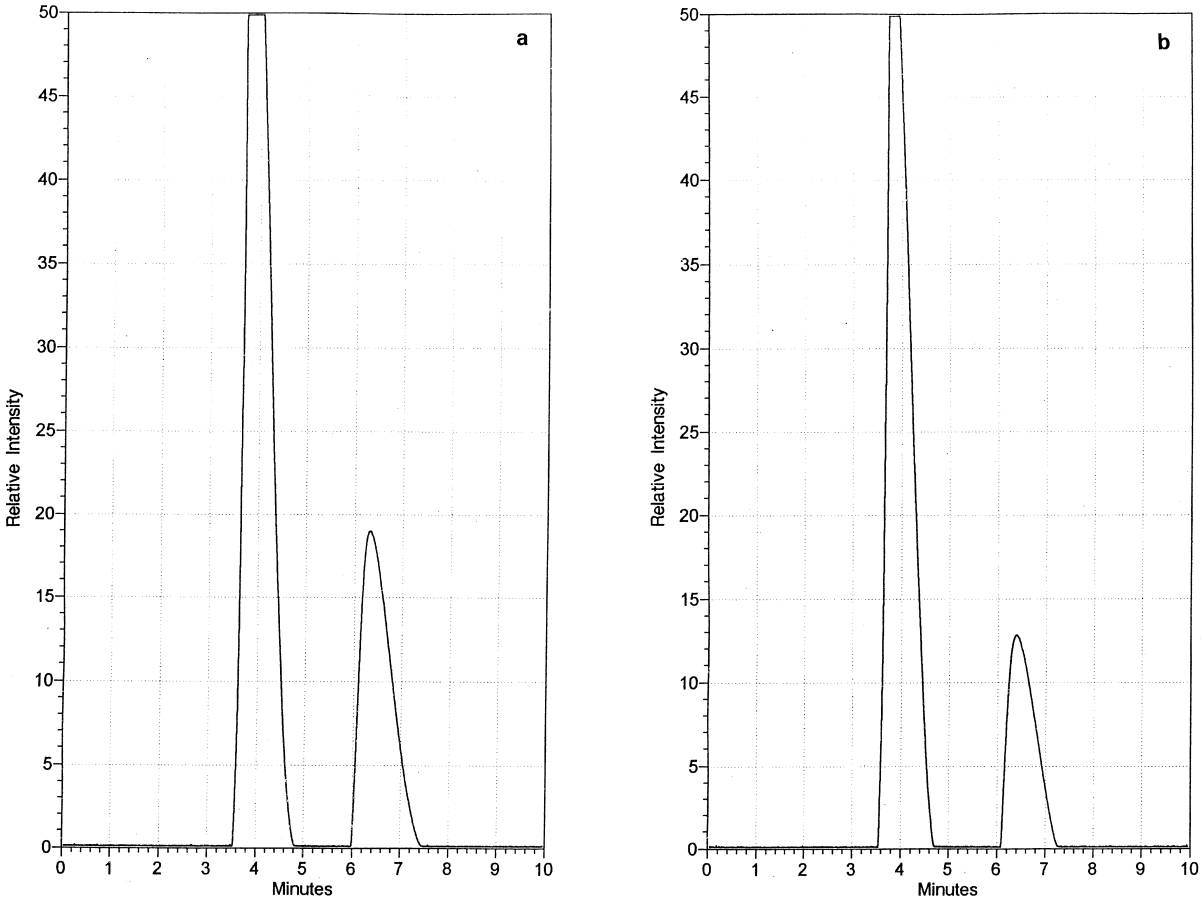

Fig. 4. Chromatograms of a 5% SDS solution containing 50% (v/v) methanol (a) and of the filtrate of the SDS–methanol solution (b). Ten μl of solution was injected for each assay. SDS peak, eluted at 6.3 min, was observed on both chromatograms.

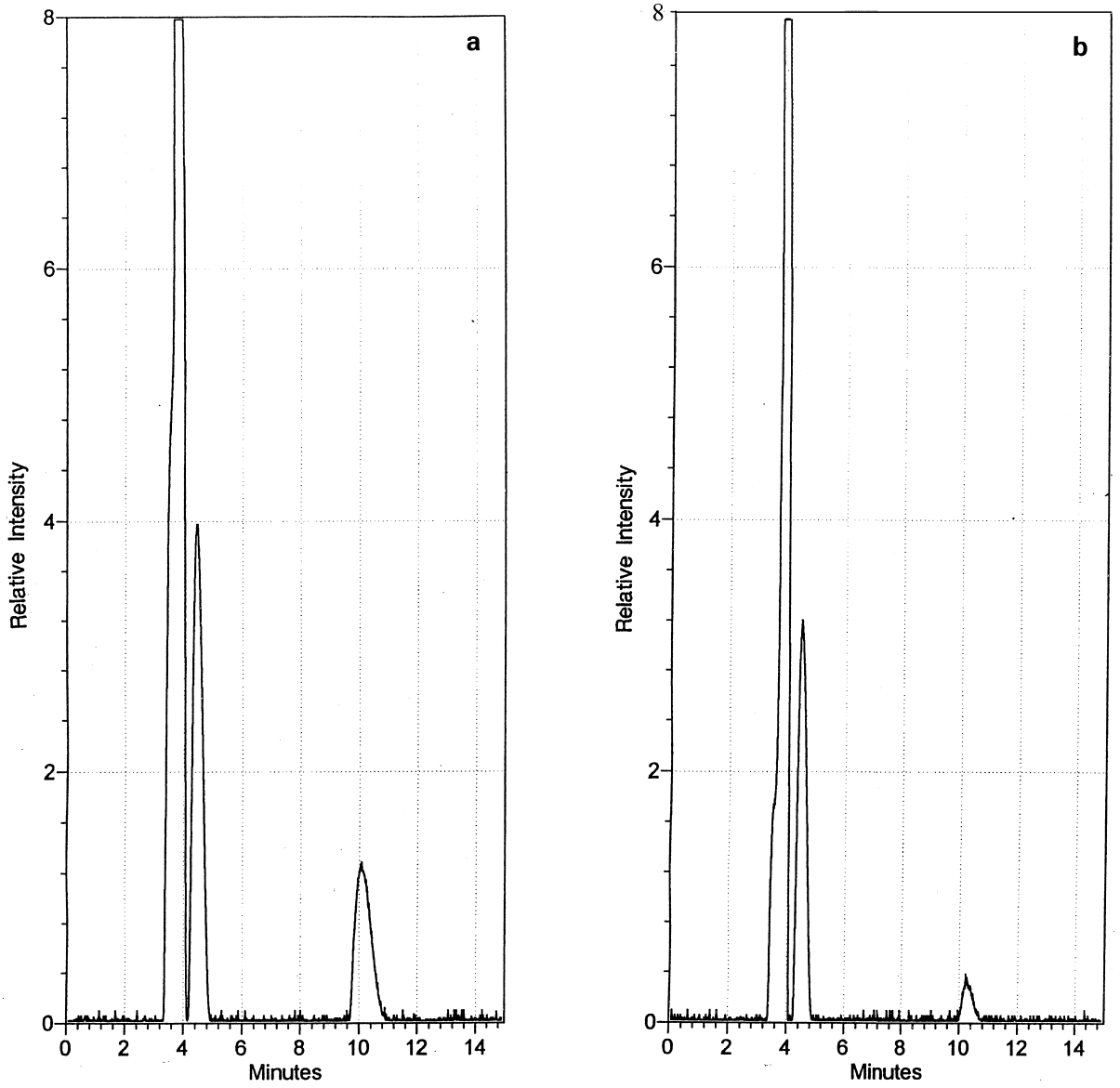

Fig. 5. Chromatograms of a 1% CTAB solution containing 50% (v/v) methanol (a) and of the filtrate of the CTAB–methanol solution (b). Ten μl of solution was injected for each assay. CTAB peak, eluted at 10.1 min, was observed on both chromatograms. The peak eluted around 4.5 min was methanol which also led to a slight shift in retention time.

tant–methanol solution]×100%. 75.6% of SDS micelles and 46.2% of CTAB micelles were dissociated with 50% of methanol. In the presence of 60% methanol, more than 90% of SDS micelles and CTAB micelles were dissociated.

To confirm that the nominal molecular diameter of other extracellular macromolecules will not be significantly altered by the presence of methanol, similar ultrafiltration experiments with lysozyme, a protein with a molecular mass of about 14 000, were conducted. The chromatograms of lysozyme solution, filtrate from ultrafiltration with a MWCO 10 000 membrane with and without methanol (50%) were shown in Fig. 7. The peak eluted at 8.6 min

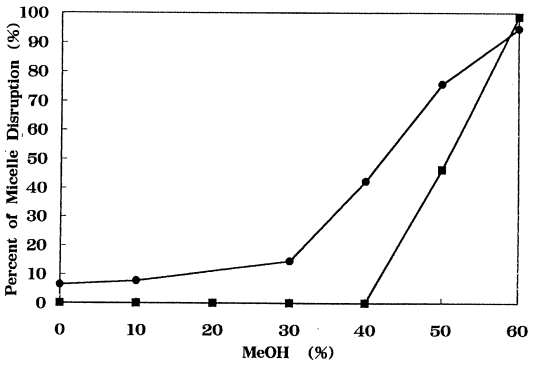

Fig. 6. Effects of methanol concentration on the degrees of SDS (●) and CTAB (■) micelle dissociation, defined as [surfactant concentration in the filtrate]/[surfactant concentration in the surfactant–methanol solution]×100%. A MWCO 3000 membrane was employed.

was identified as lysozyme. Unlike SDS or CTAB, a lysozyme peak was not observed on the chromatograms for the filtrate with or without methanol, indicating that lysozyme can be concentrated by ultrafiltration and that the presence of 50% methanol does not change the nominal molecular diameter of lysozyme to such an extent that its permeation through the membrane is not restricted. Similar behavior was also observed for poly(ethylene glycol) (PEG) 6000 with a MWCO 3000 membrane (data not shown).

These results indicate that surfactant micelles concentrated in the retentate can be separated from other macromolecules such as proteins by the addition of appropriate amount of methanol. Although the permeation behavior of polysaccharides was not studied in this report, the ultrafiltration and HPLC experiments with PEG suggested that it may also be possible to separate dissociated surfactant molecules from extracellular polysaccharides by the addition of methanol. Therefore, it is proposed that biosurfactant peaks for any cell-free fermentation broth can be identified by comparing the chromatograms of the fermentation broth, of the ultrafiltration filtrate, and of the ultrafiltration filtrate obtained from biosurfactant solution containing appropriate amount of methanol. The peaks disappear from the chromatogram of the ultrafiltration filtrate and reappear on the chromatogram of the filtrate with methanol can be identified as biosurfactants; the peaks observed only on the chromatogram of the fermentation broth but not on the chromatograms of the filtrates with or without methanol can be identified as macromolecules. The feasibility of this approach was tested for the identification of biosurfactants produced by *B. subtilis*.

B. subtilis has been shown to be effective for the production of surfactin, a highly active anionic lipopeptide biosurfactant [29]. The chromatograms of *B. subtilis* cell-free fermentation broth and of the ultrafiltration filtrate of the fermentation broth with a MWCO 10 000 membrane were shown in Fig. 8. The peaks eluted between 18 and 31 min on the chromatogram of the broth, Fig. 8a, were not observed on the chromatogram of the ultrafiltration filtrate, Fig. 8b, indicating that these peaks corresponded to surfactin micelles and/or macromolecules retained in the concentrate of ultrafiltration. To further confirm the identities of these peaks, methanol was added to the ultrafiltration concentrate to a final concentration of 50%, and the resultant solution was further concentrated by ultrafiltration. The chromatogram of the filtrate was shown in Fig. 9a. The peaks eluted between 18 and 31 min were observed again in the chromatogram, indicating that these

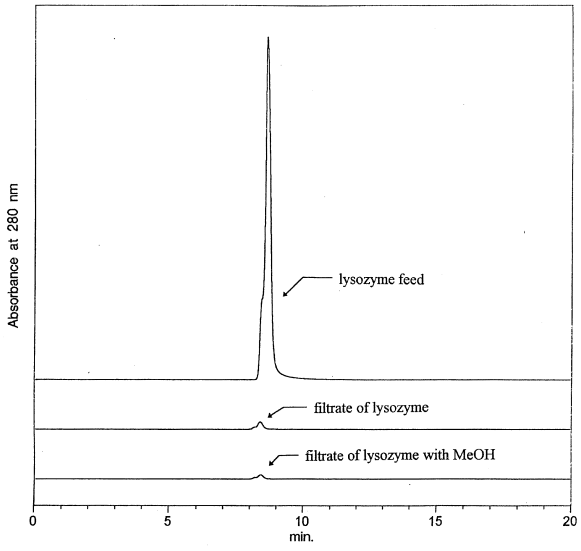

Fig. 7. Chromatograms of a lysozyme solution (0.1 mg/ml), of the filtrate of the lysozyme solution, and of the filtrate of the lysozyme–methanol solution. A MWCO 10 000 membrane was employed. Ten μl of solution was injected.

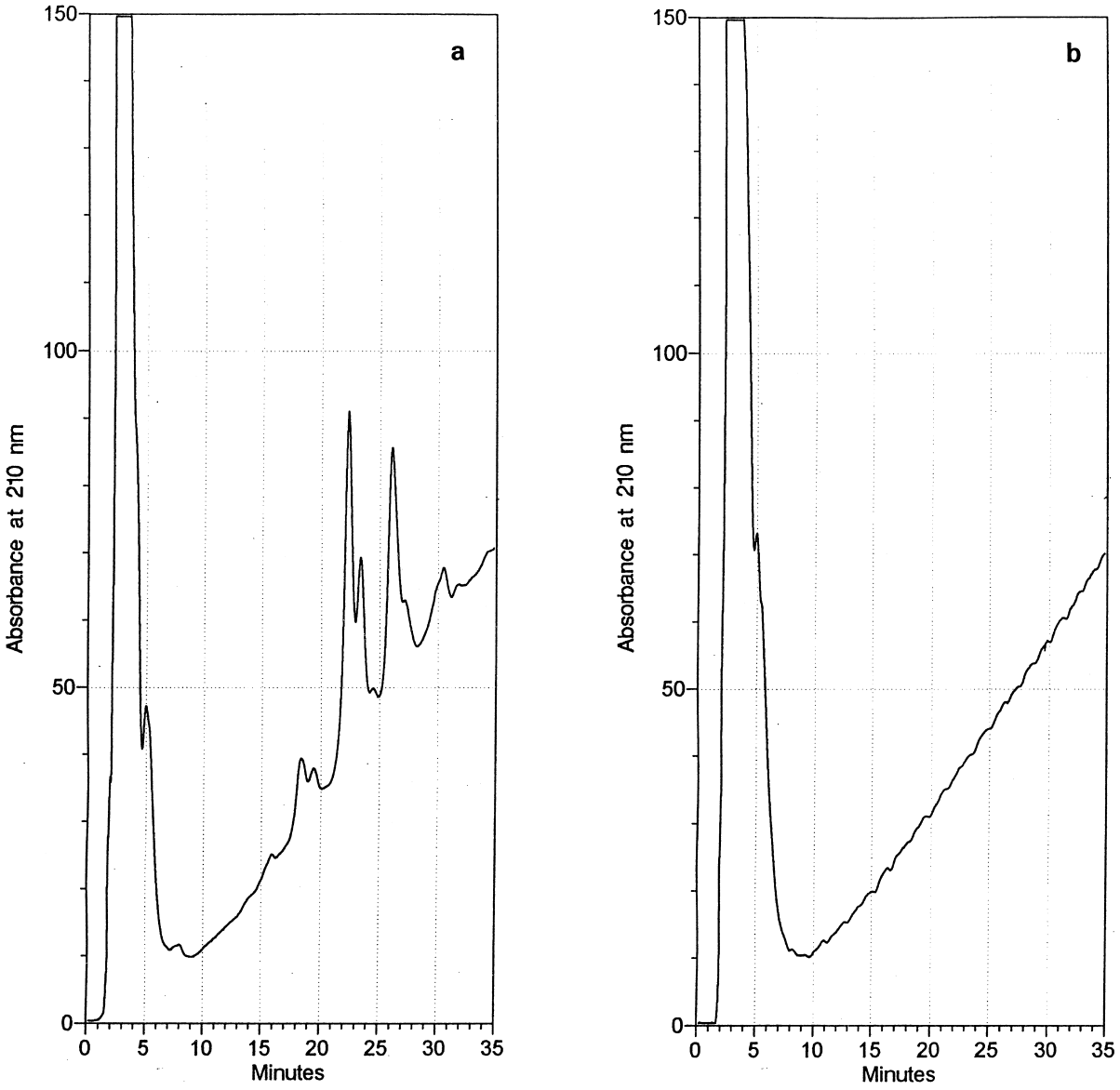

Fig. 8. Chromatograms of the cell-free *B. subtilis* fermentation broth (a) and of the filtrate of the fermentation broth (b). A MWCO 10 000 membrane was employed. Ten μl of solution was injected for each assay.

peaks corresponded to micelle-forming molecules, surfactin, instead of other extracellular macromolecules, because as shown in Fig. 7 the peak corresponding to macromolecules such as proteins should not be observed on the chromatogram of the filtrate with methanol. The chromatogram of surfactin standard from Sigma was shown in Fig. 9b. It can be observed that the peaks eluted between 18 and 31 min in Fig. 9a and b were essentially identical, further confirming that these peaks corresponded to surfactin.

The presence of more than one surfactant peaks on the chromatograms for surfactin standard was resulted from the existence of several surfactin structures produced by *B. subtilis*. Like most secondary metabolites, surfactin consists of a family of lipopep-

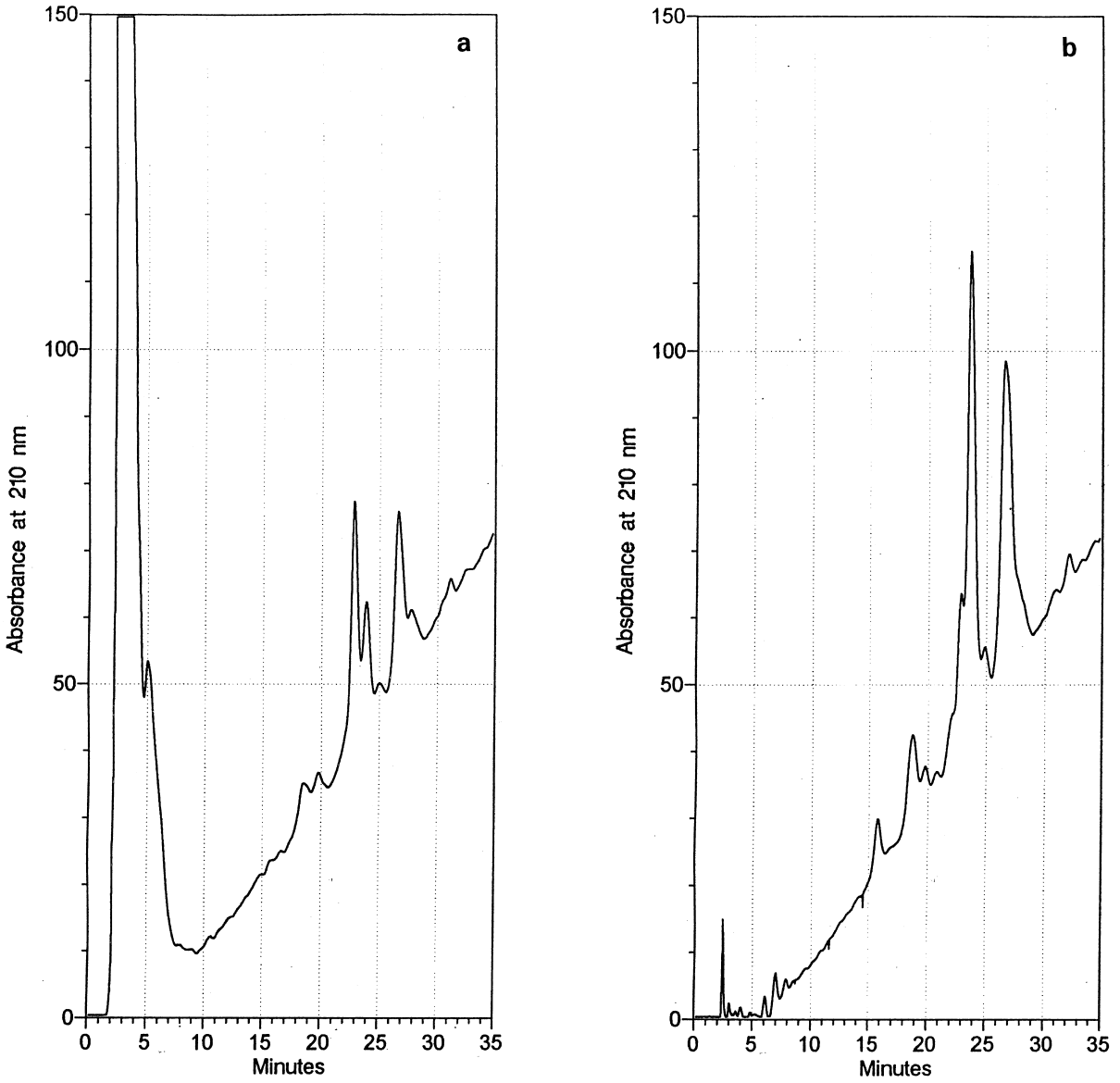

Fig. 9. Chromatograms of the filtrate of the concentrated fermentation broth containing 50% methanol (a) and of the surfactin standard, 500 mg/l, (b). A MWCO 10 000 membrane was employed. Ten μl of solution was injected for each assay.

tides with similar chemical structures. So far at least nine different surfactin structures has been identified [24,27,30]. The relative areas of the surfactin structures eluted between 18 and 23 min of the fermentation broth, Fig. 9a, are slightly different from those of the standard from Sigma, Fig. 9b. The difference in relative areas were resulting from the presence of different surfactin compositions. It has been reported that surfactin molecules of distinctive chemical structures are produced at different concentrations and ratios under different fermentation conditions such as medium formulation [30].

4. Conclusions

Surface-active compounds at concentration above the CMC tend to associate into supramolecular structures such as micelles, which can be concentrated effectively by ultrafiltration with high MWCO membranes. Surfactant micelles, formed under the influence of hydrophobic interaction, can be easily dissociated into unassociated molecules by the addition of appropriate amount of alcohol. Unlike surfactant micelles with significantly higher nominal molecular diameters, the unassociated surfactant molecules can permeate high MWCO membranes freely. Therefore, by comparing the chromatogram of the cell-free fermentation broth, such as Fig. 8a, the chromatogram of the ultrafiltration filtrate, such as Fig. 8b, and the chromatogram of the ultrafiltration filtrate of methanol–surfactant mixture, such as Fig. 9a, the peaks corresponding to that of the biosurfactants can be identified without any prior structural information of the biosurfactants. In general, it can be assumed that the peaks observed only on the chromatogram of the filtrate of methanol–surfactant mixture but not on the chromatogram of the filtrate are biosurfactant peaks.

This approach can be applied for the development of HPLC methods for any biosurfactants as long as the concentration of biosurfactant in the fermentation broth is higher than its CMC. If the concentration of the biosurfactant is below the CMC, pre-concentration by evaporation or freeze–drying is necessary to increase the biosurfactant concentration to above the CMC. This approach can also be used for the preparation of homogeneous biosurfactant samples necessary for performing chemical analyses such as NMR, FT-IR and fast atom bombardment (FAB) MS useful for the elucidation of chemical structure and for determining the physical properties of biosurfactants such as CMC [31].

Acknowledgements

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