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DEVELOPMENT OF MORE EFFECTIVE BIOSURFACTANTS FOR ENHANCED OIL RECOVERY

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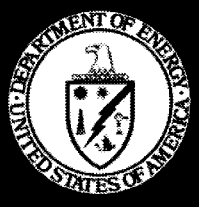

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Abstract

Core displacement experiments at elevated pressures were conducted to determine whether microbial processes are effective under conditions that simulate those found in an actual oil reservoir. The *in situ* growth of *Bacillus licheniformis* strain JF-2 resulted in the recovery of about 23% of the residual oil remaining in Berea sandstone cores after waterflooding. Oil recovery by *B. licheniformis* strain JF-2 was highly correlated to surfactant production. A biosurfactant-deficient mutant of strain JF-2 did not recover residual oil. These data show that surfactant production is an important mechanism for microbially enhanced oil recovery. We initiated work on determining the molecular genetics of biosurfactant production. A variety of deoxyribonucleic acid (DNA) sequences for genes involved in the synthesis of cyclic peptide surfactants and antibiotics were obtained. These genes were analyzed for unique regions that could be used to identify the biosurfactant genes in *B. licheniformis* strain JF-2. Primer DNA sequences were constructed by using this information to identify and clone the genes for biosurfactant production. We have also begun work to determine the nutritional factors that control the growth and biosurfactant production in *B. licheniformis* strain JF-2.

Introduction

Enhanced oil recovery due to microbial activity is a commercially viable technology in the petroleum industry with thousands of wells treated on a yearly basis in the United States (1, 2). Several, well-controlled field trials have shown that additional oil is recovered and that the economics of microbially enhanced oil recovery (MEOR) are very attractive (3,4). Although these results are promising, the implementation of MEOR still suffers from a lack of understanding of how microorganisms can recover oil. In order to develop MEOR as a commercially viable technology more fully, a greater understanding of the mechanisms of MEOR processes is needed.

After a reservoir has been waterflooded, brine, gas, and small droplets of oil remain trapped within pores of the reservoir medium. Many tertiary processes currently being employed can increase the recovery of this trapped oil. These processes include *in situ* combustion, CO_2 and chemical flooding, and selective plugging. In MEOR, the growth and metabolism of indigenous bacteria are stimulated. The bacteria produce products similar to those used in chemical flooding such as carbon dioxide, solvents, surfactants, and polymers. Since microorganisms use relatively inexpensive materials to synthesize these products, MEOR may provide a cost effective alternative to the above methods. Laboratory studies conducted by Bryant and Douglas (5) suggest that the production of gas, solvents, organic acids, and surfactants are important mechanisms for microbially enhanced oil recovery in sandstone cores. However, it is not clear whether all of these products need to be produced, and which of these products is the most effective for the recovery of oil (6).

One of the main mechanisms that limits the ultimate recovery of oil is the entrapment of oil in microscopic pores by capillary forces. Only when the interfacial tension between oil and water is lowered by a factor of about a thousand fold will significant amounts of residual oil be recovered. Biosurfactants are a potentially important mechanism for oil recovery since they can significantly reduce the interfacial tension between the oil and brine. In chemical surfactant flooding, much of the surfactant adheres to the surface of the reservoir rock near the well-bore. This decreases the concentration of the surfactant to levels below that needed to recover residual oil. A potential advantage of microbial processes is that the surfactant is produced *in situ*, near the site where it is needed to recover residual oil. However, since biosurfactant-producing organisms have other activities that could also result in oil recovery, it is difficult to determine whether surfactant production alone is a major mechanism for oil recovery by microorganisms.

The objectives of our initial work were two fold. First, core displacement studies were done to determine whether microbial processes could recover residual oil at elevated pressures. Second, the importance of biosurfactant production for the recovery of residual oil was studied. In these studies, a biosurfactant-producing, microorganisms called *Bacillus licheniformis* strain JF-2 was used. This bacterium produces a cyclic peptide biosurfactant that significantly reduces the interfacial tension between oil and brine (7). The use of a mutant deficient in surfactant production and a mathematical MEOR simulator were used to determine the major mechanisms of oil recovery by these two strains.

Materials and Methods

Organisms. *Bacillus licheniformis* strain JF-2 was obtained from our culture collection.

Media and Conditions of Cultivation. Composition of the medium used to grow *B. licheniformis* strain JF-2 in liquid culture and in the sandstone cores is shown in Table 1. The medium contained inorganic minerals and organic growth factors, a buffer, and glucose as the same energy source. Anaerobic growth of *B. licheniformis* strain JF-2 also required the addition of sodium nitrate as an electron acceptor, and maximal surfactant production by this strain required high amounts of sodium chloride.

B. licheniformis strain JF-2 and a non-surfactant-producing mutant of this strain were also grown in medium E modified by the addition of 0.5 g/l each of yeast extract and sodium nitrate (8).

Procedures for the preparation and use of anaerobic media and solutions were those of Byrant (9) and Balch and Wolfe (10).

Isolation of non-surfactant-producing mutant. A spontaneous mutant of *B. licheniformis* strain JF-2 that no longer lowered the surface tension of the medium was obtained by selection for non-hemolytic colonies on blood agar plates (11). Strain JF-2 was grown aerobically in modified medium E at 37°C, and transferred to sterile medium when the culture reached the stationary phase of growth. The culture was serially transferred in this manner twenty times to enrich for non-surfactant-producing cells. The non-surfactant-producing mutant was isolated by serial dilution and inoculation of blood agar plates. Non-hemolytic colonies were picked and restreaked to obtain a pure culture.

Core flow apparatus. Berea sandstone cores were steam-cleaned for two weeks to remove humic acids and other organic materials. After steam cleaning, the cores were dried at 125°C for 24 hours, and then placed in a vacuum dessicator to cool. Each core was wrapped with Teflon™ tape, and then inserted into a rubber sleeve. Liquid gasket material was applied to the ends of the rubber sleeve to prevent leaks. The rubber sleeve with the core was placed in a stainless steel cylindrical core holder. The core holder was connected to the core flow apparatus using stainless steel tubing and compression fittings. The components of the system were rated to operating pressures of about 35,000 kPa.

The core holder was placed inside of a constant temperature oven to maintain the temperature at 36°C. Pressure gauges were attached to monitor the confining pressure and the pore pressure. A back-pressure regulator located on the effluent side of the core was used to control the pore pressure. A stainless steel transfer vessel operated at a pressure equal to that of the pore pressure of the core was used to inject fluids into the core. A piston-driven pump was used to displace the fluid from the transfer vessel into the core. A plastic syringe located downstream of the back-pressure regulator was used to collect liquid and gas samples. Prior to use, the core apparatus was pressurized with nitrogen for several days to check for leaks.

Core flow experiments. Petrophysical data for the cores used in this study are given in Table 2. To determine whether the *in situ* generation of microbial products

Table 1. Medium components used for the growth *Bacillus licheniformis* strain JF-2 in liquid culture and in sandstone cores.

Component	(g or ml/l)
Tanner's Minerals[a]	20.0 ml
Tanner's Metals[a]	10.0 ml
Glucose	1.8 g
Yeast Extract	5.0 g
NaCl	50.0 g
Piperazine-N,N'-bis[2-ethanesulfonic acid]	4.0 g
Balch Vitamins[a]	5.0 ml
NaNO3	1.0 g

[a] Composition of solutions is given in reference 22.

Table 2. Petrophysical properties of Berea sandstone cores[a]

Core Number	3	4	5	6
Porosity (%)	19.0	14.0	17.0	17.5
Pore volume (ml)	113	85	103	105
Absolute permeability (μm^2)	0.18	0.10	0.29	0.26
Oil permeability (μm^2)	0.09	0.07	0.20	0.15
Connate water (ml)	41	30	33	35
Connate water saturation (%)	36	35	32	33
Water permeability (μm^2)	0.02	0.02	0.06	0.07
Residual oil (ml)	35	26	30	26
Residual oil saturation (%)	31	31	29	24

[a]All cores had a diameter of 5.08 cm and a length of 28.42-29.85 cm.

at reservoir pressures enhances oil recovery, cores were incubated at an initial pore pressure of about 7,000 kPa. A 50 g/l sodium chloride solution was used to waterflood each core after the incubation period.

Each core was vacuum-saturated with the 50 g/l sodium chloride solution. After saturation, core was inserted into the rubber sleeve and then placed inside of the core holder. The core holder was connected to the flow system, and the core was flooded with ten pore volumes of the 50 g/l sodium chloride solution containing 0.1 M $CaCl_2$ to stabilize clay particles, and 50 ml/l of methanol to disinfect the core apparatus. The core apparatus was incubated for twenty-four hours with the 50 g/l sodium chloride solution containing calcium chloride and methanol. The apparatus was then flushed with ten pore volumes of the respective brine solution without methanol and calcium chloride. The core was flooded with oil to connate water saturation, and then flooded with the respective brine to residual oil saturation (12).

To determine whether the disinfection procedure was effective, an uninoculated core was aseptically flooded with sterile medium and incubated. Neither the presence of viable cells in the effluent nor an increase in pore pressure was observed after five days of incubation.

A series of nutrient treatments were performed on each core after the core was flooded to residual oil saturation. Each core received from three to five pore volumes of culture medium containing a 5% (vol/vol) inoculum of a culture of *B. licheniformis* strain JF-2. The core was incubated without fluid flow until no further change in the pore pressure was observed. The core was then flooded with about five pore volumes of the respective brine solution. The amounts of oil, gas, and brine collected after each treatment were measured volumetrically. Table 3 shows the actual amounts of culture medium and brine that each core received and the incubation times of each treatment.

Analyses. Absolute permeability, porosity, pore volume, connate water saturation, residual oil saturation, and effective permeabilities to oil and water were determined as described (12, 13, 14, 17). The permeability reduction factor (in percent) was calculated from the change in permeability after each treatment (15). Pore pressure was measured using a calibrated gauge.

The amount of gas produced was estimated volumetrically by using a plastic syringe connected to the effluent flow line. Organic acids and alcohols were quantified by using high pressure liquid chromatography (HPLC) and gas chromatography, respectively (15, 16). Carbon dioxide was measured by gas chromatography, and hydrogen was measured using a gas chromatograph equipped with a mercury reduction detector (18). Glucose was measured using the glucose oxidase method (Sigma, Inc., St. Louis, MO). Since the HPLC method did not separate lactate and succinate, the succinate dehydrogenase assay (Sigma, Inc., St. Louis, MO) was used to check for the presence of succinate. Succinate was not detected.

The amounts of products produced by liquid cultures were corrected for the small amounts of these products present in the medium at the start of incubation, and for the amounts of these products produced in control cultures that lacked glucose. The amounts of fermentation products produced during *in situ* growth in cores were corrected for the small amounts of the products in the influent medium.

Table 3. Treatment regimes for microbially enhanced oil recovery experiments.

Core No.	Organism	Treatment No.	Nutrient Volume (ml)	Brine Flood Volume (ml)	Incubation Time (days)
3	*B. licheniformis* (wild-type)	1	300	400	8
		2	300	400	9
		3	300	400	4
4	*B. licheniformis* (wild-type)	1	300	400	4
		2	300	400	5
		3	300	400	4
		4	300	400	5
		5	300	400	5
5	*B. licheniformis* (wild-type) serially transferred	1	250	400	5
		2	250	400	6
		3	250	320	2
6	*B. licheniformis* (mutant)	1	300	350	4
		2	300	350	5
		3	300	350	4

Growth of liquid cultures was measured spectrophotometrically by following the change in absorbance with time. Cell concentration was determined by quantifying the amount of whole cell protein. Samples were centrifuged (12, 000 x *g*) for 2 minutes to collect the cells. The cell pellet was washed twice by resuspending the pellet in a 10 mM sodium/potassium phosphate buffer (pH 7.2) and recentrifuging. The final cell pellet was resuspended in 0.1 N NaOH and incubated at 70°C. Protein was determined colorimetrically using bovine serum albumin as the standard (19).

Surface tension of cultures and core effluents was measured using a DeNoy ring and an automated tensiometer (7). The relative amount of surfactant produced was estimated from the number of units of surfactant activity as described previously (7).

Results and Discussion

Fermentation Studies. The fermentation balance for *B. licheniformis* strain JF-2 was incomplete since this strain produced a large amount of an unknown metabolite. Of the identified products, 47.2 mmoles of lactate, 21.8 mmoles of acetate, 13 mmoles of propionate, and 21.8 mmoles of CO_2 (calculated) were produced from 100 mmoles of glucose. The carbon recovery was 41%. The unidentified product migrated between isobutyrate and butyrate on both gas chromatography and HPLC. Assuming that the unidentified peak has four carbons and has a similar detector response as butyrate, about 80 mmol were produced per 100 mmoles of glucose. This would give a carbon recovery of about 95%.

A conversion factor that relates the units of surfactant activity to the moles of biosurfactant was calculated (7). We found that the most purified fraction of the biosurfactant contained 1090 units of activity per mg (dry weight) (8). Lin et al. (20) reported a molecular weight of the JF-2 biosurfactant of 1035 g/mole. Assuming that our most pure fraction contains only the JF-2 biosurfactant, then one mole of the biosurfactant would have 1.1×10^9 units of activity. This conversion factor can be used to estimate the molar concentration of the JF-2 biosurfactant produced in core experiments by determining the number of units of surfactant activity present.

Isolation of a biosurfactant mutant. Cultures of strain JF-2 lost the ability to reduce the surface tension of the medium when they were repeatedly transferred in liquid medium. We noticed that the cultures that had been repetitively transferred had a large number of smooth colonies, while those that had been inoculated directly from an agar colony had rough colonies, with only a very small percentage of smooth colonies (<0.2%). We hypothesized that the ability to produce the surfactant was not a stable trait of JF-2, and that smooth colonies were mutants of JF-2 that did not produce the biosurfactant (21). We tested this hypothesis by determining whether smooth colonies were hemolytic and whether they had surfactant activity.

The number of rough and smooth colonies in cultures that had been transferred 15 times was compared to that found in cultures that were inoculated directly from a rough colony. Cultures that had been transferred 15 times in liquid medium had about 1.5×10^9 smooth colonies per milliliter. No rough colonies were observed ($<10^7$ colonies/ml). The surface tension of these cultures was greater than 40

mN/m. With some of the cultures, the surface tension was as high as 60 mN/m. Cultures that were inoculated directly from a rough colony had predominantly rough colonies (about 2.4×10^9 colonies/ml) and very few smooth colonies (about 1×10^7 colonies/ml). These cultures always had surface tensions below 30 mN/m. One culture that had been repetitively transferred and one that had been inoculated directly from a rough colony were plated onto blood agar medium to determine the number of hemolytic and non-hemolytic colonies. All of the colonies that grew on plates inoculated with culture that had had not been transferred were hemolytic. Blood agar plates inoculated with cultures that had been transferred 15 times had a large number of non-hemolytic colonies; about 69% of all of the colonies were non-hemolytic. These data suggest that selection for rough colony morphology was required to maintain biosurfactant production by strain JF-2, and that repetitive transfer in liquid medium is not recommended for the maintenance of the strain.

One of these non-hemolytic colonies was picked and restreaked onto blood agar medium to obtain a pure culture of a biosurfactant-deficient mutant of JF-2. When grown in liquid culture, the JF-2 mutant did not lower the surface tension of the medium below 60 mN/m, which was close to the surface tension of uninoculated growth medium. As a comparison, the wild-type strain of JF-2 consistently lowered the surface tension of the medium below 30 mN/m and had a critical micelle dilution of 16. The biosurfactant-deficient mutant of JF-2 was non-hemolytic and formed smooth colonies. Otherwise, it had the same physiological properties as the wild-type strains. The mutant grew anaerobically at 45°C in medium with 50 g/l NaCl. Fermentation end-products of the mutant were similar to those of the wild-type strain of JF-2 as were the morphology and Gram reaction. These data show that a spontaneous, biosurfactant-deficient mutant of strain JF-2 was obtained. The mutant strain will be useful as a negative control to determine the importance of surfactant production in MEOR.

Oil recovery by **Bacillus** ***strain JF-2.*** The biosurfactant-deficient mutant strain of JF-2 was used to determine the importance of biosurfactant production for oil recovery. Two cores (cores 3 and 4) were inoculated with the wild-type strain of JF-2 that had not been repetitively transferred (Table 4). One core was inoculated with a culture of JF-2 that had been transferred about 15 times (core 5) and one core was inoculated with a culture of the biosurfactant-deficient mutant strain (core 6). When the wild-type strain of JF-2 was used, 23 and 21% of the residual oil was recovered from cores 3 and 4, respectively. Analysis of the core effluents showed that small amounts of acetate (0.1 mM), butyrate (1.1 mM) and lactate (0.8 mM) were produced. Surface tensions of the effluents were below 30 mN/m. Microscopic analysis of the effluent showed that most of the turbidity in the aqueous phase was due to very small drops of oil, about the size of a bacterial cell. Effluent samples from the core inoculated with the serially transferred culture of JF-2 had a thin film of oil. When the JF-2 mutant was used, a small amount of oil (about 1 ml) was produced after the first treatment. In the second treatment, only a thin film of oil was detected. Less than 6% of the residual oil was recovered when the mutant strain was used.

Figure 1 shows that oil recovery was highly correlated (r^2=0.979) to surfactant production for core 4. A similar trend was observed for core 3.

Table 4. Experimental results obtained for cores inoculated with *Bacillus licheniformis* strain JF-2.

Core No.	Treatment No.	Culture	Initial Pore Pressure (kPa)	Volume of Oil recovered (ml)	Residual Oil Recovery (%)	Gas Production (ml)
3	1	Wild-type	7,770	3		<0.1
	2		7,840	2		1
	3		7,490	3	23	<0.1
4	1	Wild-type	7,350	1		<0.1
	2		7,490	2		<0.1
	3		7,350	2		<0.1
	4		7,350	0.5		<0.1
	5			<0.5	24	<0.1
5	1	Wild-type	7,350	<0.5		<0.1
	2	serially transferred	7,350	<0.5		<0.1
	3		7,350	ND	<3	<0.1
6	1	Mutant	7,350	1		<0.1
	2		7,350	<0.5		<0.1
	3		7,420	ND	<6	<0.1

[a] ND, not determined.

Table 4. continued.

Core No.	Surfac-tant (units)	PRF (%)	Influent pH	Effluent pH
3	180	79	6.8	7.3
	120	90	6.9	7.3
	60	0.7	6.8	7
4	132	105	7	7.1
	116	118	6.8	7.2
	79	109	6.8	7.2
	57	96	6.9	7.2
	46	91	7.1	7.4
5	NDa	262	6.8	7.5
	165	257	6.9	7.4
	6	245	6.9	7
6	53	131	6.5	7
	ND	140	6.8	7
	ND	140	6.9	7.1

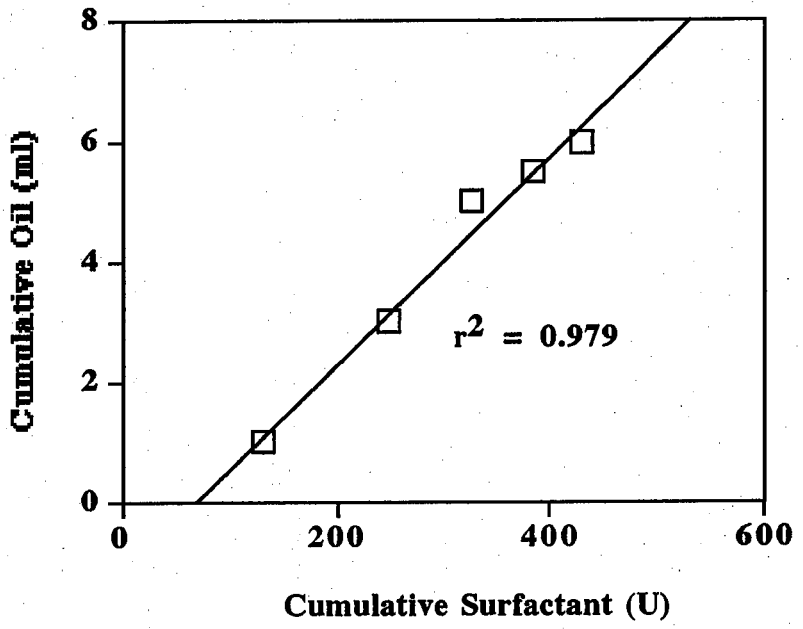

Figure 1. Correlation between the cumulative amount of oil recovered and the cumulative amount of biosurfactant produced in Core 4.

Conclusion

Our data show that the main mechanism for oil recovery by *B. licheniformis* strain JF-2 is biosurfactant production. Oil recovery is lost when the ability to produce the biosurfactant is lost. We also show that there is a strong correlation between the amount of biosurfactant produced by *B. licheniformis* strain JF-2 and the amount of oil recovered. Thus, factors that lead to greater production of the biosurfactant will result in increased oil recovery. We are currently studying the nutritional and genetic factors the control growth and biosurfactant production by *B. licheniformis* strain JF-2. This information will result in more cost effective methods to recover additional oil.

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