Single-step Cross-flow Ultrafiltration for Recovery and Purification of Surfactin Produced by *Bacillus subtilis* ATCC 21332

Muhammad Qadri Effendy Mubarak, Mohd Hafez Mohd Isa, and Abdul Rahman Hassan

***Abstract*—**Surfactin is a powerful biosurfactant that has attractive behavior produced by fermentation of various strains of *Bacillus*. However, the downstream processing is a major obstacle due to the impurities present in the fermentation broth. Downstream technique was applied by a single-step cross-flow ultrafiltration (UF) technique using a benchtop cross-flow filtration unit equipped with a hydrosart membrane with a 10 kDa molecular weight cut-off (MWCO) (HT10) and a polyethersulfone membrane with 10kDa MWCO (PES10) membranes to recover and purify surfactin from the fermentation broth. Four different transmembrane pressures (TMP) varying from 0.5 bar to 2.0 bar were applied for each filtration process. Permeate flux, rejection coefficient (R) of surfactin and protein contents both in permeates and retentates were measured during the UF to evaluate the characteristic of both membranes towards the recovery and purity of the final product. Surfactin was retained almost completely with a rejection coefficient (R) close to 1 for both membranes, with permissible purity ranging from 82% to 88%. Four different TMPs applied on the membrane had no significant effect ($P<0.05$) on R because the pore size of the membranes were smaller than surfactin micelles. In this study, HT10 achieved better recovery and purity of the final product compared to PES10. Later, FTIR analysis and surface tension measurements were conducted to assess the purity and functionality of the recovered and purified surfactin from both UF membranes used in this study.

***Keywords*—**Hydrosart membrane, polyethersulfone membrane, transmembrane pressure, rejection coefficient.

I. Introduction

Bacillus subtilis is a sporulating rod bacterium that thrives in the soil and is nonpathogenic to human beings [1], enabling its application in various fields and making it one of the most studied Gram-positive bacteria [2]. The ability of *B. subtilis* strains to produce a series of lipopeptides (surfactin, iturin and fengycin) has been documented over 60 years [3] and has created great potential for its application in various fields as an alternative surfactant to replace chemical surfactants. Surfactin is a heptapeptide linked to a β-hydroxy fatty acid chain of 13–16 carbon chains and produces a series of isoforms. It is a high-value bioproduct that offers advantageous properties for mankind. However, surfactin is an expensive lipopeptide, which makes it unable to compete effectively with chemical surfactants because the downstream process contributes up to 60% of its production cost [4] due to the complexity of fermentation broth, which contains impurities such as proteins, sugar, lipid compounds and different types of amino acids [5].

In recent years, a lot of effort has been expended in cutting down the downstream processing costs, including using foam fractionation [6], acid precipitation [7], [8] extraction using organic solvent, adsorption chromatography or a combination of these techniques. Unfortunately, these techniques give low surfactin purity (<65%), which is insufficient and improvement in downstream processing techniques and performance is hugely important. In addition, some of the approaches involving two-step treatment of fermentation broth make it impractical and less attractive for industrial application [9], [10], [11]. In addition, most of the conventional methods dealing with toxic organic solvents such as chloroform and dichloromethane make the final product suffer from the loss of biosurfactant activity. Hence, there is a demand to develop more economic and environmentally friendly method to improve current downstream processing method.

Surfactin separation efficiency from fermentation broth is the essential issue in developing commercial-scale processes. One of the alternative techniques for downstream processing is membrane filtration. There is a lot of interest in applying a membrane system for the purpose of recovery and purification of biosurfactants [9], [10], [11]. Membrane filtration using pressure-driven force applied to a membrane to dissolve and suspend species based on the size and molecular scale [4] is widely used in various chemical and biochemical processes. More importantly, the membrane approach process involves no phase change [12], which enables the molecules structure to be preserved. In much of the literature, membrane filtration meets downstream separation needs because the concentration and purification of the final product surpasses the limitations of traditional methods [13], [14].

Membrane filtration has been considered in this work

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because it is environmentally friendly and economical for the purpose of the downstream processing [10]. The excellent characteristics of UF include the minimized physical damage of biomolecules from shear effects, minimal denaturation, high recovery yield, and the avoidance of resolubilization requirement. In this study, cross-flow UF equipped with a polyethersulfone (PES) and hydrosart (HT) membrane with a molecular weight cut-off (MWCO) of 10 kDa was used for the filtration of raw fermentation broth of *B. subtilis* ATCC 21332. The final surfactin and protein concentration both in permeates and retentates were analysed to evaluate the performance of UF. The aim of this work is to evaluate which type of membrane is better in terms of the recovery and purity of the final products of retentate operated under different transmembrane pressure (TMP).

II. Materials And Methods

A. Preparation of culture media

A defined mineral salts medium (MSM) described by Cooper [15] was used as fermentation media with 4% (w/v) of glucose. All chemicals used were of analytical grade. Prepared medium was sterilized prior to fermentation.

B. Culture conditions and fermentation

Bacillus subtilis ATCC 21332 provided by Microbiology Laboratory, Faculty of Science and Technology, Universiti Sains Islam Malaysia (USIM). Stock culture was maintained on the nutrient agar. Two loopful of grown bacterial cells from the nutrient agar were transferred to 25mL of nutrient broth containing 40 g/L of glucose, followed by the incubation of the culture broth at 200 rpm at 30^{o}C for 24 h. A volume of 5mL culture broth was then transferred to five conical flasks each containing 45 mL of Cooper's medium [8] incubated under the same conditions as previously described for 16 h [11]. An inoculum size of 5% (v/v) [10] were used to inoculate a 4750 mL Cooper's media with in submerged bioreactor (Sartorius Stedim, German) with a working volume of 5 L. The fermentation conditions were set at the temperature of 30°C, agitation speed of 100 rpm, air flow rate of 1 vvm^{-1}, and pH 7 for 55 h. Culture broth samples were taken during the fermentation process at regular intervals for determination of bacterial growth and surfactin concentration.

C. Analytical methods

1. Measurement of bacterial growth

Bacterial growth was measured by determining the biomass concentration (gram dry weight per liter of culture medium) at different time intervals up to 55 h. Fixed volumes of the fermentation broth were withdrawn aseptically and transferred to centrifuge tubes for centrifugation at 10 000 rpm for 10 min. The supernatant was withdrawn and biomass left at the bottom of the tubes was dried using an oven at 105^{o}C for 24 h. The dry weight was then measured using a balance.

2. Measurement of surfactin concentration

Culture samples were withdrawn aseptically at various time intervals and centrifuged at 10 000 rpm for 10 min. Then the supernatant was filtered through a 0.2 µm nylon filter membrane. The surfactin concentration was measured using high-performance liquid chromatography (HPLC; Agilent Technologies, 1200 Series, USA) equipped with Chromolith® high performance RP-18 (100 mm × 4.6 mm, 5 µm) and detected at 205 nm with a variable wavelength detector (VWD). Mixtures of mobile phase consisted of acetonitrile (ACN) and 3.8 mM trifluoroacetic acid (TFA) solution at the ratio of 80:20 were pumped with an isocratic mode at a flow rate of 2.2 mL/min. The sample injection was set at 30 µL and the duration of each analysis was within 8 min. Standard surfactin (Sigma) with 98% purity was used as the standard.

3. Measurements of surface tension

Clarified fermentation broth and UF retentate samples were filtered with 0.2 µm nylon filter. Each sample, including standard surfactin was prepared in 5 mM of Tris buffer using deionized water. The surface tension of each sample was measured by ring method using a digital tensiometer (KRÜSS, Germany). A platinum ring was automatically submerged into each solution and then slowly pulled through the air/water interface. The ring was washed, flamed and cooled between each measurement. Each measurement was taken at room temperature.

4. Measurement of surfactin purity

The surfactin purity in the dried sample was calculated using the following equation:

$$\text{Purity}(\%) = \frac{\text{amount of surfactin determined by HPLC}}{\text{Weight of dried sample powder}} \times 98\% \quad (1)$$

5. Measurement of protein concentration

The protein concentration in fermentation broth was calculated using the following equation:

$$\text{Protein concentration (mg/L)} = \text{Weight of dried sample} - \text{Weight of surfactin determined by HPLC} \quad (2)$$

D. Recovery and purification of surfactin by UF

Fig. 1 shows schematic diagram of UF process conducted in this study. Microfiltration were applied to remove all contaminant that might appear in broth thus to prevent them from blocking the membrane pores at the UF stage. Microfiltration was applied to remove biomass and small particle present in fermentation broth. Small-scale cross-flow UF procedures were carried out using a benchtop cross-flow filtration device (Sartorius Stedim, Germany) equipped with two sets of membrane which are polyethersulfone (PES) membrane and hydrosart (HT) membrane each with molecular weight cut-off (MWCO) of 10 kDa with an effective area of 0.02 m^2. The driving force for the permeate flow was the pressure supplied by the external pump towards the system. In general, a feed volume of 250 mL was added to the reservoir and the volume was reduced to 25 mL. Later, retentates and permeates were recovered and analyzed to determine surfactin concentration, protein concentration, the rejection coefficient of surfactin by membrane (R) and the recovery of surfactin in UF retentate. Recovery is defined as:

$$\text{Recovery}(\%) = \left(1 - \left(\frac{C_p}{C_f}\right)\right) \times 100 \quad (3)$$

Where C_p and C_f are the concentration of surfactin in permeates and feed respectively.

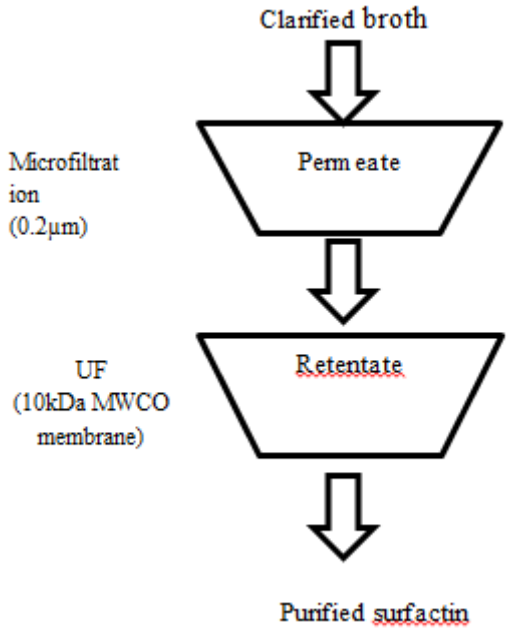

Fig. 1. Schematic representation of filtration process carried out in this study.

Throughout the UF procedure, the flow rate across the membrane was estimated by collecting permeates of interested volumes during a precisely controlled period of time. Permeate flux was calculated by using the following equation:

$$\text{Flux}\left(\text{LMH or}\frac{\text{L}}{\text{m}^2\text{h}}\right) = \frac{\text{flow rate (L/h)}}{\text{membrane area (m}^2\text{)}} \quad (4)$$

The four different TMPs for this procedure ranged from 0.5 bar to 2.0 bar was conducted manually by adjusting the valve and pump controller. TMPs of filtration process were calculated following Eq. 5. The highest TMP applied was up to 2.0 bar.

$$\text{TMP} = \left(\frac{P_{in} - P_{out}}{2}\right) - P_{permeate} \quad (5)$$

E. Statistical analysis

Data analysis consisted of calculating mean, standard deviation of the mean value and determination of the level of the significance using Student's t-test and Tukey-Kramer multiple comparison test. The differences between measurements were considered significant at the level of $P < 0.05$.

III. Results and Discussion

A. Production of surfactin by B. subtilis ATCC 21332

In various sources, *B. subtilis* ATCC 21332 is already known as a producer of surfactin [8] [10], Cooper's media with 4% (w/v) of glucose was chosen as the media because it has been designed to supply nutrients for bacterial cell growth and surfactin synthesis by *Bacillus* strains [8] [10] [16]. During the course of fermentation, the pH of the fermentation broth was maintained at pH 7 to avoid the acidification of the culture medium due to the change from aerobic to anaerobic respiration by the cell [16] where the cell grows in the absence of oxygen. Surfactin will lose its ability to solubilize if the pH drops to pH 5, causing surfactin to precipitate [17]. Fig. 2 shows bacterial cell growth and surfactin production during the fermentation of *B. subtilis* ATCC 21332. The beginning of cell growth of *B. subtilis* ATCC 21332 lasted for about 10 h, implying that the cells take time to adapt to Cooper's media. This lag phase shows almost no apparent cell growth due to the adaptation of microorganism to the new environment in which the rate of cell growth is very low. Later the cells grow exponentially between 10 h and 45 h in which the cell growth increased in a logarithmic pattern. The stationary growth phase for *B. subtilis* ATCC 21332 began after 45 h of incubation time. The final concentration of surfactin in the fermentation broth was 470 mg/l, which was higher than that obtained by Davis *et al.* [6] of 439 mg/l under the same experimental conditions. Cooper *et al.* [15] suggested that biosurfactant production by *B. subtilis* strain was closely related to microbial cell growth, while Shepard & Mulligan [18] stated that biosurfactant production mainly occurs at the end of the exponential phase or at the stationary phase of microbial growth. This study shows that the production of surfactin is very closely related to the growth of the strains where the maximum production is found at the end of the exponential growth phase.

B. Fermentation broth composition

Although the exact composition of the broth treated after centrifugation is unknown, it was thought that the fermentation broth consisted of surfactin, biomass cells, proteins, macromolecules, glucose and amino acids [19]. The final concentration of biomass, surfactin and protein in the fermentation broth of *B. subtilis* ATCC 21332 is illustrated in Table 1. The raw fermentation broth was then subjected to the single-step cross-flow UF process for recovery and purification of surfactin.

C. Total recovery and purity of surfactin

The UF technique applied was able to recover the surfactin in the retentate, thus achieving a good degree of purity. The niche of UF compared to other downstream processing techniques is its ability to segregate the solute components based on molecular weight without phase changes. The effect of increasing TMP on the flux of fermentation broth, the rejection coefficient (R) of surfactin by membrane, total recovery and purity of surfactin in the final fraction by HT10 and PES10 membranes is shown in Fig. 3 and Table 2.

Surfactin was completely rejected by both membranes, and achieving average total recovery and purity of 95.2% and 85.8%, respectively. Results obtained as shown in Table 2 suggest low-molecular-weight impurities including amino acids and other metabolites were able to pass through the pore of the membranes. The size and shape of molecules are the

most frequently considered parameters affecting the separation efficiency [20].

Even though the molecular size of surfactin is around 994 Da to 1050 Da, at concentrations above 15 mg/L [21] surfactin is able to form micelles with a size varying from 30 kDa to 100 kDa [9] [19]. At critical micelle concentrations (CMC), surfactin molecules readily associate to form supramolecular structures with nominal molecular diameters up to two to three orders of magnitude larger than single unassociated molecules [9]. In this work, surfactin micelles were sufficiently retained by using membrane with MWCO of less than 100 kDa and this is in agreement with findings previously discussed. The effectiveness of UF in this study is due to fact that the surfactin micelles are big enough to be rejected by 10 kDa membrane. Given these facts, it was agreed that the size of the surfactin micelles was above 30 kDa, as reported in previous studies [9], [21].

Insignificant differences ($P>0.05$) were observed when increasing TMP to the *R* of surfactin for both HT10 and PES10 as shown in Fig. 3. Very limited differences in the rejection coefficient on both membranes were observed due to the size of surfactin micelles was bigger than the MWCO of membranes, making surfactin unable to permeate and thus be retained completely. Fig. 4 shows that overall, both membranes were able to achieve high recovery and purity of surfactin from fermentation broth although, the membrane material had an effect on the recovery and purity of surfactin. Use of HT10 provided significantly higher ($P> 0.05$) recovery of surfactin from fermentation broth, in comparison to the use of PES10. The purity of final fraction of surfactin for HT10 is higher than that for PES10, however the difference is less than 5% because the MWCO of both membranes is small, which can effectively permeate protein contaminants, and achieving high level of purity of surfactin final fraction in membranes retentate.

TABLE I
MAJOR COMPOSITION OF FERMENTATION BROTH OF *B. SUBTILIS* ATCC 21332 (MEANS ± SD, N = 3)

Major composition of fermentation broth	*B. subtilis* ATCC 21332
Final concentration of biomass (g/l)	2.06 ± 0.02
Final concentration of surfactin (mg/l)	447.06 ± 1.25
Final concentration of protein (mg/l)	109.00 ± 2.11

TABLE II
TOTAL RECOVERY AND PURITY OF FINAL FRACTION OF SURFACTIN IN UF RETENTATE OF PES10 AND HT10.

Membrane	TMP	Total recovery (%)	Purity (%)
PES 10	0.5	94.5	83.0
	1.0	94.9	83.6
	1.5	94.2	85.9
	2.0	94.4	87.2
HT 10	0.5	96.0	83.9
	1.0	96.2	86.6
	1.5	95.1	87.0
	2.0	96.3	88.9

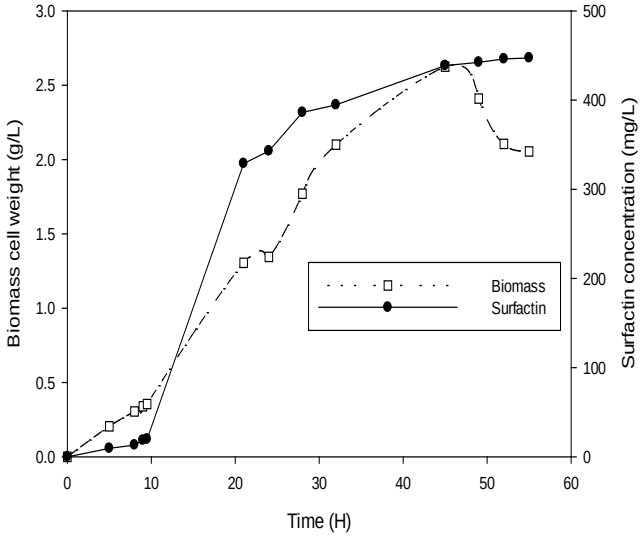

Fig. 2. Production of surfactin by *B. subtilis* ATCC 21332.

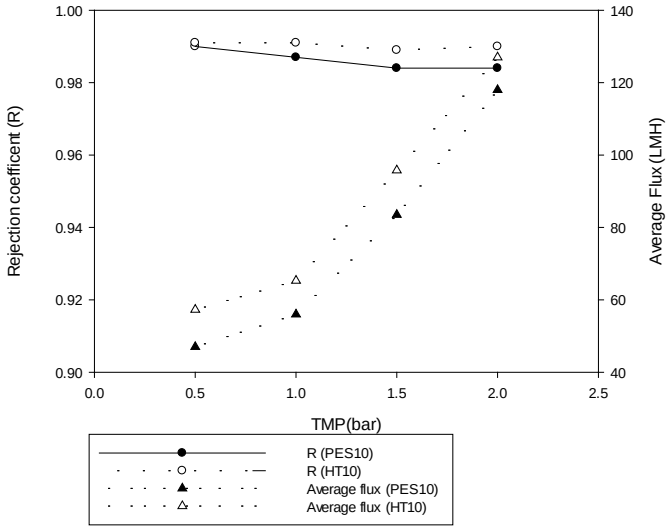

Fig. 3. Rejection coefficient (R) of surfactin and average flux of fermentation broth by using PES10 and HT10 membranes, respectively.

1. Surface tension measurements

Surface tension measurements were used to evaluate the functionality and purity of surfactin samples using standard surfactin (98% purity) as reference under similar and controlled conditions [11], [21] Fig. 4 shows the surface tension profiles of the purified surfactin using HT10 and PES10 in comparison to standard surfactin. The results show close proximity in terms of the surface activity of purified surfactin in relation to standard surfactin, which indirectly shows purity of surfactin samples and this corresponds to the earlier results on purity measurements as shown in Table 2. In addition, Fig. 4 shows the purified surfactin behaves as a very powerful biosurfactant and the presence of some contaminants did not affect the original surfactin functionality [18].

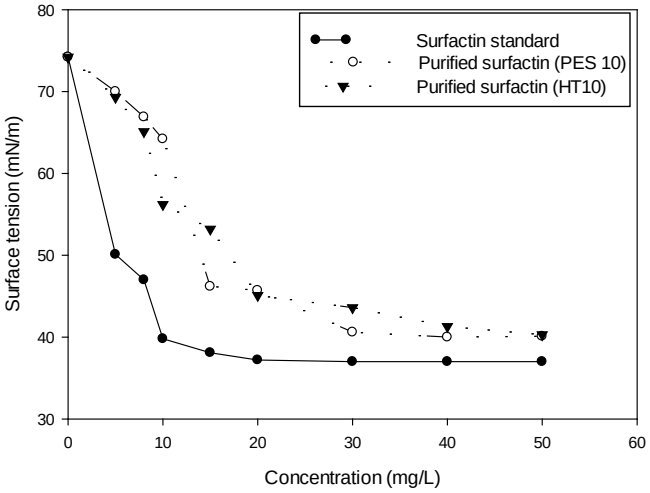

Fig. 4. Surface tension of surfactin standard, purified surfactin (HT10), and purified surfactin (PES10).

2. Structural analysis using FTIR

The molecular composition of surfactin was evaluated by FTIR. Fig. 5 (a), (b) and (c) presents the spectra of standard surfactin, surfactin purified using PES10 and surfactin purified using HT10, respectively.

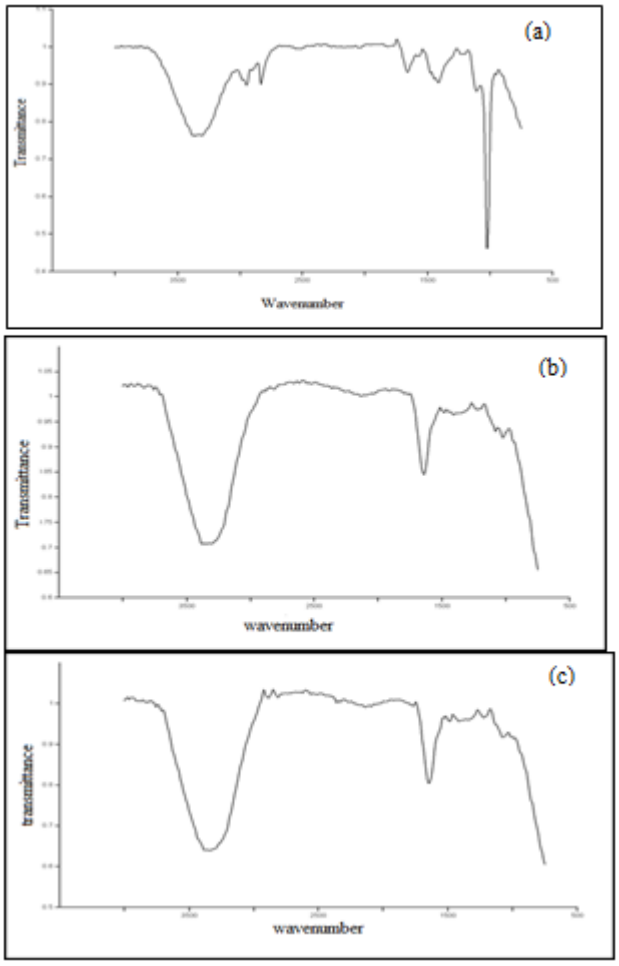

Fig. 5 FTIR spectrum of (a) standard surfactin; (b) surfactin purified using PES10; (c) surfactin purified using HT10.

The spectra showed that the same adsorption bands differed in relative areas under the various absorption bands. The most important characteristic absorption bands corresponding to functional groups typically forming part of surfactin could be observed for all samples and were located at: 1) bands characteristic of peptides at 3305 cm^{-1} (NH stretching mode); 2) the bands at 2956–2924cm^{-1}, 2869 cm^{-1} and at 1463 cm^{-1}, 1377cm^{-1} reflect aliphatic chains (–CH3, –CH2–) of the fraction; 3) the bands between 1020 and 1030 cm^{-1} reflect C–O–C vibrations (surfactin head rings); 4) the bands at 1655 cm^{-1} (C=O stretching in proteins). These results imply that the biosurfactant produced contains aliphatic hydrocarbons as well as a peptide like moiety. This FTIR spectrogram confirmed the lipopeptide nature of biosurfactants, which could correspond to surfactin structure.

IV. Conclusions

Recovery and purification of surfactin from fermentation broth of *B. subtilis* ATCC 21332 by a single-step cross flow UF technique using PES and HT membranes of 10 kDa MWCO was evaluated through investigation of permeate flux, rejection coefficient (R) and purity of surfactin at various TMP's. It is shown that surfactin was retained effectively by both HT10 and PES10 membrane with an R of almost 1, and this was due to the fact that surfactin micelles were unable to permeate both membranes at given MWCO. HT10 membrane provides slightly better recovery and purity of surfactin from fermentation broth of *B. subtilis* ATCC 21332, in comparison to PES10 membrane. In addition, product characterization analysis was conducted to evaluate the functionality and purity of surfactin in final fraction of UF retentate by surface tension and FTIR analysis with use of standard surfactin as reference under similar and controlled conditions. Recovered and purified surfactin showed close proximity of surface activity in relation to standard surfactin which indirectly shows the presence of impurities in the final fraction did not affect the original surfactin functionality. FTIR spectra confirmed that the UF retentate contains aliphatic hydrocarbons as well as a peptide-like moiety, which correspond to the structure of surfactin. Both membranes used in this single-step cross-flow UF technique lead to high recovery and purity of surfactin from fermentation broth of *B. subtilis* ATCC 21332 and results obtained in this study can further assist in improving the cost-effectiveness of downstream processing of surfactin which in turn can reduce the overall cost of surfactin production, recovery and purification.

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