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MICROBIAL ENHANCED OIL RECOVERY RESEARCH

Quarterly Report for the Period
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SUMMARY OF TECHNICAL PROGRESS

This quarterly report documents progress made in the period between October 1 and December 31, 1989.

PRODUCTION OF BIOSURFACTANT BY *BACILLUS LICHENIFORMIS* JF-2

In the previous quarterly report we described the criteria for selecting a microorganism for Microbial Enhanced Oil Recovery studies. After careful consideration we chose *Bacillus licheniformis* JF-2 because of its ability to withstand reservoir conditions and the production of a surface active lipopeptide. A preliminary study on the effect of growth conditions (temperature, pH, carbon source, salinity) has been reported previously. For these experiments the cultures were grown aerobically in shake flasks or in anaerobic test tubes. Once the proper range of cultivation conditions had been determined, further experiments were conducted to obtain the relationship between important process variables such as: i) biomass accumulation, ii) interfacial tension decrease, iii) glucose consumption and iv) accumulation of metabolic byproducts (which may act as co-surfactants or inhibit growth. However, in aerobic shake flasks, the dissolved O_2 concentration varies with time and the cells become oxygen limited for at least part of the fermentation. Similarly, experiments in anaerobic test tubes do not allow periodic sampling for glucose or biosurfactant measurement. For these reasons, detailed experiments had to be conducted in stirred tank fermenters equipped with pH control and constant sparging of air or, in the case of anaerobic experiments, O_2-free nitrogen. The effect of temperature and pH on biomass production, glucose consumption and interfacial tension against decane were determined for both aerobic and anaerobic conditions.

MATERIALS AND METHODS

Batch fermentations were carried out in a 2-liter Multigen fermentor (New Brunswick Scientific Co., New Brunswick, NJ). The working volume was 1 liter. The fermentor was inoculated with 25 ml of fresh culture. *Bacillus licheniformis* JF-2 was grown in medium E (described in previous quarterly report) with glucose instead of sucrose. The pH was kept constant by periodic addition of 1 M NaOH or 1 M HCl using an external pH controller. Anaerobic experiments were conducted in medium E supplemented with 1% NaNO3. Oxygen-free nitrogen was sparged throughout the fermentation to ensure anaerobic conditions.

Bacterial concentration was followed by periodically taking a sample (typically every 30-60 min) and measuring the optical density at 600 nm. The interfacial tension of the growth medium against decane was measured by the spinning-drop technique. The minimum interfacial tension was determined by periodically taking samples of the culture broth (usually every hour), centrifuging at 8000g for 10 min to pellet the cells and measuring the interfacial tension of the supernatant against decane. The supernatants after centrifugation of the cells were kept at -70 °C. Glucose concentration was determined by a Yellow Springs Instruments enzymatic glucose analyzer or, for some experiments by Anthrone's method using Anthrone's reagent for determination of carbohydrates (Sigma Chemical Company, St Louis, MO).

RESULTS AND DISCUSSION

Aerobic fermentations

A typical fermentation profile is shown in Figure 1. A lag phase of approximately 4-5 hours is observed before the beginning of exponential growth. A final bacterial concentration of 1 g/L is obtained before growth stopped and the optical density began to decrease. At T= 45 °C, 1% glucose as the carbon source and 4.5% NaCl, the doubling time was 90 min. Therefore, the

growth pattern is similar to that obtained in shake flasks. It can be seen in Figure 1 that the reduction in interfacial tension (IFT) occurs during exponential growth. It remains stable during early stationary phase and then increases. This increase in IFT may be caused either by the release of cellular components in the medium which cause higher IFT's or by inactivation of the surfactant (e.g. degradation). Glucose is completely utilized during the exponential phase as seen in Figure 2. The growth yield of biomass on glucose for the fermentation in Figure 1 is shown in Figure 2. The yield values lie in the range between 0.05 and 0.13 g cell dry weight/g glucose and are low compared to the yield obtained with *E. coli* or *B. subtilis* (e.g. with the values reported by Snay et al., Biotechnology Progress 5:63 1989). Since glucose was completely exhausted at the end of the exponential phase, the low yield values are not likely to result from limitations in some other nutrient. Inhibition of growth due to the accumulation of metabolic byproducts can also be ruled out since the cells were able to assimilate all the available carbon source. A likely explanation for the observed low bio-mass yield is that most of the carbon source is diverted for the production of metabolic byproducts. It is also interesting to note that higher final yields were obtained at lower temperatures: a final yield of 0.125 mg cells/mg glucose consumed was obtained at 30 ° C whereas a value of 0.028 was obtained at 53 °C.

The effects of the growth medium pH on the minimum interfacial tension of the growth medium against decane were studied. The effect of growth medium pH is shown in Table 1. The large increase in interfacial tension as the pH is raised from 7 to 8 is probably due to a decrease in surfactant production since basic pH has little effect on interfacial tension. The effect of growth temperature on the minimum IFT is shown in Figure 3. The minimum interfacial tension is obtained within the temperature range of 45 °C and 50 °C. Overall, the interfacial tension of the fermentation broth exhibits a relatively small variation with temperature in the 30-50 °C. However, as the temperature is raised to 53 °C, a large increase in the interfacial tension is observed and is accompanied by severe decreases in yield and biomass production (not shown). The increase in IFT is not due to surfactant deactivation since the surfactant is stable at temperatures up to 100 °C.

Anaerobic fermentations

The time profile for the growth of *Bacillus licheniformis* JF-2 under anaerobic conditions, 5% NaCl and T=45 °C is shown in Figure 4. The fermentation is characterized by a very long lag phase, typically 2-3 days before the onset of growth. The long lag is consistent with, and explains some earlier results of MEOR experiments in cores. In that work it was found that growth and oil recovery were evident seven days after incubation, apparently due to the large lag before the onset of Biomass increase.

Further experiments showed that the lag phase could be reduced to 10-20 hours by starting the fermentation with acclimated cells grown under anaerobic conditions. Typical bacterial concentrations of 0.8 mg/ml were obtained before growth ceased and the optical density began to decrease. A typical doubling time of 5 hours was observed. Addition of 0.1 % yeast extract in the growth medium did not have an effect on the doubling time of the organism. Growth in the fermenter was slow compared to test tubes (see previous quarterly report), possibly because the cells may be susceptible to liquid shear.

It can be seen in Figure 4 that the reduction in interfacial tension occurs during exponential growth. It remains stable during early stationary phase and then increases giving a profile very similar to that of the aerobic fermentations. The surfactant is also either degraded or affected by the release of cellular components in the medium. As shown in Figure 5, glucose is not completely utilized during anaerobic fermentation of *B. licheniformis* JF-2. Typically, between 40 to 95 % of the glucose is used depending on the composition of the growth medium. The yield of biomass on glucose consumed does not vary much with time. Values around 0.1 mg cells/mg glucose consumed were calculated.

The effect of temperature on the minimum interfacial tension and final bacterial concentration is shown in Table 2. As the temperature is decreased to 30 ° C or increased to 50 °C,

a drastic increase in minimum interfacial tension is observed. The final bacterial concentrations values show that at 30ºC, the cells grew well but little surfactant was produced whereas at 50ºC, the high interfacial tension value can be attributed to very poor growth.

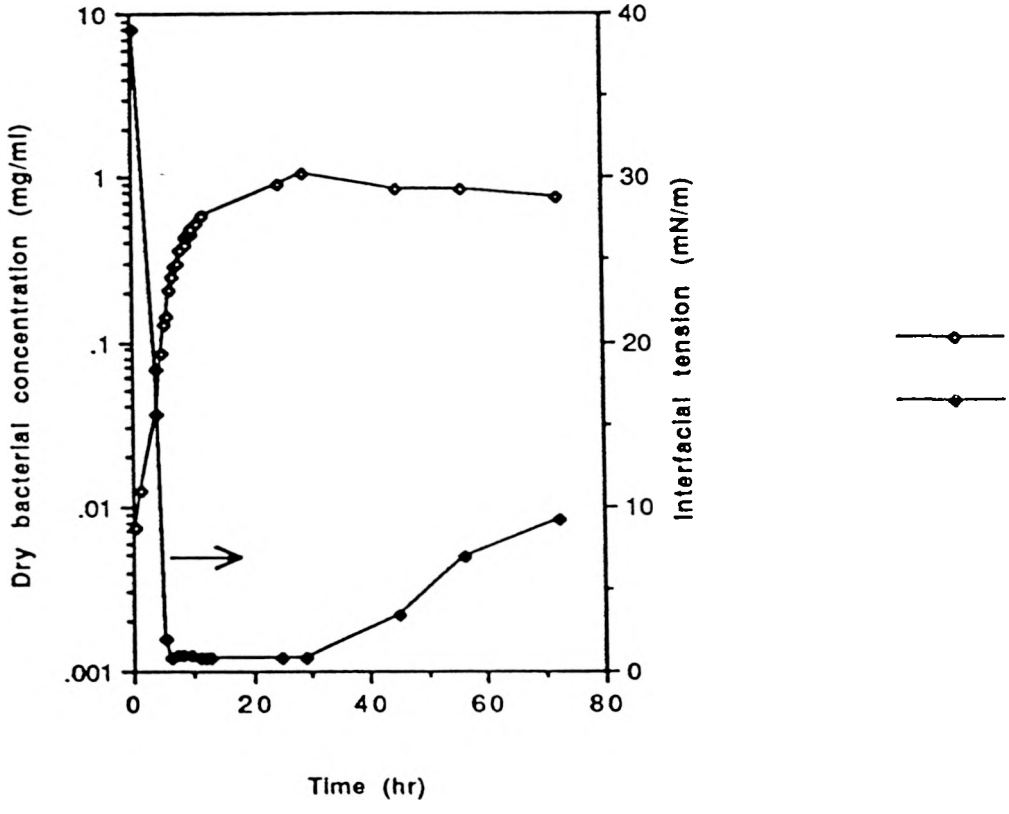

Figure 1: Typical fermentation profile of Bacillus licheniformis JF-2 grown in a 1-liter batch fermentor under aerobic conditions at constant pH. B. licheniformis JF-2 was grown in medium E supplemented with 1 % glucose and 5 % NaCl. The temperature was kept constant at 45 ºC.

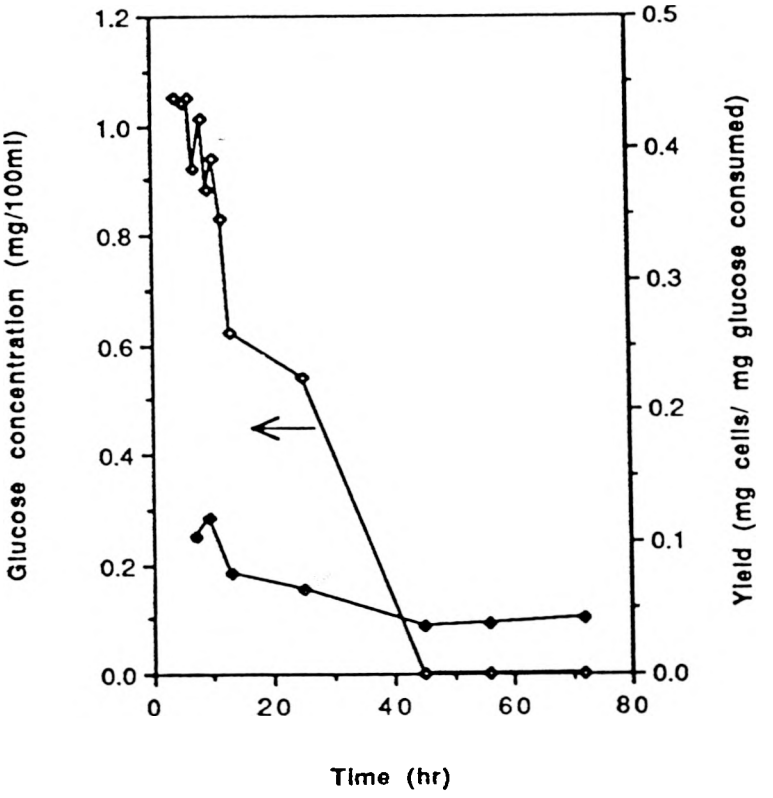

Figure 2: Glucose concentration and yield of biomass on glucose consumed during a batch fermentation of B. licheniformis JF-2 under aerobic conditions. The growth medium was medium E supplemented with 1 % glucose and 5 % NaCl. The temperature was kept constant at 45 ºC

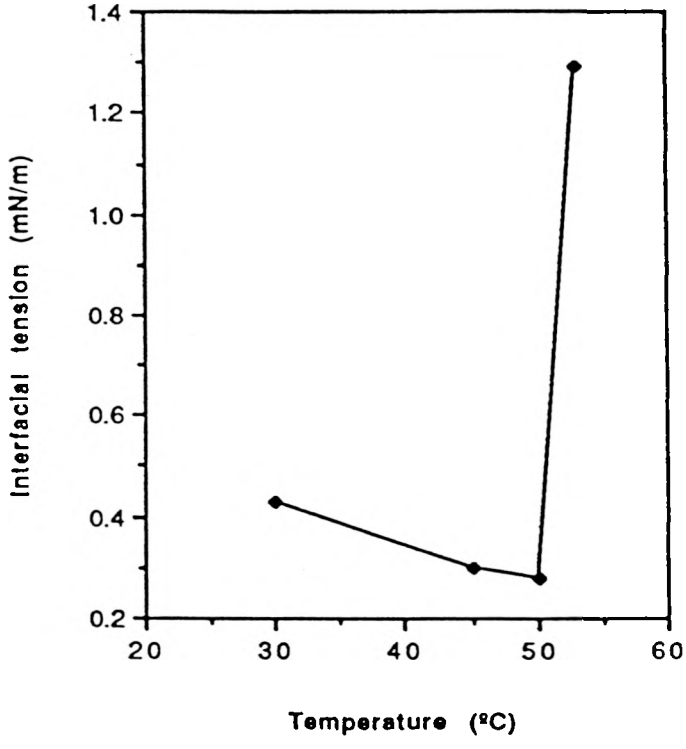

Figure 3: Minimal interfacial tension obtained during an aerobic batch fermentation of Bacillus licheniformis JF-2 as a function of temperature. The organism was grown in medium E supplemented with 1 % glucose and 5 % NaCl.

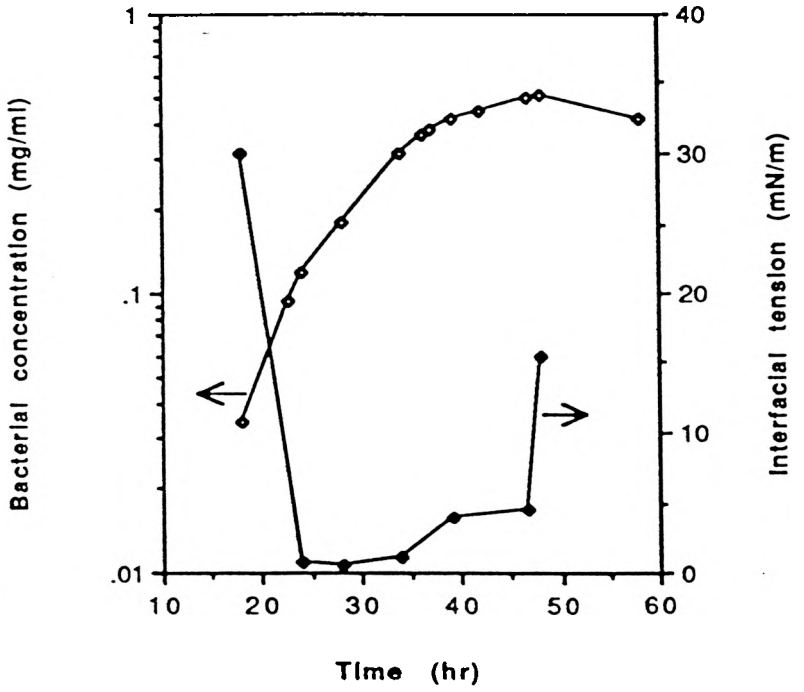

Figure 4: Typical fermentation profile of Bacillus licheniformis JF-2 grown in a 1-liter batch fermentor under anaerobic conditions at constant pH in medium E supplemented with 1 % glucose, 5 % NaCl and 1 % $NaNO_3$. The temperature was kept constant at 45 ºC.

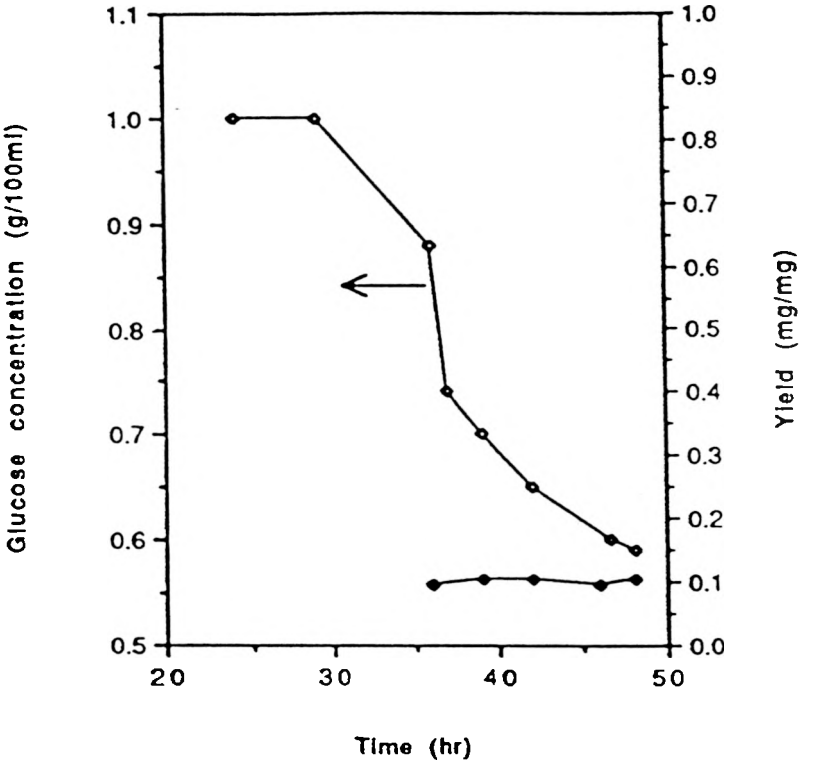

Figure 5: Glucose concentration and yield of biomass on glucose consumed during a batch fermentation of B. licheniformis JF-2 under anaerobic conditions at 45 ºC. The organism was grown in medium E supplemented with 1 % glucose, 5 % NaCl and 1 % $NaNO_3$.

pH	interfacial tension (mN/m)
6	0.50
7	0.30
8	3.65

Table 1: Minimal interfacial tension during an aerobic batch fermentation of B. licheniformis JF-2 as a function of the pH of the growth medium.

T (°C)	Minimum interfacial tension (mN/m)	Final bacterial concentration (mg/ml)
30	5.81	0.71
45	0.51	0.83
50	8.06	0.20

Table 2: Minimal interfacial tension and final bacterial concentration obtained during an anaerobic batch fermentation of B. licheniformis JF-2 as a function of the temperature.