

## Recovery of Biosurfactants by Ultrafiltration

Catherine N. Mulligan<sup>a</sup> & Bernard F. Gibbs<sup>b\*</sup>

<sup>a</sup>Biochemical Engineering and <sup>b</sup>Protein Engineering Sections, National Research Council of Canada, Biotechnology Research Institute, 6100 Royalmount Ave., Montreal, Quebec, Canada H4P 2R2

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### ABSTRACT

*Ultrafiltration was used in a one-step method to purify and concentrate biosurfactants—surfactin and rhamnolipids—from culture supernatant fluids. The ability of surfactant molecules to form micelles at concentrations above the critical micelle concentration allows these aggregates to be retained by relatively high molecular weight cut-off membranes. Lower molecular weight impurities such as salts, free amino acids, peptides and small proteins are easily removed. Various molecular weight cut-off membranes were examined for the retention of surfactin and rhamnolipids (mol. wts 1036 and 802 respectively). Amicon XM 50 was the superior membrane for retention of surfactin and a 160-fold purification was rapidly achieved. The YM 10 membrane was the most appropriate for rhamnolipid recovery. Ultrafiltration can play an important role in biosurfactant purification as large volumes of media can be processed rapidly at extremely low cost.*

**Key words:** ultrafiltration, surfactin, rhamnolipid, biosurfactant purification.

### 1 INTRODUCTION

Although biosurfactants are biodegradable and very effective, commercial interest remains low because they are present at only low concentrations during fermentation.<sup>1</sup> As most of these compounds are lipid-based, classical recovery methods such as precipitation, crystallization and solvent extraction have been used.<sup>2</sup> Other methods, such as *in-situ* recovery, are being developed to reduce solvent requirement and product degradation.<sup>3</sup>

\* To whom correspondence should be addressed.

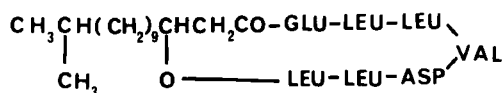


Fig. 1. Structure of *B. subtilis* surfactin. Amino acids are represented as: ALA—alanine, VAL—valine, GLU—glutamic acid, and LEU—leucine.

In the case of the cyclolipopeptide surfactin, produced by *Bacillus subtilis*, foam flotation and fractionation minimize end-product inhibition and concentrate the surfactant. After foam collapse and cell removal, acid precipitation followed by solvent extraction has been used for purification.<sup>4</sup> Similar recovery processes are required for surface active rhamnolipids R-1 and R-2 from *Pseudomonas aeruginosa*.<sup>5,6</sup> Adsorption and ion-exchange chromatography have also been used in pilot-plant studies.<sup>7</sup> These are key examples of solvent- and labour-intensive processes.

In this study, ultrafiltration was evaluated as a method to concentrate surfactin (Fig. 1) and rhamnolipids from the collapsed foam. Surfactant molecules form micelles at concentrations higher than the critical micelle concentration (CMC), and the remaining molecules remain unassociated.<sup>8–11</sup> Micelles would be retained by high molecular weight cut-off membranes.

Biosurfactants have very low CMCs, ideal for ultrafiltration. The CMC of the cyclolipopeptide has been reported<sup>4</sup> as  $0.025 \text{ g dm}^{-3}$  with a molecular weight of 1036.<sup>12</sup> The rhamnolipids have CMCs<sup>13</sup> of  $0.050\text{--}0.200 \text{ g dm}^{-3}$  with molecular weights for R-1 and R-2 of 744 and 802, respectively.<sup>5,6</sup>

Various molecular weight cut-off membranes were evaluated for their ability to concentrate and purify the biosurfactants.

## 2 MATERIALS AND METHODS

### 2.1 Microorganisms

*Bacillus subtilis* ATCC 21332, was maintained at 4°C on 4% glucose, mineral salt medium<sup>4</sup> agar plates. *Pseudomonas aeruginosa* ATCC 9027 was maintained on *Pseudomonas* agar P (Difco).

### 2.2 Cultivation conditions

After 3 days growth in  $100 \text{ cm}^3$  of 4% glucose and mineral salt medium supplemented with  $3.2 \times 10^{-4} \text{ mol dm}^{-3}$   $\text{FeSO}_4$ ,  $50 \text{ cm}^3$  of *B. subtilis* was transferred into  $500 \text{ cm}^3$  ( $2 \text{ dm}^3$  flask). After 6 h of growth, inoculum ( $0.5 \text{ dm}^3$   $10.0 \text{ dm}^{-3}$ ) was added to a  $20 \text{ dm}^3$  Bioengineering fermenter. The following cultivation conditions were used: aeration at  $20 \text{ dm}^3 \text{ min}^{-1}$ , pH control at 6.7, 100 rpm agitation and 37°C. Foam was collected and collapsed in a flask on the air exhaust line.<sup>4</sup> *Pseudomonas aeruginosa* was grown in a similar manner in proteose peptone medium.<sup>14</sup> Cells were removed by centrifugation in a Beckman centrifuge at  $12\,000 \text{ g}$  for 10 min.

### 2.3 Ultrafiltration

Cell-free foam fractions were concentrated by an Amicon magnetically stirred ultrafiltration cell, containing a YM 10, YM 30, XM 50, XM 100 or XM 300 membrane (mol. wt cutoffs of 10 000, 30 000, 50 000, 100 000 and 300 000 daltons respectively). A pressure of 172 kPa was used.

### 2.4 Analytical methods

Surface tension, CMC, and amino acid concentration were determined on the permeate and the retentate throughout ultrafiltration. Surface tension was determined by the de Nouy method with a Fisher Tensiomat Model 21. The CMC was determined by measuring the surface tension at various dilutions.<sup>15</sup> The logarithm of the dilution was plotted as a function of the surface tension. The CMC is the point at which the surface tension abruptly increases. The reciprocal of CMC is an indication of relative concentration.

The amount of surfactin was determined by amino acid analyses. A 10  $\mu\text{m}^3$  aliquot was dried and acid hydrolysed for 2.5 h at 150°C in a PICO-TAG amino acid analysis system. The residue was redissolved in 200  $\mu\text{m}^3$  of sodium buffer and injected on a Beckman System 6300 high performance analyser equipped with a Beckman Model 7000 data station. All buffers and ninhydrin reagents were purchased from Beckman. The concentration of surfactin was calculated by multiplying the lipopeptide concentration ( $\text{mol dm}^{-3}$ ) by the molecular weight (1036).

### 2.5 Chemical isolation of surfactin

Surfactin was isolated by adding concentrated hydrochloric acid to the collapsed foam after cell removal.<sup>4</sup> Dichloromethane (1:1, v/v) was added to the suspension in a separatory funnel and shaken vigorously. The aqueous (bottom) layer was removed and extracted twice more as described above. The organic layer was pooled and evaporated. The residue was redissolved in water (pH 8.0) and filtered through Whatman No. 1 paper to remove undissolved impurities. Concentrated HCl was again added to the filtrate and extracted with dichloromethane (1:1, v/v) three times and evaporated as described.

### 2.6 Viscosity measurement

The viscosity of the surfactin solution was measured by the Couette principle using a Contraves Low Shear 30 rotational rheometer. Temperature was controlled at 26°C by a Contraves Rheotherm 115 water bath.

### 2.7 Glucose and phosphate measurements

Glucose and inorganic phosphates were analysed using a Waters high performance liquid chromatograph (HPLC), equipped with a Digital Model 350 computer. For the glucose analysis, a Shodex DC613 column was used. With a mobile phase of acetonitrile–water (70:30) at a flow rate of 0.8  $\text{cm}^3 \text{min}^{-1}$  (50°C), glucose was detected by a Waters 401 differential refractometer.

Phosphates were detected by a Waters 430 conductivity detector, mobile phase of

gluconate ( $16 \text{ g dm}^{-3}$ ), boric acid ( $18 \text{ g dm}^{-3}$ ), and sodium tetraborate  $\cdot 10\text{H}_2\text{O}$  ( $25 \text{ g dm}^{-3}$ ), at a flow rate of  $0.75 \text{ cm}^3 \text{ min}^{-1}$  ( $45^\circ\text{C}$ ). The analyses were performed using a Waters ICPAK A column.

### 2.8 Mass spectrometry

Mass spectra were obtained in the positive ion mode on a VG Analytical ZAB-HS double focusing mass spectrometer. The accelerating voltage was 10 kV and the fast xenon atom beam was operated with an emission current of 1 mA at 8 kV. Mass spectra were recorded with an integrated data acquisition system and calibration was performed with CsI. Spectra for samples are an average of 10 scans.

## 3 RESULTS AND DISCUSSION

Five different ultrafiltration membranes were evaluated for their ability to retain surfactin and remove impurities. A  $10 \text{ cm}^3$  sample of collapsed foam was concentrated to *c.*  $1 \text{ cm}^3$  in each case. The results are shown in Table 1. Surfactin retention by the three lower molecular weight cut-off membranes was superior to the remaining two. This implies that between 50 and 100 molecules aggregate to form micelles. The permeates (YM 10, YM 30 and XM 50) contained only the surfactin molecules which were unassociated. This was confirmed by the relatively high surface tension measurements. Because of the low CMC of the surfactant, only small fractions of the molecules pass through these membranes. Although the CMC has been reported<sup>4</sup> as  $0.025 \text{ g dm}^{-3}$ , a concentration of  $0.011 \text{ g dm}^{-3}$  was determined by amino acid analysis and surfactant dilutions.

Threonine, serine, glycine and alanine were found in the foam fraction, in addition to the surfactin amino acids. Retention of these impurities decreased as the molecular weight cut-off increased (Table 1). Although ultrafiltration improved the surfactin amino acid composition, no significant difference was seen between the membranes.

**TABLE 1**  
Purification of *B. subtilis* Surfactin by Ultrafiltration

Membrane	Retention of surfactin (%)	Surfactin amino acids in retentate (%)	Purification factor	Viscosity of retentates (cP)	Surface tension of permeates ( $\text{mN m}^{-1}$ )
None <sup>a</sup>	0.0	92.9	1.0	1.0	27.8
YM 10	98.2	96.6	9.9	1.2	34.6
YM 30	96.8	96.7	9.9	1.3	31.7
XM 50	98.2	96.9	9.8	1.2	31.9
XM 100	73.8	97.1	7.8	1.2	30.5
XM 300	28.0	97.6	2.9	1.0	30.6

<sup>a</sup>Data represent the characteristics of the surfactin solution before ultrafiltration.

The retention of other impurities by the membranes was also verified. Glucose ( $10 \text{ g dm}^{-3}$ ) and inorganic phosphate ( $4.2 \text{ g dm}^{-3}$ ) were two major components in the growth medium. None of the glucose and 10% of the phosphates were retained by each of the membranes. Ultrafiltration efficiently removed free amino acids, small peptides, proteins and medium components from the product.

In a larger-scale experiment,  $7 \text{ dm}^3$  of collapsed foam was passed through a YM 30 membrane. A purification factor of 160 was achieved with 90% retention of the surfactant. A surfactin concentration of  $51.8 \text{ g dm}^{-3}$  was obtained. The purity (52.6%) of the dried surfactin was superior to that of the chemically purified product (31.6%). Mass spectrometry (Fig. 2) confirmed the structure of the surfactin. The spectrum agrees with the original identification of the compound.<sup>12</sup> The protonated molecular ion ( $\text{MW} + \text{H}^+$ ) is seen at  $\text{M}/\text{Z} = 1037$ .

Concentrating surfactin up to 10-fold (Table 1) did not significantly increase viscosity. However, as the surfactin concentration increased by 160-fold, the viscosity became significant ( $4.1 \text{ cP}$  at  $51.8 \text{ g dm}^{-3}$ ), retarding the filtration rate. In view of the above, surfactin concentrations should be limited to *ca.*  $20 \text{ g dm}^{-3}$ .

Retention of rhamnolipids from *P. aeruginosa* by ultrafiltration membranes was examined. The YM 10 (Table 2) was the most effective membrane as only a small fraction of the biosurfactant passed through this membrane. These rhamnolipid micelles are smaller than the surfactin aggregates.

In summary, ultrafiltration is a simple technique for surfactin purification. Membranes with relatively high molecular weight cut-offs (i.e. 50 000) can be used as aggregates of 50–100 molecules are formed at a concentration above the CMC. Impurities can be easily removed with minimum surfactin loss. Recovery costs are dramatically reduced, as large volumes of solvents are not required. In addition, this method requires only a fraction (*ca.* 2%) of the time required for the quickest previously published method.<sup>4</sup> This technique is not restricted to lipopeptide and

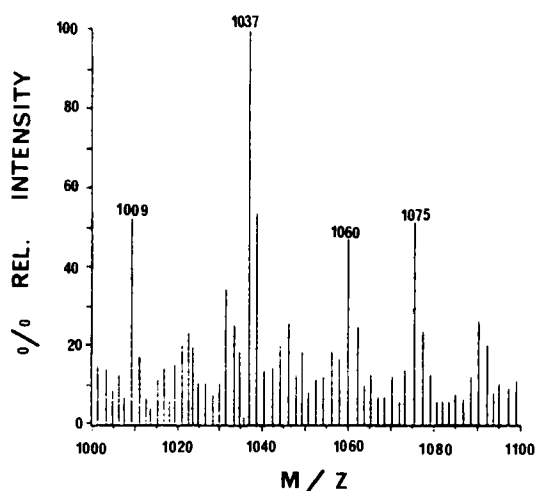


Fig. 2. Mass spectrum of purified *B. subtilis* surfactin. The protonated molecular ion is seen at 1037. The spectrum agrees with the original authors<sup>12</sup>

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