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Recovery and separation of surfactin from pretreated fermentation broths by physical and chemical extraction

Huei-Li Chen, Ruey-Shin Juang *

Department of Chemical Engineering and Materials Science, Yuan Ze University, Chung-Li 32003, Taiwan

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Abstract

The recovery of surfactin from fermentation broths with the culture of *Bacillus subtilis* ATCC 21332 by physical and chemical extraction was studied, in which the broths were pretreated by acid precipitation and, if necessary, the precipitate was further dissolved in NaOH solution. The physical solid–liquid and liquid–liquid extractions were performed with different organic solvents (ethyl acetate, *n*-hexane) and at different times of extraction run. It was shown that better extraction was obtained with ethyl acetate than *n*-hexane. The extraction could be improved by increasing the times of extraction run under a given volume of the organic solution. For chemical liquid–liquid extraction of surfactin with Aliquat 336 (5–200 mM) in *n*-hexane, the efficiency was improved. The amounts of inorganic salt in the strip solution were also optimized. Surfactin recovered after physical or chemical extraction was finally characterized by mass and NMR spectroscopies. The results of chemical extraction showed that surfactin would readily bind with the quaternary ammonium cations of Aliquat 336.

Keywords: Surfactin; Recovery; Fermentation broths; Extraction; Ethyl acetate; *n*-Hexane; Aliquat 336

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, lipopeptides, polysaccharide–protein complexes, phospholipids, fatty acids, and neutral lipids [1,2]. They also have a strong effect on interfacial rheological phenomena and mass transfer [3]. Biosurfactants have several advantages, such as low critical micelle concentration and high biodegradability, over chemical synthetic surfactants and, thus, are especially well suited for environmental applications such as bioremediation and the dispersion of oil spills [4,5]. In addition, biosurfactants are thought to be potential candidates to replace chemically synthetic surfactants in the future, particularly in food, cosmetic, and health care industries, as well as in industrial cleaning of products and in agricultural chemicals [6,7].

Surfactin, a cyclic lipopeptide produced by several strains of *Bacillus subtilis*, is one of the very powerful biosurfactant [7,8]. It has a cyclic lipopeptide with β-hydroxy fatty acids linked to a heptapeptide (L-Glu-L-Leu-D-Leu-L-Val-L-Asp-D-Leu-L-Leu) [3,9]. Generally speaking, surfactin has an exceptional surface-active power because it lowers the surface tension of water from 72 to 27 mN/m at a concentration even as low as 20 μM [8].

It is recognized that downstream processing in many biotechnological processes is responsible for up to 60% of the total production cost [10]. From the economic point of view, most biosurfactants would have to involve whole-cell spent culture broths or other crude preparations [1]. The most commonly used and cheap methods for the recovery of biosurfactants from fermentation broths are acid precipitation, foam separation, or the combination of both [1]. However, they usually lead to relatively low biosurfactant purity (<50%). An alternative method for biosurfactant recovery is solvent extraction [10]. In practice, a wide variety of organic solvents including methanol, ethanol, butanol, diethyl ether, *n*-pentane, acetone, acetic acid, chloroform, and dichloromethane have been used, either single or in combination, for this purpose [1]. The most effective solvent would be the mixture of chloroform and methanol in various ratios, which facilitates adjustment of the polarity of extraction solvents to the target extractable material [11]. However, chloroform is highly toxic compound regarded as harmful to the environment and for human health [2]. Thus, cheap and low-toxicity solvents are highly desired for biosurfactant extraction from the view-

* Corresponding author. Tel.: +886 3 4638800; fax: +886 3 4559373.
E-mail address: rsjuang@saturn.yzu.edu.tw (R.-S. Juang).

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Nomenclature

A	volume of aqueous solution (mL)
Aliquat 336	trioctylmethylammonium chloride
LLE	liquid–liquid extraction
O	volume of organic phase (mL)
SLE	solid–liquid extraction

point of industrial applications. In recent years, ethyl acetate and *n*-hexane, which are the representatives of polar and nonpolar solvents, have been used to recover biosurfactants produced by *B. subtilis* [12–14]. Such types of low cost, less toxic, and easily available solvents could be employed to cut the recovery cost and minimize the environmental hazards.

In this work, the fermentation broth was pre-precipitated and the precipitate was obtained by centrifuging and oven-drying. In most cases, the precipitate was further dissolved in alkaline solution. Surfactin was then recovered by solid–liquid extraction (SLE) and liquid–liquid extraction (LLE) by the solvents *n*-hexane and ethyl acetate (physical extraction). Because physical extraction is sometimes not selective enough and has low recovery yield [15], LLE using a commercial basic extractant (chemical extraction) was also carried out for comparison. Higher recovery and purity were simultaneously targeted because the possibility of using such solvent extraction for practical applications was examined. Factors affecting extraction performance including types of organic solvent (ethyl acetate, *n*-hexane), times of extraction run, extractant concentration (0.5–200 mM) were investigated. The effect of added concentration of sodium chloride or ammonium sulfate (0.5–15 g/50 mL) on the stripping in chemical extraction system was also studied. Finally, the characteristics of the final product obtained after physical and chemical extraction were identified and compared.

2. Materials and methods

2.1. Microorganisms and culture condition

Here, *B. subtilis* ATCC 21332 was selected to produce surfactin. The nutrient broth (NB) medium contained 3 g/L beef extract, 5 g/L peptone, and the mineral salt (MS) medium at pH 7. The MS medium consisted of 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 [16]. 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.

Culture of *B. subtilis* ATCC 21332 was taken from −80 °C frozen stock and transferred onto agar medium for pre-culture. The *Bacillus subtilis* culture (1 mL) was inoculated into 250-mL flask containing 100 mL of NB medium at 30 °C with 200 rpm of agitation. After growing up to late exponential phase (near 14 h), the NB medium containing *B. subtilis* cells was inoculated and fermented in 5-L fermenter (Firstek Scientific Co., Taiwan) with 4-L working volume at 30 °C and 200 rpm for another 4 days. The aeration was controlled at a rate of 1.0 vvm. The dissolved oxygen concentration maintained 80% during the fermentation (2–4 days). The medium pH was maintained at 7.0 by automatic addition of 1 N NaOH or HCl solution to the fermenter according to the signal received from the pH electrode.

The fermentation broth was pretreated as follows. First, the liquor inside the fermenter was centrifuged at 10,000 × *g* to remove biomass impurities; the remaining supernatant was further treated and precipitated by adding 1 M HCl to a pH of around 4. The yellowish precipitate (crude powder) was obtained by centrifuging at 10,000 × *g* for 15 min and oven-drying at 37 °C for 2 days. In most situations, the dried precipitate was further dissolved in NaOH solution at pH 11 in order to yield a homogeneous solution. It was found that the crude precipitate had a surfactin purity of about 53% according to the method described below.

2.2. Assay surfactin concentration

Culture samples were taken after centrifuging at 12,000 × *g* for 15 min to remove the biomass, and surfactin concentration in the clarified supernatant was measured with HPLC equipped a reverse phase C18 column (5 μm, Merck) at 30 °C [17]. The samples were subjected 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 [18]. Each concentration analysis was at least duplicated under identical conditions. The reproducibility is mostly within 5%.

Surfactin powder purchased from Sigma Co. served as the standard (98% purity as per label claim). The purity of surfactin in the dried sample was calculated by

$$\text{purity}(\%) = \left(\frac{\text{amount of surfactin determined by HPLC}}{\text{weight of dried sample powder dissolved in the solution}}\right) \times 98 \quad (1)$$

The purities of surfactin in the recovered product and in the fermentation broth would be used to calculate the recovery yield of surfactin.

2.3. Physical extraction

In SLE, 0.1 g of the dried precipitate was contacted with 25 mL of the organic solvent, *n*-hexane or ethyl acetate (Mallinckrodt Co.). The solution mixture was agitated using a magnetic stirrer at 250 rpm for 24 h at 30 °C. Preliminary experiments had shown that the extraction studied was complete within 18 h. Then, it was centrifuged at 6000 × *g* for 10 min and the solvent was collected. For a given volume of the organic solution, the extraction was performed several times by fresh organic solution when organic solution was divided into the corresponding parts. After evaporation of the organic solution, surfactin solid