Isolation and characterization of a surfactant produced by *Bacillus licheniformis* 86

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**SUMMARY**

Surfactant (BL86) was isolated from foam produced during growth of *Bacillus licheniformis* 86 by acid-precipitation followed by extraction into tetrahydrofuran or methanol. The surfactant is anionic and dissolves in tetrahydrofuran, methanol, chloroform, dichloromethane, xylene, toluene, and alkaline water. The surfactant lowers the surface tension of water to 27 dynes/cm, and achieves the critical micelle concentration with as little as 10 μg surfactant/ml. Its interfacial tension can reach 0.36 dynes/cm when measured in 4% sodium chloride against n-hexadecane. The surfactant is stable from pH 4.0 to 13.0, at temperatures ranging from 25 to 120 °C, and in salt solutions ranging from 0 to 30% NaCl. Preliminary analytical results indicate that the surfactant is a mixture of lipopeptides different from previously reported *Bacillus* produced surfactants.

**INTRODUCTION**

Surfactants are molecules that tend to concentrate at the phase-boundary and alter the interfacial properties. A surfactant is an amphipathic molecule having two functional parts: a polar, hydrophilic head group, and a non-polar, lipophilic tail. The character of the surfactant is determined by the balance between its hydrophilic and lipophilic components. In solution, surfactant molecules tend to aggregate either with each other (micelle formation) or between phases of different polarity, such as oil/water. Surfactants are characterized by their surface tension (ST) reducing ability, critical micelle concentration (CMC), Gibbs surface excess, interfacial tension (IFT), and hydrophilic-lipophilic balance (HLB) [5,9,10,17,23,24].

There are two broad classes of surfactants: chemically synthesized surfactants and biologically produced surfactants, biosurfactants. Chemically synthesized surfactants are usually classified according to the nature of the polar group: cationic, anionic, and nonionic types. Although there are ionic and nonionic biosurfactants, usually they are categorized by their chemical composition and/or the producing organism. Five categories of biosurfactants have been reported: (1) glycolipids, (2) lipopolysaccharides and polysaccharide-lipid complexes, (3) lipopeptides, (4) phospholipids, and (5) fatty acids and neutral lipids [5,10,17,20,23,24]. Theory suggests that the natural role of microbial surfactants may involve adhesion to substrate and nutrients emulsification, desorption from surfaces, antibacterial and antifungal activities, and receptors for bacteriophage [20].

Several surfactants produced by different species and strains of *Bacillus* have been reported [5,10,20,22,23,24]. Many of these are lipopeptides [22]. The best characterized lipopeptide surfactant is surfactin (also named subtilysin and serolysin) and is produced by some strains of *Bacillus subtilis* [1,3,6]. Surfactin was patented in 1972 [2]. Suggested uses for surfactin include inhibition of stationary growth in bouillon of microorganisms belonging to the genus *Mycobacterium*, inhibition of fibrinogen-thrombin reaction (i.e., inhibition of fibrinogen clot formation), increasing the antifungal activity of antifungal agents, treating or preventing hypercholesterolemia, and inhibiting loss of activity of various active substances. Surfactin has also been used in promotion of plasmin-catalyzed and trypsin-catalyzed fibrinolysis [15], as a cytolytic agent in haemolysis (i.e., lysis of erythrocytes) [3], and as an antibiotic in lysis of protoplasts and spheroplasts derived from several bacterial species [3]. A surfactin like lipopeptide surfactant, lichenysin, is produced by *B. licheniformis* JF-2 [13,14] and was patented in 1985 [16] as an enhanced oil recovery agent.

Other peptidelipids are produced by strains of *B. subtilis*. For instance, NLF-I, which has a promoting effect on the lysis of Gram-negative bacteria (e.g.,
**Pseudomonas aeruginosa** [21] and Mycosubtilin [18,19] and Bacillomyces C [4], which are used as antifungal agents (antibiotics). *Bacillus circulans* produced the peptidolipid, NLF-II and the peptidolipid has been shown to promote lysis of Gram-negative bacteria [21].

We are currently investigating a novel lipopeptide surfactant produced by *B. licheniformis* 86. The surfactant has been designated surfactant BL86. This paper describes the initial isolation and characterization of the surfactant.

**MATERIALS AND METHODS**

**Microorganisms.** *Bacillus licheniformis* 86 was isolated by and obtained from J.E. Zajic (Petroleum Bioresources, Inc., El Paso, TX as strain PBR 1177). *Bacillus subtilis* 21332 and *B. licheniformis* 39307 were obtained from the American Type Culture Collection. The microorganisms were stored lyophilized and working stock cultures were maintained on Nutrient Agar (Difco) slants at 4 °C.

**Culture conditions.** The organisms were grown aerobically on Cooper's medium [6]. Small scale fermentations were performed using a 2-l fermentation vessel (MultiGen, New Brunswick Scientific) with a 1-l working volume. The cultures were grown at 30 °C, for 20 h, agitated at 275 rpm and air sparged (1.0 vvm). Large scale fermentations (16 l) were performed for production of *B. licheniformis* 86 surfactant and *B. subtilis* 21332 surfactin using a 20-l fermentor (L.H. Fermentation Series 21332 and *B. licheniformis* 86 were performed using a 2-1 fermentation vessel (MultiGen, New Brunswick Scientific) with a 1-1 working volume. The cultures were grown at 30 °C, for 20 h, agitated at 500 rpm and air sparged (2.0 vvm). The foam produced by the growing cultures, which contained the surfactants, was continuously collected.

**Surfactant isolation.** Bacterial cells were removed from the surfactant-containing foam by centrifugation (13000 × g, 10 °C, 15 min) for the 1.6-l fermentations and by continuous centrifugation (Sorvall KSB-R continuous flow system, 17210 × g, 5 °C, until the supernatant was clear) for the 16-l fermentations. The supernatant was then subjected to acid precipitation by adding concentrated HCl to a final pH of 2.0 and allowing the precipitate to settle at 4 °C. The acid precipitate was recovered by centrifugation (11000 × g, 4 °C, 20 min). The pellet was washed 4 times with acid-water (pH 2.0, by HCl) and lyophilized overnight. The surfactant was extracted from the powder into methanol for the 1.6-l fermentations and tetrahydrofuran (THF, spectrograde) or dichloromethane for the 16-l fermentations of *B. licheniformis* 86 or *B. subtilis* 21332, respectively. Surfactin was further purified as described by Cooper et al. [6].

The THF extracted surfactant BL86 was further processed by drying, using vacuum distillation at 40 °C, and washing 3 times in 5 ml of *n*-hexane each. The dried powder was an off-white color. The material was stored desiccated below 0 °C. In some cases the acid precipitate was neutralized to pH 7.0 prior to lyophilization. Tetrahydrofuran was replaced by methanol in extracting the surfactant BL86 in several preparations.

**Surface and interfacial tension measurements.** The surface tension measurements were done using a plate tensiometer (Universal Transducer Readout model SC1001, Gould Statham). Critical micelle dilution (CMD -1) and CMC were determined by measuring the ST of 15 ml samples of a serial (1 : 1) dilution in water of the collected, cleared foam or the dissolved surfactant BL86, respectively. To evaluate the salt stability of surfactant BL86, the ST of 1 mg of surfactant (in 100 μl methanol)/15 ml alkaline-water was measured for 0, 5, 10, 15, 17.5, 20, 22.5, 25 and 30% NaCl solutions.

The IFT measurements were done using a Spinning Drop Interfacial Tensiometer model 5000 (Gaertner Scientific Corporation).

**Thin layer chromatography.** One dimensional thin layer chromatography (TLC) was performed using silica gel G (Fischer Brand Redi/Plate). Samples of surfactant BL86 and surfactin were dissolved in methanol at a concentration of 25 mg/ml and 13 μl of each were spotted on the TLC plates. The solvent system was chloroform: methanol: 28% ammonium hydroxide, 65:25:4 [6]. Spots were visualized by sulfuric acid charring at 125 °C.

**Reverse phase high performance liquid chromatographic analysis.** Reverse phase high performance liquid chromatography (HPLC) was performed using a 30 cm C18 μ-Bondapak column (Waters) at 25 °C. The mobile phase was a 60 to 90% linear gradient of acetonitrile in 0.01 M ammonium acetate, pH 4.8. The eluted peaks were detected by following UV absorbance at 210 nm and by a mass detector at 60 °C (ACS light scattering vapor phase detector).

**RESULTS**

**Isolation and characterization of the surfactant from *B. licheniformis* 86 fermentations**

The 1.6-l fermentation results are presented in Table 1. On the average 284 mg of methanol purified surfactant BL86 was produced during the 20 h fermentations. The cell mass production was relatively consistent for the four fermentations. However, the amount of surfactant produced varied considerably ranging from 0.6 to 1.2 mg surfactant/g dry cell consumed. There appeared to be no obvious reason for the variability. The calculated volumetric productivity using these data was 22 mg/l/day.

Surfactant BL86 was recovered from the foam produced during the fermentation. Surface tension measurements on the collected foam prior to any manipulations showed a surface tension of 27 dynes/cm and had a