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# Improved performance of cross-flow ultrafiltration for the recovery and purification of $Ca^{2+}$ conditioned lipopeptides in diafiltration mode of operation

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ABSTRACT

This is for the first time that an attempt was made to enhance the performance of cross-flow ultrafiltration (CFUF) for the recovery and purification of lipopeptides by size-conditioning their micelles with $Ca^{2+}$ ions and operating the system in diafiltration mode. In this study, polyether sulfone (PES) membranes with MWCOs 10, 30 and 50 kDa were evaluated for recovery and purification of lipopeptides produced by *Bacillus* sp. CFUF studies performed under batch mode showed poor recovery ($68 \pm 2\%$) of $F_2$ fraction comprising Fengycin isoforms, whereas $F_1$ fraction comprising a mixture of surfactin and iturin isoforms showed a moderate recovery of $83.5 \pm 2.4\%$ in 50 kDa MWCO membrane. To improve the recovery, lipopeptides were conditioned with $Ca^{2+}$ ions. The CFUF purification of this size-conditioned lipopeptide solution under diafiltration mode was carried out in 50 kDa PES membrane. Continuous irrigation of $Ca^{2+}$ to lipopeptide solution under diafiltration mode of CFUF resulted in the increased recovery of lipopeptides, $F_1$ (surfactin+iturin) fraction about $90.5 \pm 2.1\%$ and $F_2$ about $95.3 \pm 2.0\%$. Further, the colored impurities with MW range of 2334–3506 Da were effectively removed from the solution. The results of CFUF purification of $Ca^{2+}$conditioned lipopeptide solution under diafiltration mode indicate that this process can be readily scaled up to the commercial level, workable even with higher MWCO membranes.



## 1. Introduction

Surfactin, iturin and fengycin are lipopeptide type biosurfactants that are primarily synthesized by *Bacillus* sp. Based on the nature of species, they are synthesized either as a single compound [1] or in conjunction with one or more other types of lipopeptides [2–4]. Marine *Bacillus circulans*, for example, is capable of synthesizing all these three lipopeptides [4], however, at relative concentrations based on the nature of medium and culture conditions. Different applications of lipopeptides have emerged based on the desired level of purity of the final product. Crude lipopeptides can straightaway be used in MEOR [5] and other bioremediation related applications [6,7], where the overall economy of the process is the most important concern. On the other hand, partially purified fractions (about 60–80% pure) can suit applications in microemulsion based nanoparticle synthesis [8,9], laundry [10] and food industry [11]. However, the requirement for ultra-high pure product is indispensable, if the lipopeptides are to be considered for pharmaceuticals and human healthcare applications [12–14]. Being versatile molecules and with many outstanding properties such as antibacterial, anticancer and antiviral [13,15] pertaining to human healthcare applications, they are required to be produced and purified in a more economical way. In attempts to improve the lipopeptide production, approaches such as the use of cheaper substrates to reduce the cost of raw materials [10,16], process optimization and intensification to improve the yield and productivity [1,3,17], and genetic engineering of organisms to improve the product synthesis have been successfully implemented to a great extent. On the contrary, downstream processing steps have not been so effective in achieving the desired level of purity in lipopeptides for an intended application. More often than not, the presence of mixture of lipopeptides in the crude product makes their recovery and purification more complicated because of the narrow differences in their molecular weights, existence in various isoforms (surfactin isoforms – 994 to1064 Da, iturin isoforms – 1043 to 1112 Da, fengycin – 1435 to 1580 Da), complex molecular structures and random aggregation characteristics of lipopeptide monomers.

It is indeed this self-aggregation ability of lipopeptides [18,19] which aids the formation of supramolecular structures such as micelles and vesicles that is exploited in the membrane purification of lipopeptides. The conformation of surfactin micelles in

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water has been investigated adequately by researchers [18–20]. Osman et al. [21], studied the structural transformation of surfactin micelles under various induced extrinsic environmental conditions. Particularly, addition of $Ca^{2+}$ caused the structural transformation of surfactin monomers, first to an alpha-helix conformation at 0.3 mM $Ca^{2+}$ and then to beta sheets at 0.5 mM $Ca^{2+}$. Similar observations of strong variation in the conformation of surfactin with $Ca^{2+}$ have been reported by Vass et al., Li et al. observed that the addition of $Ca^{2+}$ ions promoted the formation of larger surfactin micellar aggregates. Most recently, Jauregi et al. [22] assessed the size of surfactin-mycosubtilin mixed micelles using DLS techniques to make the most appropriate choice of the MWCO for membrane purification of lipopeptides.

Different types of membranes (PES, cellulose acetate, cellulose ester) having different MWCO ranges (10–300 kDa) have so far been investigated for the recovery and purification of lipopeptides using a single stage or a two stage UF processes [23–25]. As already mentioned, the requirement for further purification by HPLC or ion-exchange process depends solely on the desired application of the final product. It is clearly evident from various investigations on the UF purification of lipopeptides that the loss of biosurfactants as encountered with the use of higher MWCO membranes is so inevitable that it not only affects the process economics (poor recovery of lipopeptide) but also makes it unrealistic for scale-up.

In the current study, we propose a novel strategy to minimize the loss of lipopeptides during CFUF purification using higher MWCO membranes and at the same time we aim to achieve a nearly-complete removal of impurities by proper conditioning of lipopeptide solution. Accordingly, we set the following objectives to evaluate (i) the performance of three different MWCO membranes (10 kDa, 30 kDa and 50 kDa) for lipopeptide recovery and purification under batch mode of CFUF operation, (ii) the size distribution of micelles with the addition of $Ca^{2+}$ ions, and finally (iii) CFUF purification of $Ca^{2+}$ conditioned lipopeptide solution in a 50 kDa membrane operated under a continuous diafiltration mode of operation.

## 2. Materials and methods

### 2.1. Chemical standards

Surfactin and iturin were purchased from Sigma Aldrich, St. Louis, USA. All other chemicals and solvents were purchased from Merck, India. The solvents used were of HPLC grades

### 2.2. Preparation of crude lipopeptide solution

Lipopeptide per-se synthesized by *Bacillus* sp., and processed to a crude form as lyophilized powder as described elsewhere [17] was used in the present study. A stock lipopeptide solution of concentration 10 g $L^{-1}$was prepared in double distilled water, whose pH was adjusted to 8.5 using 2 N NaOH and was stored at 4 °C until use.

### 2.3. Ultrafiltration studies – Batch mode

A Millipore Labscale tangential flow filtration (TFF) system was used for UF studies. Biomax polyethersulfone (PES) membranes of different molecular weight cutoffs (MWCOs) 10 kDa, 30 kDa and 50 kDa, each of area 50 $cm^2$ were used to investigate the filtration efficiency of lipopeptide solutions at two transmembrane pressures (TMPs), 103.4 kPa and 172.4 kPa, respectively. For batch CFUF studies, the initial volume of lipopeptide solution (500 ml of 1 g $L^{-1}$ lipopeptide, pH 8) was reduced to a final volume of 50 ml (designated as $CFUF_1$ mode) and the filtration was resumed after the retentate was treated with 9 volumes of 60% methanol for 15 min (designated as $CFUF_2$ mode). Permeate flux was calculated based on the time taken for collecting every 50 ml of permeate as given below:

Flux = volume of filtrate/(time × membrane area) (L $m^{-2}$ $h^{-1}$)

The final permeate samples were stored at 4 °C for HPLC and MALDI-TOF analysis.

### 2.4. CFUF-diafiltration mode

#### 2.4.1. Conditioning of micelles

$Ca^{2+}$ was chosen for micelles conditioning studies, as it was reported that divalent cations such as $Ca^{2+}$ and $Mg^{2+}$ showed more affinity towards lipopeptides than monovalent cations ($Na^+$ and $K^+$) [26,27]. Further, the addition of divalent cations to surfactin solution increased the overall size of the micelles [28].

About 1 g $L^{-1}$ lipopeptide solution was used for conditioning experiments. The concentration of $Ca^{2+}$ ions added as $CaCl_2$. $2H_2O$ in the final lipopeptide solutions was in the range of 0.2–8 mM. All samples were vortexed for 30 min and further filtered using 0.22 μm millipore filter.

#### 2.4.2. Diafiltration

For UF studies under the dialysis mode of operation, $Ca^{2+}$-conditioned and -unconditioned lipopeptide solutions were tested for their retention by 50 kDa PES membrane. Lipopeptide solution was conditioned by adding an optimal concentration of $Ca^{2+}$ ions (which was found to be 2 mM based on the DLS studies as shown later), followed by mixing for 30 min in the UF cell. $Ca^{2+}$ solution (at pH 8.0) prepared separately was fed continuously to the UF system at a rate equal to the permeate removal rate. The CFUF was run in diafiltration mode and as many as 6 volumes (i.e. $6 \times 500 = 3$ L) of $Ca^{2+}$ containing dialyzate were passed. The permeate and retentate samples were then stored for further analysis of lipopeptide by HPLC and MALDI-TOF.

### 2.5. Analytical techniques

#### 2.5.1. UV–vis spectrometry

The $OD_{600}$ of the samples were measured using Chemito Double-Beam UV–vis spectrophotometer (model: SPECTRASCAN UV 2600)

#### 2.5.2. HPLC analysis

Lipopeptide concentrations of crude sample and retentates were analyzed using reverse phase high performance liquid chromatography (RP-HPLC) with a Zorbax Eclipse reverse phase column (C18, 5 μm, 4.6 (ID) 250 mm (L)) on an Agilent 1100 series HPLC instrument equipped with a diode array detector (DAD) system. The column was eluted with isocratic pumping of 60% acetonitrile containing 0.1% TFA at a flow rate of 1 ml $min^{-1}$. About 20 μL of methanol-extracted lyophilized powder samples were injected into the column.

#### 2.5.3. MALDI-TOF analysis

Samples were analyzed by a 4800 MALDI-ToF/ToFTM analyzer (Applied Biosystems Inc, MDS SCIEX, USA) equipped with a nitrogen UV laser (337 nm) and operated at 10 Hz for the desorption and ionization of molecules. DHB (2,5-dihydroxybenzoic acid) (Sigma, USA) was used as a matrix compound. Matrix stock solution of a concentration 10 mg $ml^{-1}$ was prepared in acetonitrile, methanol and water (9:9:2). Equal volumes of sample and matrix were mixed thoroughly for 5 min in a vortex mix and were spotted on a target plate. Plates were then dried and placed inside the sample cabinet of the instrument. A voltage of 20 kV was