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Separation of surfactin from fermentation broths by acid precipitation and two-stage dead-end ultrafiltration processes

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Abstract

Separation and recovery of surfactin from fermentation broths with the culture of *Bacillus subtilis* ATCC 21332 by two-stage ultrafiltration (UF) or nanofiltration (NF) processes was studied, in which the broths were pre-treated by acid precipitation and then the precipitate was dissolved in NaOH at pH 11. Experiments were performed at different initial concentrations of surfactin (210–3620 mg/L), concentrations of added micelle-destabilizing solvent ethanol (0–44%, v/v), membrane molecular-weight cut-off (MWCO, 1–300 kDa), and transmembrane pressures (86.184–517.106 $\times 10^3$ Pa (12.5–75 psi)). Under the concentration ranges tested, surfactin micelles could be efficiently destroyed when more than 33% (v/v) ethanol was added to the broth. The UF membranes with MWCO less than 100 kDa were found to be suitable for the retention of surfactin micelles, and the NF membrane with a MWCO less than 1 kDa was suitable for the retention of surfactin monomers. Finally, the separation strategy involving two-stage membrane filtration (UF or NF) processes was proposed. Depending on the forms of the final product, it was shown that more than 72% of recovery yield and more than 83% of purity could be achieved.

Keywords: Surfactin; Separation; Fermentation broths; Acid precipitation; Two-stage UF processes

1. Introduction

Biological molecules exhibiting particularly high surface activity are classified as biosurfactants. They generally include a wide variety of chemical structures such as glycolipids, lipopetides, polysaccharide–protein complexes, phospholipids, fatty acids, and neutral lipids [1,2]. In terms of physicochemical properties such as surface activity as well as pH and heat stability, many biosurfactants are comparable to synthetic chemical surfactants [3]. Biosurfactants have some advantages, such as low critical micelle concentration (CMC) and high biodegradability, over synthetic surfactants and, therefore, are particularly well suited for environmental applications such as bioremediation and the dispersion of oil spills [4,5]. Biosurfactants are also thought to potential candidates to replace chemical surfactants in the future, especially in the food, cosmetic, and health care industries, industrial cleaning of products, and in agricultural chemicals [6].

Surfactin, a cyclic lipopeptide produced by several strains of *Bacillus subtilis* [7], is a very powerful one of biosurfactant [8]. Its biological activities are related to its ability to interact with phospholipida and cations [9]. Surfactin is a heptapeptide (L-Glu-L-Leu-D-Leu-L-Val-L-Asp-D-Leu-L-Leu) linked to a β-hydroxy fatty acid comprising mainly 14 or 15 carbon atoms; also, it has exceptional surface-active power because it lowers the surface tension of water from 72 to 27 mN m^{-1} at a concentration even as low 20 μM [8].

Nowadays, more and more high-value bioproducts are produced by fermentation bringing new challenges to industrial recovery and purification steps, which count not only for labile nature of most of these molecules but also for the economy of the process. Downstream processing in many biotechnological processes is usually responsible for up to 60% of the total production cost [10]. Due to economic considerations, most biosurfactants would have to involve whole-cell spent culture broths or other crude preparations. The most commonly employed surfactin recovery techniques from fermentation broth include acid precipitation, foam separation, or the combined both [1]; however, they often give low surfactin purity (<60%). Some methods including extractions with organic solvents, adsorption

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chromatography, and thin-layer chromatography have been reported [11]. In general, the extraction suffers from the use of toxic solvents (e.g., dichloromethane, chloroform, etc.) or the loss of biosurfactant activity. Moreover, the expensive nature of the latter two methods makes them practically less attractive. Hence, it is desired to develop a more economic and environmentally friendly method to improve recovery yield and purity.

It has been accepted that membrane meet downstream separation needs in pharmaceutical and biological process such as concentration and purification, surpassing the limitations of traditional techniques [12,13]. Ultrafiltration (UF) and nanofiltration (NF) are pressure-driven membrane separation techniques for dissolved and suspended species based on size and molecular scale [12]. The characteristics of UF and NF that make them excellent for many applications include the minimized physical damage of biomolecules from shear effects; minimal denaturation; high recovery yield; the avoidance of re-solubilization problems because solutes are retained in the solution phase; high throughput; and cost effectiveness [13]. At concentrations above the critical micelle concentration, surfactant molecules will 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 single unassociated molecules. Thus, surfactin micelles can easily be retained by UF membranes with sufficiently low MWCOs. In fact, one-stage UF process has been applied for the recovery of surfactin produced by *Bacillus subtilis* from complex fermentation medium [14–16].

In a word, the aim of this work was to separate and recover surfactin from acid-precipitated fermentation broths by a two-stage dead-end membrane filtration process. Higher recovery yield and purity of surfactin were simultaneously targeted. Factors affecting filtration performance such as the initial concentration of surfactin (210–3620 mg/L), the concentration of added (0–46%, w/v) micelle-destabilizing solvent ethanol (0–44%, v/v), membrane MWCO (1–300 kDa), and membrane pressure (86.184–517.106 $\times 10^3$ Pa (12.5–75 psi)) were investigated. Three different separation strategies were also proposed and screened.

2. Materials and methods

2.1. Microorganisms and culture condition

In this work, *Bacillus subtilis* ATCC 21332 was selected to produce surfactin. The nutrient broth (NB) medium consisted of 3 g/L beef extract, 5 g/L peptone, and the mineral salt (MS) medium at pH 7. The MS medium contained 40 g/L glucose, 50 mM NH_4NO_3, 30 mM KH_2PO_4, 40 mM Na_2HPO_4, 7 μM $CaCl_2$, 0.8 mM $MgSO_4$, 4 μM $FeSO_4$, and 4 μM tetrasodium salt of EDTA [17]. The pH was regulated at 7 by adding 0.1 M HCl or NaOH. Prior to use, the MS medium and deionized water (Millipore, Milli-Q) were sterilized in autoclave at 121 °C for 15 min. All inorganic chemicals were offered from Merck Co. as analytical reagent grade.

Table 1
Components classified in the raw fermentation broth [14][a]

Macromolecules	Mid-molecules	Small molecules
Surfactin micelle (30,000–100,000)	Surfactin monomer (1036)	MS medium (80–400)
Polysaccharides		Alcohols (46)
Peptides		Phthalic acid (150)
Proteins		Amino acid (200)
		Glycine (75)
		Serine (105)
		Threonine (119)
		Phosphate (100)
		Alanine (89)

[a] The numeral in the parentheses indicates the molecular weight in g/mol.

Culture of *Bacillus subtilis* ATCC 21332 was taken from −80 °C frozen stock and transferred onto agar medium for preculture. The *Bacillus subtilis* culture (1 mL) was inoculum into 250-mL flask containing 100 mL of NB medium at 30 °C with 3.33 Hz (200 rpm) of agitation. After growing up to late exponential phase (near 14 h), the NB medium containing the *Bacillus subtilis* cells was inoculum and fermented in 5-L fermenter with 4-L working volume at 30 °C and 3.33 Hz (200 rpm) for another 4 days.

As liquor inside the fermenter was centrifuged at 10,000 × *g* to remove biomass impurities, the supernatant was called the raw broth. It was reported that the raw broth with culture of *Bacillus subtilis* ATCC 21332 contains macromolecules, mid-molecules, and small molecules, as listed in Table 1 [14]. On the other hand, the raw broth was further treated by acid precipitation; that is, the addition of 1 M HCl to a pH of around 4. The yellowish precipitate (crude powder) was obtained by centrifuge at 10,000 × *g* for 15 min and oven-drying at 37 °C for 2 days. The powder was then dissolved in NaOH at pH 11, and the resulting solution was called the treated broth. It was found that the crude powder had a surfactin purity of about 55% according to the method described below.

2.2. Assay surfactin concentration

Culture samples were taken after centrifuge at 12,000 × *g* for 15 min to remove the biomass, and surfactin concentration in the clarified supernatant was measured by reverse phase C18 HPLC equipped with a Merck C18 column (5 μm) at 30 °C [18]. The samples were subject to filtration through a Millipore filter (0.45 μm) before analysis. A mixture of 3.8 mM trifluoroacetic acid (20%, v/v) and acetonitrile was used as the mobile phase, and the flow rate was 1.0 mL/min. An aliquot of the sample (20 μL) was injected and analyzed using an UV detector (Jasco 975, Japan). The wavelength was set at 205 nm [15]. Each concentration analysis was at least duplicated under identical conditions. The reproducibility is mostly within 5%.

Surfactin powder purchased from Sigma Co. was served as the standard, in which a purity of 98% was claimed by the manufacturer. The purity of surfactin in the dried sample was