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Anaerobic Production of a Biosurfactant by *Bacillus licheniformis* JF-2

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***Bacillus licheniformis* JF-2 anaerobically produced a biosurfactant when grown in a glucose-mineral salts medium containing yeast extract and $NaNO_3$. Surface tension of the medium was reduced from 70 to 74 mN/m to as low as 28 mN/m due to the production of an anionic biosurfactant.**

Interest in biosurfactants has increased because of their potential applications in enhanced oil recovery and as specialty chemicals. Many microorganisms produce biosurfactants (8, 19, 22), and several of these biosurfactants have been found to significantly reduce the interfacial tension between oil and brine to less than 0.01 mN/m (10, 19), making them potential candidates for enhanced oil recovery processes. However, these biosurfactants are produced by aerobic organisms. Aerobic biosurfactant-producing organisms would not be well suited for in situ microbially enhanced oil recovery processes since most oil reservoirs are anaerobic (6).

Little is known about anaerobic biosurfactant producers. La Riviere (15) showed that a 7-day-old culture of *Desulfovibrio desulfuricans* had a surface tension of 50 mN/m. Similar reductions in surface tension were observed when various clostridial species were used (12). No attempt was made to determine the nature of the surface tension-lowering activity in these cases. Cooper et al. (9) found that *Clostridium pasteurianum* produces an extracellular neutral lipid that lowers the surface tension of water (72 mN/m) to about 55 mN/m. In this report, we show that *Bacillus licheniformis* JF-2 produces under strictly anaerobic conditions a biosurfactant that significantly lowers the surface tension of the medium (<30 mN/m). The potential use of this bacterium and its biosurfactant in enhanced oil recovery is discussed.

Strain JF-2 was isolated from water injection brine obtained from an oil well in Carter County, Okla., and was tentatively identified as a strain of *B. licheniformis* (4). Strain JF-2 was grown in medium E (6) containing glucose instead of sucrose and supplemented with 0.1% $NaNO_3$ and 0.1% yeast extract (Difco Laboratories, Detroit, Mich.). Medium E is a mineral medium containing 5% NaCl (6). Strain JF-2 was grown in liquid culture at 40°C as previously described (14).

Anaerobic cultures were prepared by three methods: aseptically bubbling oxygen-free nitrogen gas through each flask of medium for 10 min (method 1); boiling the medium under a stream of oxygen-free nitrogen (method 2); or bubbling the medium for 1 h with oxygen-free nitrogen and adding 1 mM dithiothreitol (method 3). All flasks were sealed with no. 6 black rubber stoppers. Subsequent manipulations of the anaerobic medium were performed by the Hungate technique as modified by Bryant (4). Oxygen was removed from the nitrogen by using a heated copper column (4). To some flasks, a 2.5% (wt/vol) cysteine hydrochloride–2.5% (wt/vol) $Na_2S \cdot 9H_2O$ reducing solution (5) was added at a final concentration of 0.05% (wt/vol) of each component. Addition of resazurin (final concentration, 0.0001% [wt/vol]) to some flasks showed that reduced medium was obtained for each method.

The effectiveness of the biosurfactant produced by JF-2 was determined by measuring the surface tension with a Fisher Autotensiomat ring detachment apparatus as previously described (14). The surface tension of distilled, deionized water was measured each time to check the accuracy of the ring calibration and the condition of the ring. Relative amounts of the biosurfactant in different cultures were measured by the reciprocal critical micelle dilution (CMD^{-1}) (22). Samples of the culture were serially diluted, and the surface tension of each dilution (10-ml volume) was measured. The CMD^{-1} is the reciprocal of the dilution at which a sharp increase in surface tension is observed or the reciprocal of the dilution needed to reach the critical micelle concentration.

A crude biosurfactant preparation was obtained by centrifuging (10,000 × *g*, 10 min, 4°C) a stationary-phase culture to remove the cells and adjusting the pH of the spent medium to 2 with 1 N HCl (7). The acidified liquid was kept at 4°C overnight, and the precipitate that formed was collected by centrifugation (17,300 × *g*, 30 min, 4°C). The precipitate was dissolved in distilled water, the pH was adjusted to 7.0 with 1 N NaOH, and the solution was lyophilized. The lyophilized material was designated the acid precipitate.

The acid precipitate was analyzed by thin-layer chromatography with Baker microcrystalline cellulose-F plates and the solvent system described by Cooper et al. (7). The components were visualized by staining them with ninhydrin (5 mg of ninhydrin in a 50 ml butanol–50 ml acetone mixture) and heating them at 100°C for 5 min or by staining them with rhodamine B (250 mg of rhodamine B in 100 ml of absolute ethanol) and observing them under UV light.

Samples were periodically taken to determine fermentation products produced during anaerobic growth of JF-2. Samples were centrifuged (10,000 × *g*, 10 min, 4°C) to remove cells and then frozen at −20°C in sealed tubes until analyzed. Acetoin and 2,3-butanediol were measured by the colorimetric assays of Neish (16). The presence of *n*-alcohols (methanol, ethanol, propanol, and butanol) and volatile fatty acids was determined by gas chromatography (18). Samples for volatile fatty acids analysis were acidified by adding 0.2 ml of 6 N HCl to 1.8 ml of sample.

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TABLE 1. Biosurfactant production by aerobically and anaerobically grown *B. licheniformis* JF-2

Cultivation condition[a]	Surface tension (mN/m)[b]	Growth (A_{480})	CMD^{-1}[a]
Aerobic	30	1.50	4
Anaerobic			
Method 1	31	1.05	4
Method 2	32	1.05	ND[c]
Method 3	27	0.50	ND

[a] See text for details. Values for surface tension and absorbance are averages of values obtained from three different cultures except for the anaerobic culture prepared by method 2 which was a single determination. The CMD^{-1} values were obtained from a single culture.

[b] The surface tension of uninoculated medium was 66 to 70 mN/m. The surface tension immediately after inoculation was similar to that of the uninoculated medium.

[c] ND, Not determined.

Cultures of JF-2 grown aerobically or anaerobically in modified medium E exhibited low surface tensions (Table 1), indicating that a biosurfactant was produced. The presence of nitrate and yeast extract was required for anaerobic growth of JF-2 in modified medium E (data not shown). Surface tensions of the anaerobic cultures were as low as those obtained for aerobic cultures and close to the values reported for other good biosurfactant-producing organisms (8, 19, 22). The anaerobic medium prepared for each method was reduced as indicated by the complete reduction of resazurin. The CMD^{-1} value was about four times the amount required for micelle formation. Anaerobic cultures of JF-2 grew well, reaching an A_{480} of 0.5 to 1.05 within 40 to 48 h. Aerobic cultures generally grew faster, reaching an absorbance of 1.5 within 24 to 30 h.

The addition of 2.5% (wt/vol) cysteine hydrochloride–2.5% (wt/vol) $Na_2S \cdot 9H_2O$ solution inhibited both growth and biosurfactant production of JF-2. It is known that sulfide inhibits nitric oxide and nitrous oxide reductase (20), which could explain why JF-2 grew poorly anaerobically in modified medium E with nitrate when cysteine-sulfide reducing solution was added. In anaerobic cultures containing this reducing solution in which some growth occurred, the surface tension of the medium was reduced from 69.5 to 47 mN/m. In addition, low surface tensions were obtained in medium containing dithiothreitol (Table 1), which indicates that the biosurfactant was produced even under strictly anaerobic conditions.

Measurements of surface tension during growth of JF-2 showed that anaerobically grown cells produced the biosurfactant during the exponential phase of growth (data not shown) as was found for aerobically grown cells (14).

Serial transfer of JF-2 in anaerobically prepared media showed that the biosurfactant was produced under strictly anaerobic conditions for about 18 generations (data not shown). However, the minimum surface tension obtained by the culture increased with successive transfers from 28 to 44 mN/m after transfer four (24 generations). Similar increases were observed for aerobically grown cultures treated in an identical manner. The reason behind the increase in surface tension due to transfer may be the selection of variants that do not produce the biosurfactant or produce lower amounts of the biosurfactant. Serial transfer of the culture three to four times increased the proportion of cells that produced round colonies rather than volcano-shaped colonies on medium E. Liquid cultures inoculated with cells from round colonies had higher surface tensions (about 45 mN/m) than those inoculated with cells from volcano-shaped colonies (<30 mN/m). A few round colonies were often observed on plates of medium E streaked with a well-isolated volcano-shaped colony, indicating that the round colonies are variants of JF-2 and not contaminants. Plasmids were not detected in cells obtained from cultures with low surface tensions (<30 mN/m) when using procedures that resulted in the isolation of plasmids from *Escherichia coli* or *Haemophilus influenzae* (13). Thus, the loss of surface tension-lowering ability was not correlated with the loss of a plasmid.

Acetate was the main fermentation acid produced by the anaerobically grown cultures and was found at a concentration of 2 mM. Acetoin and 2,3-butanediol were detected at concentrations of 5 and 800 μg/ml, respectively. Methanol, ethanol, propanol, and butanol were not detected. The addition of up to 10 mg of acetoin or 2,3-butanediol per ml did not affect the surface tension of uninoculated medium E.

The aerobically or anaerobically produced biosurfactant was ionic in nature and was precipitated from the medium with acid (Table 2). Thin-layer chromatography of the acid precipitates obtained from aerobic and anaerobic cultures showed the presence of one major component with an R_f value of 0.45 in each case, suggesting that the biosurfactant produced anaerobically was the same as that produced aerobically. This component reacted with ninhydrin and rhodamine B, indicating the presence of a free amino group and a lipid moiety. The acid precipitates obtained from aerobic and anaerobic cultures (25 mg) were twice extracted with 10 ml of chloroform, and the chloroform-soluble and insoluble materials were analyzed by thin-layer chromatography. The chloroform-soluble material in each case had one major component that had identical mobility and staining properties as that found in the acid precipitate. Surface tension measurements of the chloroform-soluble material after it was reduced to dryness and dissolved in 10 ml of H_2O (pH 7.0) showed that it contained the surface tension-lowering activity. Very little material was recovered in the chloroform-insoluble fraction. The biosurfactant was also soluble in dichloromethane.

We showed above that *B. licheniformis* JF-2 produces a biosurfactant which significantly lowers the surface tension of the medium when grown anaerobically in a glucose-yeast extract-mineral salts medium with $NaNO_3$ (Table 1). The surface tensions of JF-2 cultures were much lower than those reported for other anaerobic biosurfactant producers (9, 12, 15). Alcohols can act as cosurfactants to enhance the effectiveness of surfactants (2, 11, 21). Since alcohols are a common bacterial end product, it may have been possible

TABLE 2. Effect of acid precipitation on the surface tension of aerobically and anaerobically grown cultures of *B. licheniformis* JF-2[a]

Culture	Fraction	Surface tension (mN/m)
Aerobic	Spent medium	30
	Acid precipitate	27
	Supernatant	59
Anaerobic	Spent medium	31–33
	Acid precipitate	27
	Supernatant	54

[a] *B. licheniformis* JF-2 was grown aerobically and anaerobically (method 2), and the biosurfactant was obtained by acid precipitation as described in the text. The supernatant is the liquid obtained after acid precipitation and centrifugation of the spent medium.

that the low surface tensions were due to alcohol-biosurfactant interactions. However, the surface tension-lowering activity was removed from the medium by acid precipitation (Table 2), which suggests that it is an ionic compound, and JF-2 produces only small amounts of alcohols under anaerobic conditions. Thus, it is unlikely that the observed surface tension reductions were affected by solvent production. The biosurfactant produced by JF-2 appears to be quite similar to surfactin, a lipoprotein surfactant produced by *Bacillus subtilis* (1, 3, 7). Both compounds are ionic, are soluble in dichloromethane, contain a free amine group and a lipid moiety, and have similar surface tension-lowering abilities and critical micelle concentrations (7, 14, 17).

B. licheniformis JF-2 and the biosurfactant that it produces have many properties that are desirable for enhanced oil recovery operations. Strain JF-2 grows in medium with NaCl concentrations up to 10% (wt/vol) over a wide range of temperatures (up to 50°C) and pH values (4.6 to 9.0) and is not inhibited by the presence of crude oil (14) as is *B. subtilis* (7). JF-2 grows on water-soluble substrates such as sucrose and glucose and does not use oil. The biosurfactant produced by JF-2 was not affected by the pH, temperature, or the NaCl or calcium concentration found in many oil reservoirs (14). The JF-2 biosurfactant reduces the surface tension of 5% NaCl solutions to 27 mN/m (14; this work) and has a critical micelle concentration of about 0.02 mg/ml (17), values which are as low or lower than the values reported for other good biosurfactants (7, 10, 22). The whole broth of JF-2 cultures significantly lowers the interfacial tension of octane–5% NaCl brine mixtures to about 0.05 mN/m (17). The fact that the biosurfactant is made anaerobically at temperatures and salinities found in many reservoirs, has a low critical micelle concentration, and generates low interfacial tension when dilute biosurfactant concentrations are used without alcohols or other cosurfactants suggests that JF-2 is suitable for in situ microbially enhanced oil recovery process.

The loss of surfactant-producing capability of JF-2 by repeated culturing may present problems when cultures of JF-2 are scaled up to obtain inocula for field trials or large quantities of the biosurfactant. However, this problem can be minimized by maintaining the culture on agar plates so that the volcano-shaped colonies are selected and by minimizing the number of liquid transfers. Large quantities of the acid precipitate (0.1 to 0.7 g/liter) can be obtained from a 50-liter fermentor operated as described by Cooper et al. (7) by using the following scale-up protocol. A 100-ml culture is inoculated with cells from a volcano-shaped colony. The 100-ml culture is used to inoculate a 5-liter fermentor which is then used to inoculate the 50-liter fermentor.

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