Biochemical Engineering Journal 61 (2012) 78–83

Contents lists available at SciVerse ScienceDirect

Biochemical Engineering Journal

journal homepage: www.elsevier.com/locate/bej

Recovery and separation of surfactin from pretreated *Bacillus subtilis* broth by reverse micellar extraction

Ruey-Shin Juang*, Huei-Li Chen, Shu-Chi Tsao

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

ARTICLE INFO

Article history:
Received 13 August 2011
Received in revised form
11 November 2011
Accepted 29 December 2011
Available online 4 January 2012

Keywords:
Separation
Surfactin
Fermentation
Liquid–liquid extraction
Reverse micelles
Cationic surfactants

ABSTRACT

The recovery and purification of surfactin from the fermentation broth of *Bacillus subtilis* ATCC (American Type Culture Collection) 21332 by extraction and stripping (back extraction) using reverse micelles was studied. Prior to extraction, the broth was precipitated by acid (HCl) at pH 4 and the precipitate was then dissolved in alkaline (NaOH) solution at pH 11. The effects of the type and concentration of surfactant, phase volume ratio, initial aqueous pH, and the presence of co-solvents on the extraction as well as the effects of the type and concentration of added inorganic salt and ethanol on the stripping were studied. It was shown that, in the absence of co-solvents, the extraction efficiency of 3.0 g/L surfactin from the pretreated broth by reverse micelles of 40 mM tri-*n*-octylamine (TOA) and 5 mM Aliquat 336 in *n*-hexane was more than 92%. The concentrations of the added inorganic salt (ammonium sulfate and sodium chloride) in the strip solution were also optimized. The purity of recovered surfactin from the TOA reverse micelles was higher than 90% when the strip solution contained 0.43 M of sodium chloride in water.

1. Introduction

Surfactin, a cyclic lipopeptide produced by several strains of *Bacillus subtilis*, is known as a very powerful biosurfactant [1]. It essentially 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. Surfactin has exceptional surface-active power because it can lower the surface tension of water from 72 mN/m to 27 mN/m at a concentration even as low as 20 μM. Hence, the application potentials of surfactin in cosmetic, pharmaceutical, food, and petroleum refinery industries as well as in environmental engineering have been presented [1–3].

We have reported previously [2] that surfactin produced by *B. subtilis* ATCC (American Type Culture Collection) 21332 was recovered at a 97% yield, after an acid precipitation of the culture medium at pH 4. However, surfactin purity was as low as 55%. When the precipitate was dissolved in NaOH at pH 11 and the resulting solution containing 0.3–1.0 g/L of surfactin was treated by traditional extraction with the solvents *n*-hexane and ethyl acetate (i.e., physical extraction), 21% and 95% of surfactin could be recovered, respectively. However, the purity of surfactin extracted from both solvents was merely in the range of 58–60%. On the other hand, Sen and Swaminathan [3] have studied the recovery and purification of surfactin from the cell-free fermentation broth using a batch stirred ultrafiltration (UF) process. The purity of surfactin obtained through solvent extraction and UF was found to be 70%, as calculated from the critical micelle concentration of 0.017 g/L against that of 0.013 g/L for surfactin (Sigma, 98% pure) used as the reference standard. Moreover, Chen et al. [4] have applied a two-stage batch UF process for the separation and recovery of surfactin from pretreated fermentation broth, and indicated that the recovery of surfactin and its purity is 87% and 83–85% under optimal conditions.

In recent years, many techniques have been developed in biotechnology to achieve a highly efficient and economical separation process. One novel separation technique with the ability to scale up easily, to be operated continuously, and to be highly selective is the liquid–liquid extraction using microemulsions. Reverse micelles are spontaneous aggregates of amphiphilic molecules in non-polar media, and capable to solubilize water and hydrophilic proteins. The extraction with reverse micelles is an attractive and effective method for downstream processing of biological products, as many biochemicals including amino acids, proteins, enzymes, nucleic acids, antibiotics, and steroids can be solubilized within such solutions and be recovered without loss of their activity.

Several studies have shown that proteins can be solubilized within the reverse micelles in active form [5–7]. This method is more suitable for separating proteins than traditional liquid–liquid extraction or other methods that were applied in

* Corresponding author at: Department of Chemical Engineering and Materials Science, Yuan Ze University, 135 Yuan-Tung Road, Chung-Li 32003, Taiwan.
Tel.: +886 3 4638800x2555; fax: +886 3 4559373.
E-mail address: rsjuang@saturn.yzu.edu.tw (R.-S. Juang).

1369-703X/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
doi:10.1016/j.bej.2011.12.008

Nomenclature

A	volume of the aqueous phase (mL)
Aliquat 336	tri-*n*-octylmethylammonium chloride
O	volume of the organic reverse micellar phase (mL)
TOA	tri-*n*-octylamine
W_0	water content (mol/mol)

the past because the transfer of proteins into organic solvents often results in irreversible denaturation and loss of biological activity although they are useful in large-scale and continuous processing [8]. The whole extraction process by reverse micelles contains two basic steps, extraction (forward) and stripping (backward extraction). In the extraction, a protein is transferred from an aqueous phase into a reverse micellar organic phase and, in the stripping the protein is released from the reverse micelles and transferred into an aqueous phase to be recovered. The partition of proteins between a bulk aqueous phase and reverse micellar phase depends on several factors [5]. Of these factors, pH and ionic strength of the aqueous phase are the dominant parameters in reverse micellar extraction process. By controlling these variables, the activity of the extracted protein can be maintained or enhanced.

The aim of this work was to examine the possibility of using reverse micellar extraction for the recovery and purification of surfactin from *B. subtilis* fermentation broth that had been treated after centrifugation, acid precipitation, and then alkaline dissolution. Such a pretreatment process is needed to remove some suspended and dissolved impurities; otherwise, the unwanted emulsions may be readily formed through agitation during liquid–liquid extraction operation [2]. Reverse micellar extraction method is expected to be economical and effective because surfactin is a biosurfactant. Two basic extractants Aliquat 336 (tri-*n*-octylmethylammonium chloride) and tri-*n*-octylamine (TOA), which have been commercially applied for metallic anions and organic acids in traditional liquid–liquid extraction systems, were used as the surfactants. High recovery and high purity were simultaneously targeted here because of the consideration for future practical applications.

2. Materials and methods

2.1. Microorganisms and culture conditions

B. subtilis ATCC 21332 was used to produce surfactin. The compositions of nutrient broth (NB) medium and mineral salt (MS) medium as well as the culture conditions have been reported previously [2,4,9]. After *B. subtilis* grew up to the late exponential phase (near 14 h), the MS medium containing *B. subtilis* was inoculated and fermented in a 5 L fermenter (working volume, 4 L) at 25 °C and 200 rpm for another four days.

The fermentation broth was centrifuged at 10,000 × *g* for 15 min to remove biomass and other impurities. The resulting supernatant was adjusted to be a pH around 4 by adding 1 M HCl; it was then precipitated. The yellowish precipitate (crude powder) was obtained by centrifuging at 10,000 × *g* for 15 min and oven-drying at 37 °C for two days. The crude precipitate was found to have a purity of surfactin of about 53% according to the method described below. The dried crude precipitate was further dissolved in a solution at pH 11, which was adjusted by adding 0.1 M NaOH, in order to yield a homogeneous solution. In most cases, an initial surfactin concentration of around 3 g/L was prepared because this was the concentration usually obtained from the raw broths.

2.2. Assay surfactin concentration

Culture samples were taken after centrifuging at 10,000 × *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 [10]. 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, whose 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 [11]. Each concentration analysis was at least duplicated under identical conditions. The reproducibility of the measurements was mostly within 5%.

The 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 thus calculated by

$$\text{purity}\ (\%) = \left(\frac{\text{UV signal of a given sample determined by HPLC}}{\text{UV signal of the standard at the same weight determined by HPLC}}\right) \times 98 \quad (1)$$

The purity of surfactin in the recovered product and in the fermentation broth was used to calculate the recovery of surfactin.

2.3. Water content and surface tension measurements

The water content in the organic reverse micellar phase (W_0) was determined by Karl-Fischer titration using a volumetric titrator (Mettler Toledo DL-38), defined as the molar concentration ratio of water to surfactant in the reverse micellar phase. The Karl-Fischer reagents used were CombiTitrant 5 (one-component reagent) and Combisolvent (methanol-free solvent with one-component reagent), which were purchased from Merck Co. A 1.0-mL solution was injected using a syringe and the coefficient of variation was within ±5%.

The surface tension was measured at 25 °C by a FACE surface tensiometer (Kyowa CBVP-A3, Japan), which is constructed along the lines of the Whilhelmy method [12].

2.4. Forward extraction and stripping (backward extraction)

The experimental procedures of reverse micellar extraction were essentially the same as traditional liquid–liquid extraction [2], except that Aliquat 336 (Aldrich Co.) and TOA (Merck Co.) were present in the organic reverse micellar phase instead of the bulk organic phase (*n*-hexane). Factors affecting extraction performance including the concentration of surfactant (TOA, 5–100 mM; Aliquat 336, 0.5–200 mM), phase volume ratio, and initial aqueous pH were investigated at 25 °C. After phase separation, the upper organic phase was collected and used for the followed stripping experiments. The concentration of surfactin in the aqueous phase was determined with HPLC, and the efficiency of surfactin extraction was calculated by

$$\text{extraction}\ (\%) = \left(\frac{\text{amount of surfactin in the organic reverse micellar phase}}{\text{initial amount of surfactin in the feed sample}}\right) \times 100 \quad (2)$$