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Properties of the biosurfactant produced by *Bacillus licheniformis* strain JF-2

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SUMMARY

The activity of the biosurfactant produced by *Bacillus licheniformis* strain JF-2 was quantified using a unit defined as the amount of the acid-precipitated biosurfactant that lowered the surface tension by 10 mN/m. One unit was equivalent to 37 μg/ml of the acid-precipitated biosurfactant. Acid precipitation was very effective in the removal of the biosurfactant from the spent medium. Among the solvents tested methanol was the most efficient in extracting the surfactant activity from acid-precipitated material. Thin-layer chromatography of the acid-precipitated biosurfactant revealed four components, two of which contained a lipid moiety and one of which contained an amino group. The methanol-soluble fraction also contained these four components. Studies suggested that all four components were needed for full activity. The lowest interfacial tensions against octane were observed when NaCl concentrations were 50 g/l or greater. Calcium concentrations greater than 25 g/l significantly increased the interfacial tension

INTRODUCTION

Interest in biosurfactants has increased because of their potential applications in enhanced oil recovery and as specialty chemicals. Many microorganisms are known to produce biosurfactants [4,14,15] and several of these biosurfactants reduce interfacial tension between oil and brine to less than 0.01 mN/m [14,15] making them potential candidates for enhanced oil recovery. These biosurfactants are produced by aerobic organisms which are not well suited for in situ microbially-enhanced oil recovery processes since most oil reservoirs are anaerobic [3]. Biosurfactant production by anaerobic organisms has not been well studied. Several anaerobic bacteria have been shown to produce biosur-

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factants, but the observed reductions in surface tension (45 to 50 mN/m) [7,8,13] were much higher than those observed for aerobic organisms (27 to 30 mN/m) [5,6,14].

We have shown that under strictly anaerobic conditions *Bacillus licheniformis* strain JF-2 produces a biosurfactant which significantly lowers the surface tension of the medium (<30 mN/m) [9]. In this study, we report the further characterization of the biosurfactant produced aerobically by strain JF-2, since it is the same as the biosurfactant made anaerobically [9] and large amounts are readily obtained in crude form by collecting the foam from an aerobically-operated fermentor [6].

MATERIALS AND METHODS

Conditions of cultivation

Bacillus licheniformis strain JF-2 was grown aerobically in medium E as described previously [9]. To determine the efficiency of acid precipitation in the removal of surfactant activity from the culture medium, 100 ml cultures were grown as described before [9]. Large amounts of the biosurfactant were obtained by collecting the copious quantities of foam produced by a 5-liter culture of strain JF-2 grown aerobically at 37°C in medium E using a 5-liter fermentor with an agitation rate of 600 rpm and an aeration rate of 6 liter per min [6].

The surfactant was acid-precipitated with 6 N HCl as described previously [6,9] except that the precipitate was lyophilized.

Surface and interfacial tension measurements

The surface tension was measured using a DuNuoy ring detachment apparatus as described previously [9,10]. The critical micelle dilution (CMD^{-1}) was determined by serially diluting the crude extract until a sharp increase in surface tension was observed [9,10]. The CMD^{-1} is the reciprocal of the dilution at which the sharp increase in surface tension is observed. Interfacial tensions were determined using the spinning drop tensiometer [12].

Quantification of surfactant activity

A series of solutions, with different concentrations (1 μg/ml to 1 mg/ml) were made by dissolving the acid precipitate in distilled water (pH 7.0). The surface tension of each solution was determined and plotted against the acid precipitate concentration to obtain a standard curve.

The fractions containing surfactant activity were serially 1:2 diluted with distilled water (pH 7.0) until the surface tension of the fraction was in the linear portion of the standard curve. This surface tension value was subtracted from the surface tension of distilled water (72 mN/m), because distilled water was used as the diluting agent, and the resultiing value was then divided by 10 mN/m to obtain the number of units of the surfactant. One unit of activity is defined as the amount of surfactant required to lower the surface tension of water by 10 mN/m.

The efficiency of a particular treatment in removing the surfactant was determined by comparing the number of units in the fraction before and after treatment.

Solvent extraction of the surfactant activity

One hundred mg of the crude precipitate was suspended in 40 ml of the solvent and filtered through a 0.45 μm membrane filter. The solvent-soluble and solvent-insoluble fractions were dried under a stream of N_2 and dissolved in 20 ml of distilled water (pH 7.0). The surfactant activity in each fraction was determined. Chloroform, dichloromethane and methanol were tested using this method. For methanol extraction, 200 mg of the acid precipitate was used.

Thin-layer chromatography (TLC)

The acid precipitate or the various fractions obtained after solvent extractions were analyzed by TLC using silica gel G plates (20 by 5 cm or 20 by 20 cm). A 10 μl sample of a 20 mg/ml solution was applied to the plate and allowed to dry. The plates were developed with a solvent mixture containing chloroform-methanol-28% NH_4OH (65:35:5: vol/vol) [6]. Plates were allowed to dry and were then stained with either ninhydrin (in methanol/water, 1:1 vol/vol) or rhodamine B (0.25 g in 100 ml abso-

lute ethanol) or were sprayed with concentrated sulfuric acid and placed at 100°C until charred. The plates stained with ninhydrin were placed at 100°C for 4–5 min and then viewed directly for presence of amino acids. The plates sprayed with rhodamine B were dried and observed under ultraviolet light for the presence of lipids, and plates charred with sulfuric acid were viewed directly for the presence of organic compounds.

RESULTS

Surfactant properties

We previously showed that strain JF-2 cell-free culture fluids had an interfacial tension of 0.05 mN/m against octane at 32°C [12] and that the interfacial tension increased as alkane number increased and as the temperature increased above 60°C. The effects of increasing NaCl and calcium concentrations at different temperatures on the interfacial tension are shown in Fig. 1 and 2, respectively. The lowest interfacial tensions (< 0.1 mN/m) were observed with NaCl concentrations of 5 percent or greater. Lower NaCl concentrations resulted in much higher interfacial tensions. The lowest interfacial tensions were observed at 50°C. High calcium concentrations (2.5% or greater) resulted in very high interfacial tensions (greater than 2 mN/m) at all temperatures tested. We previously showed that the lowest surface tensions were observed when the acid precipitate was dissolved in 5% NaCl at 50°C and the surface tension was not affected by adding up to 0.3 mg per liter calcium. These data showed that the JF-2 surfactant was effective at the temperature and sodium and calcium concentrations found in many oil reservoirs.

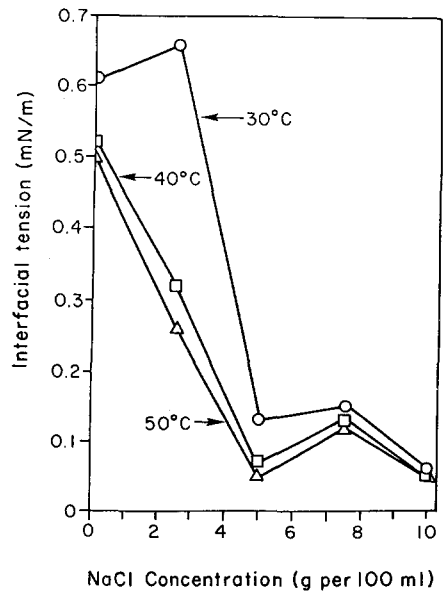

Fig. 1. Effect of NaCl concentration on the interfacial tension of the JF-2 surfactant against octane at different temperatures. The acid precipitate (35 mg per ml) of the JF-2 surfactant was dissolved in deionized water (pH 7.0) with the indicated concentration of NaCl.

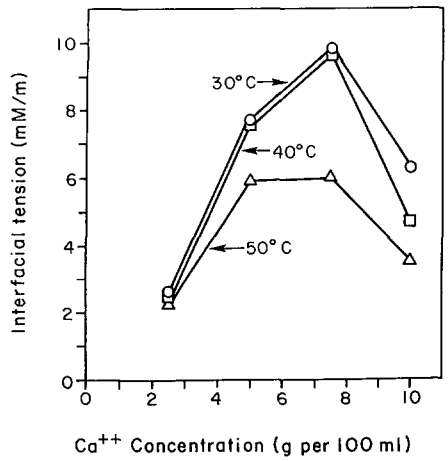

Fig. 2. The effect of calcium concentration on the interfacial tension of the JF-2 surfactant against octane at different temperatures. The acid precipitate (35 mg per ml) of the JF-2 surfactant was dissolved in 5% NaCl solution. $CaCl_2$ was added to give the indicated concentration of the calcium ion.

The CMD^{-1} of the acid precipitate varied from 20 to 100 μg per ml and the yields varied significantly (Table 2) depending on the batch of cells and other unknown variables in preparation of the samples.

Quantification of surfactant activity

Fig. 3 shows the effect of the JF-2 surfactant concentration on the surface tension of distilled water. At low JF-2 surfactant concentrations (less than 60 μg/ml), there was a linear relationship between surface tension and concentration. Above 100 μg/ml of

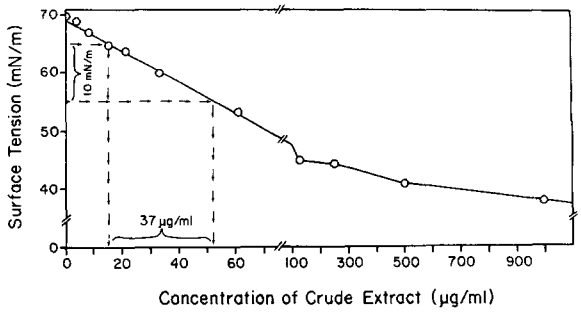

Fig. 3. Quantification of Surfactant Activity. The surface tension of different concentrations of the acid precipitate dissolved in distilled water (pH 7.0) was measured.

JF-2 surfactant, increasing concentrations did not lead to a proportional decrease in surface tension. The linear portion of the curve was used to quantify the surfactant activity of the various fractions. A unit of surfactant activity is equivalent to 37 μg/ml of the acid precipitate.

Efficiency of acid precipitation in removing surfactant activity

Table 1 shows that the presence of the cells contributed to the surface activity of the culture, since removal of the cells from the culture fluid resulted in about a 32% loss of surfactant activity. Although only 64.2% of the total surfactant activity of the culture was recovered by acid precipitation, acid precipitation was very effective in the removal of the surfactant activity from the cell-free medium. Ninety-four percent of the surfactant activity present in cell-free medium was recovered in the acid-precipitated fraction.

Table 1

Efficiency of acid precipitation in removing surfactant activity from the culture medium

Fraction	Units/ml[a]	Total units[b]	Percent Revovery
JF-2 Culture	24	2400	100
Cell-free medium	16.4	1640	68
Supernatant	4	400	1.7
Precipitate	15.4	1540	64.2

[a] Units were calculated using Fig. 3. Surface tension of medium E before and immediately after inoculation was 69–72 mN/m.
[b] The volume of each fraction was 100 ml.

Thin-layer chromatography of the acid precipitate (Fig. 4) indicated that the crude extract contained four components as detected by sulfuric acid charring. Two components with R_f values of 0.6 and 0.5 stained with rhodamine B, indicating the presence of lipids. The component that remained at the origin stained with ninhydrin, indicating the presence of an amino group, possibly an amino acid. The component moving with the solvent front did not stain with either rhodamine B or ninhydrin, but was observed when the plate was charred with concentrated sulfuric acid.

Methanol was the most effective solvent among those tested in extracting the surfactant activity from the acid precipitate (Table 2). The methanol-insoluble fraction had a dry weight of 185 mg. Thus, the methanol-soluble fraction contained only about 7.5% (15 mg) of the material originally present in the acid precipitate on a weight loss basis, but contained almost all of the surfactant activity.

Thin layer chromatography showed that the chloroform-soluble fraction contained one component with an R_f of 0.97 which was observed only with sulfuric acid charring (Table 2). The dichloro-

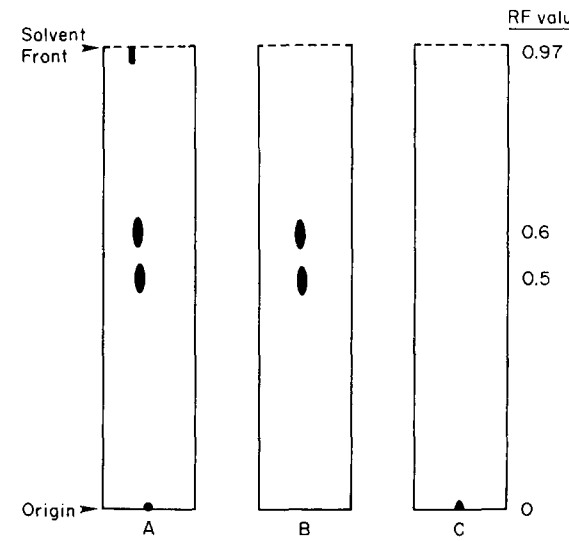

Fig. 4. Thin-layer chromatography of the acid precipitate. Ten μl of a 20 mg/ml solution of the acid precipitate was applied to the origin of each plate (5 by 20 cm). (A) Stained with sulfuric acid. (B) Stained with Rhodamine B. (C) Stained with ninhydrin.

Table 2

Efficiency of various solvents in extracting the surfactant activity[a]

Fraction	Units/ml[a]	Total Units	Percent Recovery	Components[b] (R_f)
Acid precipitate 1	26	2 600	100	0,0.5,0.6,0.97
Chloroform soluble	29	580	23	0.97
Dichloromethane soluble	73	1 450	56	0.55,0.97
Acid precipitate 2	880	17 500	100	0,0.5,0.6,0.97
Methanol soluble	820	16 400	94	0,0.31,0.45,0.97

[a] Units were calculated using Fig. 3. Acid precipitate 1 and 2 were prepared from different fermentor cultures of strain JF-2. [b] The component's presence in the fractions was detected by thin layer chromatography and differential straining as described. The R_f values are 0.97 for A, 0.6 for B, 0.5 for C and origin (0) for D (See Fig. 3).

methane-soluble fraction contained this component plus possibly the two components of the acid precipitate with R_f values of 0.5 and 0.6 since the spot was almost as large as both combined and had an average R_f value of 0.55. This spot stained with Rhodamine B indicating the presence of lipid. The methanol-soluble fraction contained four components which had similar shapes and staining properties as the components in the acid precipitate. However, the R_f values of the two Rhodamine B positive spots (0.31 and 0.45). were different than those found in the acid precipitate (R_f values of 0.5 and 0.6). It may be that these components were modified during the methanol extraction.

The methanol-soluble fraction contained four components as did the acid precipitate and contained almost all of the activity in the acid precipitate (Table 2). The dichloromethane-soluble fraction contained only three of the components in the acid precipitate, but had only 56% of the activity. The chloroform-soluble fraction had only one of the components and had only 23% of the activity. These data suggest that the presence of all four components is required for full recovery of the activity.

To test this possibility, the chloroform-soluble and insoluble fractions were recombined and the total number of units was determined before and after sonication (Table 3). The recombined fraction contained more activity than was expected from the

Table 3

Effect of recombination on the surface tension activity[a]

Fraction	Surface Tension (mN/m)	Units per ml	Total Units	Expected Units[b]
Chloroform soluble	55	0.10	2.1	– – –
Chloroform insoluble	51	0.09	1.7	– – –
Combined fractions				
not sonicated	49	0.12	2.3	1.9
sonicated	42	0.17	3.4	1.9

[a] One hundred mg of the acid precipitate was extracted with chloroform as described in the Materials and Methods section. The chloroform-soluble and insoluble fractions were diluted in distilled water until the surface tension was in the linear region of Fig. 3. Ten ml of the diluted chloroform-soluble and insoluble fractions were combined and the surface tension was measured before and after a 4-h sonication period. Units were calculated according to Fig. 3. Each fraction was 20 ml. [b] Calculated from the number of units present before they were combined.

amount of activity present in the individual fractions. After sonication, the combined fraction contained about 48% more activity than the untreated combined fraction. There was about 1.8 times more total activity in the combined fraction after sonication than was expected from the amount of activity present in the individual fractions. Sonication of the diluted fractions did not affect activity (data not shown). The surface tension of the combined fraction before or after sonication was lower than the surface tension of either of the two individual fractions. The increase in activity and the reduction in surface tension observed after recombination suggest that several components present in the acid precipitate are required for activity and that there may be a synergistic interaction between these components, which requires energy input to optimize.

DISCUSSION

Arima et al. [1] purified, crystallized, and characterized the biosurfactant, surfactin, produced by *Bacillus subtilis*. Surfactin is a lipopeptide that inhibits fibrin clot formation and lyses erythrocytes and several bacterial spheroplasts and protoplasts [2,11]. Surfactin is an effective surfactant and lowers the surface tension to as low as 27 mN/m [6] and the interfacial tension against hexadecane to less than 1 mN/m [6]. Cooper et al. [6] found that surfactin is precipitated from the spent medium of the *B. subtilis* culture by reducing the pH to 2.0 and can be further purified by dichloromethane extraction and reprecipitation with acid. The biosurfactant produced by *Bacillus licheniformis* strain JF-2 is also precipitated from the spent medium by lowering the pH to 2.0. Thin layer chromatography and differential staining showed that the JF-2 crude biosurfactant contains as major components a lipid and an amine-containing compound. Both surfactin and the JF-2 biosurfactant reduce the surface tension of water from 72 mN/m to 27 mN/m [6,9,10] which is as low a value as ever reported for a biosurfactant [4,15]. Further biochemical analysis of one of the components of the JF-2 surfactant shows that its amino acid composition and infrared spectrum is similar to surfactin (G.E. Jenneman and W.R. Finnerty, personal communication). These data strongly suggest that surfactin and a component of JF-2 surfactant are quite similar if not structurally identical. However, the JF-2 acid precipitate contains additional components, as shown by TLC and extraction with different solvents. These components of the acid precipitate have not been characterized, but the data suggest that there may be some needed interactions between all of the components for full activity of the JF-2 surfactant.

The data obtained from the extraction of the JF-2 surfactant with different solvents suggests that several components are required for full activity and that there may be some needed interactions between these components. Chloroform extracted only one component and only 23% of the activity was recoverd. Dichloromethane extracted two components and 56% of the activity. Methanol extracted all four of the components present in the crude extract and 94% of the activity. Recombination experiments definitely showed that reconstituted surfactant preparation is more active than the individually extracted components. Further work on the molecular nature of the components is needed before definitive conclusions can be made as to the function of the individual components and the nature of their interaction. Cooper and Goldenberg [5] found that *B. cereus* produced a monoglyceride biosurfactant which enhanced the activity of the polyhexosamine emulsifier produced by this organism.

Our studies on the JF-2 surfactant show that it has many useful properties for enhanced oil recovery [9,10,12]. The JF-2 biosurfactant is more effective at high NaCl concentrations since the lowest interfacial tensions occurred when the NaCl concentration was 5% (w/v) or greater. These are optimal conditions for the growth of the organism [10] and high salt concentrations are found in many oil reservoirs [3]. The very low critical micelle concentration shows that the JF-2 biosurfactant is effective at dilute concentrations and very low interfacial tensions were obtained (<0.1 mN/m). Lower interfacial tensions may occur when higher biosurfactant concentrations or cosurfactants are used. Strain JF-2 and other surfactant producing orga-

nisms are currently being used in a field trial of microbially enhanced oil recovery (R.S. Bryant, T.E. Burchfield, D.M. Dennis, and D.O. Hitzman. 1988. Microbial enhanced water flooding: Mink unit project. SPE/DOE 17341. Presented at the Joint SPE/DOE symposium on Enhanced Oil Recovery, April 18-20, Tulsa, OK). Preliminary results show a sustained 13% increase in oil recovery and a reduction of the surface tension of the produced brine indicating that in situ microbial biosurfactant production is effective in recovering additional oil. Also, 30 weeks after injection strain JF-2 was isolated from the brine obtained from the production wells. This is conclusive evidence that strain JF-2 can grow and produce its surfactant throughout an oil reservoir.

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