DOE/BC/15113-3

(OSTI ID: 807189)

Development of more effective biosurfactants for enhanced oil recovery

Semi-Annual Report

Report Start Date: October 1, 2000

Report End Date: March 31, 2001

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Date of Report: April 30, 2001

DE-AC26-98BC15113

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ABSTRACT

Bacillus mojavensis JF-2 (JF-2) grew and synthesized biosurfactant under anaerobic conditions in sand-pack columns in the presence of other competing organisms. The surface tension of the column effluent was 38 mN/m when the columns were injected with an inoculum size of 10^8 cells/ml. A slightly higher surface tension (44 mN/m) was obtained when the inoculum size was 10^4 cells/ml. Growth with glucose gave the fastest growth rate for *Bacillus mojavensis* JF-2 (JF-2) and monosaccharides in general were the preferred carbon source. The fastest growth rate occurred with a glucose concentration of 77 mM. Fructose appeared to produce the highest growth yield. The sample preparation steps used for high performance liquid chromatography (HPLC) analysis resulted in a biosurfactant recovery (from culture supernatant) of about 70%. The presence of Proteose peptone #2 (PP2) in the medium led to increased biosurfactant production under anaerobic conditions. The biosurfactant was present after extended incubation suggesting that production of biosurfactant was not confined to the exponential phase of growth under anaerobic conditions. Although it is not known exactly when maximum production of biosurfactant occurred, the data suggests that biosurfactant production occurred during stationary phase since maximal growth was reached by 48 hrs.

EXECUTIVE SUMMARY

The overall goal of this research is to develop effective biosurfactant production for enhanced oil recovery in the United States. Our current research addressed this goal in two manners. One, by studying the growth and biosurfactant production of *Bacillus mojavensis* JF-2 (JF-2) in sand packed columns and two, by optimizing the nutrient formulation of the medium. We found that JF-2 grew and synthesized biosurfactant under anaerobic conditions in sand-pack columns in the presence of other competing organisms. We also determined that an inoculum size (10^8 cells/ml) is best for biosurfactant production in the sand columns. Growth with glucose gave the fastest growth rate for *Bacillus mojavensis* JF-2 (JF-2) and monosaccharides in general were the preferred carbon source. The presence of Proteose peptone #2 (PP2) in the medium led to increased biosurfactant production under anaerobic conditions. The biosurfactant was present after extended incubation suggesting that production of biosurfactant was not confined to the exponential phase of growth under anaerobic conditions. We now know the type of nutrients and inoculum size needed to produce biosurfactants effectively in systems that mimic actual reservoir conditions.

INTRODUCTION

Surfactants are surface active agents that contain both hydrophilic and hydrophobic components. As a result, they can be useful for enhanced oil recovery (EOR). Conventional production technologies are only able to recover approximately 30 to 50% of oil originally in place. The target of EOR is to increase oil reserves by improving oil recovery. However the large capital or high chemical/energy cost of current EOR technologies limits their application. An alternative technology to improve oil recovery is to use microorganisms, called microbially enhanced oil recovery (MEOR).

MEOR capitalizes on naturally occurring substances and processes; and, since environmental compatibility is becoming an increasingly important factor in the selection of industrial chemicals, MEOR processes could result in both economical and environmentally friendly methods. However, biosurfactants are not a currently feasible alternative to chemically synthesized surfactants as a result of the potentially high production costs.

The purpose of this work is to increase biosurfactant production by *Bacillus mojavensis* JF-2 by understanding growth and biosurfactant production in the sand packed columns and by optimizing the nutrient formulation.

Chapter 1

Bacillus mojavensis JF-2 growth and biosurfactant synthesis under anaerobic conditions in sand-pack columns.

Abstract

Bacillus mojavensis JF-2 (JF-2) grew and synthesized biosurfactant under anaerobic conditions in sand-pack columns. Biosurfactant production was confirmed using surface tension measurements. The surface tension of the column effluent was 38 mN/m when the columns were injected with an inoculum size of 10^8 cells/ml. A slightly higher surface tension (44 mN/m) was obtained when the inoculum size was 10^4 cells/ml. However, JF-2 was not able to grow in the presence of the particular crude oil used in this experiment.

Introduction

Oil is an essential energy source and continued economic growth increases the demand for oil. Conventional production technologies are able to recover approximately 30 to 50% of oil originally in place (2). The target of enhanced oil recovery (EOR) is to increase oil reserves by improving oil recovery. However the large capital or high chemical/energy cost of current EOR technologies limits their application (1). An alternative technology to improve oil recovery is to use microorganisms, called microbially enhanced oil recovery (MEOR).

It has been shown that MEOR methods hold promise as an economical alternative to conventional EOR process. MEOR capitalizes on naturally occurring substances and processes; and, since environmental compatibility is becoming an increasingly important factor in the selection of industrial chemicals, MEOR processes could result in both economical and environmentally friendly

methods. With further research, it may be possible to produce large amounts of useful products such as biosurfactants from inexpensive and renewable resources.

There are a number of ways in which microorganisms may affect the mobilization of oil within reservoirs (10). The accumulation of biomass and polymers synthesized by the microorganisms themselves can plug the most permeable regions redirecting the recovery fluid to previously bypassed zones. The production of polymers can increase the viscosity of the injected fluids and thus positively affect the mobility rates. The capillary number may be increased by a reduction in interfacial tension due to surfactant production. The gas produced by the microorganisms can cause a local repressurization within the reservoir. The production of acids will dissolve the limestone matrices and improve oil recovery by changing the porosity. JF-2 is able to synthesize a biosurfactant significantly decreases both the surface tension and the interfacial tension between oil and water and it is potentially useful in enhanced oil recovery (4, 5, 9).

In previous experiments, metabolic indicators (such as the loss of glucose, and/or the production of metabolic byproducts) were not detected in the column effluent of sand-pack columns saturated with oil and inoculated with JF-2 (see previous report). While some glucose loss was detected, it was not possible to associate this loss with microbial growth. No viable bacteria were detected in the effluent. In an effort to clarify the reasons for this, several hypothesis were tested:

- JF-2 is not able to grow in a sand environment due to a contact inhibition with the sand.

- The sand contains some competing microorganisms which prevent JF-2 from growing.

- JF-2 growth is inhibited by the presence of particular crude oil that was used.

The purpose of the following studies was to test these hypothesis to determine what factor was responsible for the inhibition of JF-2 growth. First we determined if JF-2 was able to grow in the sand-pack environment under anaerobic conditions by measuring metabolic by-products synthesized from glucose and by plating the effluent onto medium to see if JF-2 was present. Secondly, we determined if the presence of this particular crude oil was inhibitory to JF-2.

Materials and Methods.

Microorganisms and growth conditions

Bacillus mojavensis JF-2 (ATCC # 39307) was grown anaerobically in medium E. Medium E contained (per liter): 2.7g KH_2PO_4, 13.9 K_2HPO_4, 50g NaCl, 10g glucose, 1 g yeast extract, 1 g $NaNO_3$, 1 g $(NH_4)_2SO_4$, 30 g Proteose Peptone #2, and 10 ml of a metal solution. The metal solution (a modified Wolin's trace metal solution) contained: 1 g EDTA, 3 g $MnSO_4 \cdot H_2O$, 0.1 g $FeSO_4 \cdot 7H_2O$, 0.1 g $CaCl_2 \cdot 2H_2O$, 0.1 g $CoCl_2 \cdot 2H_2O$, 0.1 g $ZnSO_4 \cdot 2H_2O$, 0.01 g $CuSO_4 \cdot 5H_2O$, 0.01 g AlK $(SO_4)_2$, 0.01 g H_3BO_3, 0.01 g $Na_2MoO_4.7H_2O$, 25 g $MgSO_4$. Bacterial enumeration was performed by plating onto medium E containing 1.5 g/l of agar. The serial dilution was performed using a sterilized solution containing 2.7 g KH_2PO_4, 13.9 g K_2HPO_4, and 50 g NaCl (pH of 6.8).

Sand-pack preparation

Plexiglass columns were filled with sand (quartz with a density of 2.65 kg/l) and packed with continuous vibrations to ensure a homogenous packing density and avoid the formation of layers. The columns were sealed with plastic stoppers and a butyl rubber septum was used to allow aseptic and anaerobic additions to the columns and to maintain anaerobic conditions. The ends were capped by using polypropylene filters with a pore size of 20 µm.

Each column was weighed before and after packing with sand. It will give the sand weight. Knowing the density of sand, the pore volume was determined by calculated the difference between the column volume and the sand volume. The pore volume was also determined by weighing the columns before and after the brine flooding. From the brine density (ρ_b= 1.05196), and the difference in weight before and after the flooding, the liquid pore volume was calculated. These two techniques gave similar estimates for the pore volume that differed by only 1-1.4% (Table 1). To ensure anaerobic conditions, the columns were flushed 3 times with nitrogen gas and then placed in an anaerobic chamber for 24 hours.

Brine saturation

The columns were injected with brine (degassed nanopure water with 5% NaCl using positive pressure of the fluid reservoir to push fluid into the columns. Two pore volumes were injected through the columns to saturate them and remove the gas trapped inside.

Columns treatments

A 200 ml solution of medium E inoculated with JF-2 was incubated under anaerobic conditions at 40^{o}C for 20 hours. The surface tension was 30 mN/m. The cell concentration was determined using a Petroff Hausser counting chamber (1/400 square mm, 1/50 mm deep) (3). The cells were pelleted by centrifugation and resuspended in sterile medium E to give a cell concentration of 10^4 or 10^8 cells/ml.

Two columns (1 and 2) were treated with one pore volume of medium E which contained a cell concentration of 10^4 cells/ml. A third column (3) was treated with one pore volume of medium E containing a cell concentration of 10^8 cells/ml. A fourth column (4) was treated with sterile medium E and served as the control. Each column was flooded by using a positive pressure of nitrogen gas to push he liquid into the column. The sand-packs were incubated at 40^{o}C.

Pressure measurements

The pressure inside the columns was measured by using a Cole-Parmer digital gauge. JF-2 produces CO_2 and N_2 as end product of its metabolism, so gas production was used as an indicator of in situ growth (Table 2). When the rate of increase in the gas pressure stopped, the columns were flooded with two pore volumes of brine, and samples were collected into 30 ml syringes. The effluent was collected in 20 ml samples. The liquid effluent was frozen until subsequent analysis.

JF-2 growth in the presence of crude oil

Serum bottles were filled with 50 g of sand and flushed with nitrogen for 5 minutes to ensure anaerobic conditions. Each bottle received 30 ml of medium E and 1 ml of oil. The bottles were inoculated with JF-2 and incubated for 20 hours at 40^{o}C. The positive controls consisted of inoculating medium E with JF-2 in the absence of sand and oil. The negative control consisted of an uninoculated medium with sand and oil. The sand was previously sterilized at 120^{o}C during 20 minutes. The experiment was done in triplicate

Balch tubes containing 20 ml of medium E supplemented with 1 ml of crude oil were inoculated with JF-2. The oil was previously sterilized at 120^{o}C during 20 minutes. The positive control consisted of tubes without oil. The tubes were all kept under anaerobic conditions. This was performed in triplicate.

Colony Hybridization

Cells were transferred to a nylon membrane from a petri dish by placing the nylon membrane on the surface of the petri dish. The cells were then lysed by placing the membrane in a solution of 0.5 N of NaOH, and allowed to stand for 10 minutes at room temperature. The membrane was transferred first to a filter paper soaked with 0.5 M Tris HCl (pH 7.5) for 5 minutes, then to a filter paper soaked

with a solution containing 0.5 M Tris HCl (pH 7.5) and 1.5 M NaCl for 5 minutes, and finally transferred to a filter paper soaked with 2xSSC solution (20xSSC solution is 173.3 g of NaCl and 88.2 g of sodium citrate per liter at a pH 7.8). To immobilize the DNA onto the membrane, the membrane was baked under vacuum for 2 hours at 80^{o}C.

The membrane was placed in a hybridization glass tube (Fisher Biotech) containing 20 ml prehybridization solution (DIG Eazy Hyb, Boehringer Mannheim) per 100 cm^2 of membrane surface area, and incubated in Hybridization Incubator (Fisher Biotech) at 37-42°C for 2 hours. The prehybridization solution was discarded and the prehybridization solution containing the labeled probe srfA1 (5-25 ng/ml) was added, and incubated in Hybridization Incubator at 37-42°C overnight. At the end of the hybridization, the hybridization solution was poured into a tube. The membrane was washed in 2x washing solution (300 mM NaCl, 30 mM sodium citrate, 0.1% SDS, pH 7.0) at room temperature for 10 min. The membrane was washed again in 0.5x washing solution (75 mM NaCl, 7.5 mM sodium citrate, 0.1% SDS, pH 7.0) at 68°C for 30 min.

After hybridization and post-hybridization washes, the membrane was equilibrated in washing buffer (100 mM maleic acid, 150 mM NaCl; pH 7.5; 0.3% (v/v) Tween®20) for 1 minute. The membrane was blocked by gently agitating it in blocking solution (100 mM maleic acid, 150 mM NaCl; pH 7.5; 1% (w/v) Blocking reagent [Boehringer Mannheim]) for 30-60 minutes. The blocking solution was removed and the membrane was incubated in the antibody solution (the Anti-Digoxigenin-AP in blocking solution [1:100000 v/v], Boehringer Mannheim) for 30 minutes. After the antibody solution was discarded, the membrane was washed in washing buffer for 30 minutes. The washing buffer was removed and the membrane was equilibrated in detection buffer (100 mM Tris-HCl, 100 mM NaCl; pH 9.5) for two minutes. The membrane was placed between

two sheets of acetate (PhotoGene Development Folders, Gibco BRL) and 0.5 ml (per 100 cm^2) of the Chemiluminescent substrate (CSPD® 1:100 in detection buffer, Boehringer Mannheim) was then added on top of the membrane, scattering the drops over the surface of the membrane. With a damp tissue, the top sheet of plastic was wiped gently to remove any bubbles present under the sheet and to create a liquid seal around the membrane. The filter was incubated for 5 minutes. The semi-dry membranes were sealed in acetate sheets. The membrane was incubated at 37°C for 15 minutes. For detection of the Chemiluminescent signal, the membrane was exposed to Lumi-Film (Boehringer Mannheim) for 15-20 minutes.

Analytical measurements

The surface tension was measured using a Fisher Tensiometer model 215. The surface tension of nanopure water was measured as a standard (73 mN/m). All the samples were measured at room temperature.

The concentration of glucose was measured by phenol sulfuric method (3). The absorbance was read at 488 nm against the blank prepared without glucose. The concentrations of glucose were determined in the samples from a standard curve prepared by plotting the absorbance of standards versus the concentration of glucose.

Nitrate concentrations were determined by using a Dionex Ion Chromatography system with an AS4A-SC 4-mm particle-size column, a model CD 20 conductivity detector, and a mobile phase of 1.8 mM sodium carbonate and 1.7 mM sodium bicarbonate delivered at 2ml/min.

Acetate and butanediol concentrations were determined using a gas chromatography equipped with a flame ionization detector and a glass column. The samples as well as the standards were diluted in a solution of 30 mM of oxalic acid.

The concentration of biosurfactant in the samples was quantified by HPLC analysis. A C18 column was used with a mobile phase of 70% methanol and 30% of 10 mM phosphate buffer. The HPLC was run at a flow rate of 1 ml/min and the injection volume was 20 µl. A UV detector was used with a wavelength set at 210 nm. Sample preparation is described in the previous chapter of this report.

Results and Discussion

Sand-pack experiments

The first experiment was conducted to determine if JF-2 was able to grow and produce biosurfactant in a sand environment under anaerobic conditions. It was also important to determine if inoculum size influenced biosurfactant production. The columns were inoculated with two different cell concentrations of JF-2: two columns (1 and 2) were treated with a cell concentration of 10^4 cells/ml and a third column (3) was treated with a cell concentration of 10^8 cells/ml. The fourth column was treated with uninoculated medium E and served as a control. After two days of incubation, there was not any further increase in pressure within the columns (Table 2). After 5 days of incubation, the columns were flooded with brine. The first column was allowed to incubate for a longer period of time (11 days) in order to determine if a longer incubation time was necessary for biosurfactant production.

The surface tension measured in the two first columns was reduced to 44 mN/m, which indicated the production of biosurfactant. The surface tension of the third column where the inoculum size was higher, 10^8 cells/ml reached a value of 38 mN/m. HPLC analysis was not able to detect the presence of biosurfactant. Significant depletion of glucose and nitrate was observed in all the columns indicating metabolic activity. This suggested that JF-2 grew and metabolized its substrates inside the sand-pack columns. The end -product analysis revealed the production of acetate and butanediol in all the columns, even in the uninoculated

column (Table 3), potentially due to contamination. The metabolic activity in column 4 was probably due to organisms present in the sand grains.
The effluent from each column was plated onto medium E to determine the viable cell concentration in the effluent of each column. The effluent cell concentration was $11.5x10^4$, $8x10^4$, $15x10^5$ and $16.4x10^3$ cells/ml for columns 1, 2, 3 and 4, respectively. The cells were transferred onto a membrane and lysed to expose the DNA and to hybridize it with a probe corresponding to one of the genes involved in the biosynthesis of biosurfactant to determine if JF-2 was growing inside the columns. The concentraiton of JF-2 in the column effluent was $8.5\ x10^3$ for the column 1, $5\ x\ 10^3$ for the column 2, $8\ x\ 10^4$ for column 3 and $4\ x\ 10^2$ for column 4. This showed that JF-2 represented about 2 to 7% of the viable cell population and the presence of this other bacteria did not prevent JF-2 from growing and synthesizing its biosurfactant. The surface tension in the fourth column did not decrease and the number of JF-2 cells observed was very low. These data suggest that the cell concentration is important for biosurfactant synthesis and that biosurfactant production depends on the presence of JF-2.

Previous work (under aerobic conditions) showed that biosurfactant synthesis in JF-2 occurs during the exponential phase of growth and when cells reach the stationary phase the biosurfactant concentration starts to decrease until it completely disappears from the culture broth (5, 7, 8). However as shown with the first column, which was incubated for 11 days, the surface tension was the same as that found in the column incubated for only 5 days of incubation. The anaerobic conditions may prevent the internalization of the biosurfactant molecules by the microorganisms.

The spatial distribution of metabolic end-products such as glucose, nitrate, acetate and butanediol was examined to determine if these products are uniformly present or present along a gradient within the column. The columns have a total liquid volume of about 68 ml. During the brine flood, the effluent was collected in

3 separate samples (20 ml), each corresponding to about a third of the liquid volume of the column. The analyses showed that the distribution of products along the column decreased slightly (Table 3). For example, in column 1, the first sample had a glucose concentration of 2.9 mM and the second sample had a glucose concentration of 2.5 mM. Similarly, the acetate concentration at the first sample was 17.4 mM and 12.8 mM in the second sample. The butanediol concentration was 7.6 mM and 6.1 mM respectively. These data suggest uniform growth of JF-2 throughout the column.

JF-2 growth in the presence of crude oil

From the data given above, it was determined that the presence of sand or competing microorganisms did not inhibit JF-2 growth. So, the second experiment was conducted to determine if the particular oil used in the experiment was actually responsible for inhibiting growth of JF-2. As JF-2 was isolated from oil-field injection brine (5,7) it was evident that JF-2 was able to grow in the presence of crude oil. However, in this case, the oil in use did appear to inhibit growth of JF-2. Each time oil was added to the medium inoculated with cells, no bacterial growth occurred and no metabolic activity was detected since neither glucose nor nitrate were depleted (Table 4). Since no growth was detected in bottles using sterilized sand and oil, the inhibition was not due to competition from other microorganisms. This data confirmed that that particular crude oil used in the sand packs inhibited JF-2 growth.

Conclusion

JF-2 is able to grow under anaerobic conditions in a sand environment and produce its biosurfactant. These qualities make it suitable for understanding the MEOR processes in laboratory sand-pack columns. It is able to synthesize its biosurfactant at a detectable level if the inoculum size is above 10^8 cells/ml. However the experiment revealed that the particular crude oil used in our

experiment inhibited JF-2 from growing, and this phenomenon was not due to a competing microorganism. However, other crude oils can be used which are not inhibitory.

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Table 1. Petrophysical data for the sand-packed columns.

Column #	Weight of sand (g)	Volume of sand (cm^3)	Pore volume (cm^3)	Weight of brine (g)	Pore volume (cm^3)
1	357.02	134.7245	67.1	71.1	67.6
2	358.98	135.4642	66.3	70.5	67.0
3	354.93	133.9358	67.9	72.2	68.6
4	353.29	133.4260	68.4	72.9	69.3

Table 2. Change in pressure in sand-packed columns after inoculation.

Column #	Time				
	20 hours	48 hours	4 days	5 days	11 days
1	3.6	7.1	6.4	6.4	6.2
2	3.5	9.6	8.5	8.0	
3	8.7	11.7	11.3	10.6	
4	1.7	7.4	5.2	5.2	

Table 3. Substrate consumption and product formation after growth of JF-2 in sand-packed columns.[a]

Column #	Effluent Fraction	Surface Tension (mN/m)	Glucose (mM)	Nitrate (mM)	Acetate (mM)	Butanediol (mM)
1	First	44	2.9	ND	17.4	7.6
	Middle	47	2.5	ND	12.8	6.1
2	First	44	4.8	ND	18.4	5.9
	Middle	48	3.4	ND	16.5	5.5
3	First	38	3.3	ND	17.6	9.0
	Middle	41	3.3	ND	18.8	8.4
4	First	60	4.2	ND	16.9	4.9
	Middle	58	2.8	ND	13.3	3.0

[a]Initial glucose concentrations were 59 to 64 mM and initial nitrate concentration was 15 to 16 mM.

Table 4. Effect of sand, oil and competing organisms on the metabolic activity of JF-2.

Treatment	Glucose concentration (mM)		Nitrate concentration (mM)	
	Initial	Final	Initial	Final
JF-2	53.7	20	13.8	0.5
JF-2 + oil	53.5	51	14.7	13.8
JF-2 + sand	53.6	18	14.5	1.3
JF-2 + sand and oil	52	53	14.7	13.8
JF-2 + sterile sand	51.3	26	14.9	2.8
JF-2 + sterile sand and oil	51	54	13	15
Sand + oil	55	48	16	15.7

CHAPTER 2

Anaerobic Growth and biosurfactant production of *JF-2*

Abstract

Growth with glucose gave the fastest growth rate for *Bacillus mojavensis* JF-2 (JF-2) and monosaccharides in general were the preferred carbon source. The fastest growth rate occurred with a glucose concentration of 77 mM. Fructose appeared to produce the highest growth yield. The sample preparation steps used for high performance liquid chromatography (HPLC) analysis resulted in a biosurfactant recovery (from culture supernatant) of about 70%. The presence of Proteose peptone #2 (PP2) in the medium led to increased biosurfactant production under anaerobic conditions. The biosurfactant was present after extended incubation suggesting that production of biosurfactant was not confined to the exponential phase of growth under anaerobic conditions. Both surface tension reduction data and HPLC analysis failed to detect significant amounts of biosurfactant during exponential growth. However, after 18 days, a significant amount was detected in the culture supernatant of medium containing Proteose peptone. Although it is not known exactly when maximum production of biosurfactant occurred, the data suggests that biosurfactant production occurred during stationary phase since maximal growth was reached by 48 hrs.

Introduction

Javahari *et. al*, (1), reported both the successful growth of, and biosurfactant production by, JF-2 under anaerobic conditions. This biosurfactant effectively reduces both surface tension and interfacial tension and is potentially useful in enhanced oil recovery (2,3,4). Consequently, it is important to

understand the factors that affect the production of biosurfactant. To do so, first requires the improvement of growth since biosurfactant production is linked to growth (1,2,5). The purpose of the following study was to enhance anaerobic growth of JF-2 (ATCC 39307), with the ultimate goal of improving biosurfactant production under anaerobic conditions.

Previously we reported that the addition of Proteose peptone improved the growth of JF-2 under anaerobic conditions. A method for quantifying biosurfactant using HPLC was also developed. With the HPLC method and with surface tension measurements, it was now possible to determine when biosurfactant is produced and the effect of medium additions on biosurfactant production. Although it had not been possible at this time to exactly determine when biosurfactant is maximally produced, the effect of some medium additions on biosurfactant production has been determined.

Methods and Materials

Medium and Solutions.

A modified medium E (ME_2) was used. ME_2 contained the following components per 900 mls: TES buffer (N-tris(hydroxymethyl)methyl–2-aminoethansulfonic acid) (22.9g); sodium chloride (50g); sucrose (10g); yeast extract (1g); sodium nitrate (1g); dibasic potassium phosphate (1.0g); ammonium sulfate (1g); magnesium sulfate (0.25g) and 100 mls of a metal solution. The metal solution was a modification of Wolins metal solution (6) and was contained the following components per liter: EDTA (1g); $MnSO_4 \cdot H_2O$ (3g); $FeSO_4 \cdot 7H_2O$ (0.1g); $CaCl_2 \cdot 2H_2O$ (0.1g); $CoCl_2 \cdot 2H_2O$ (0.1g); $ZnSO_4 \cdot 7H_2O$ (0.1g); $CuSO_4 \cdot 7H_2O$ (0.01g); H_3BO_4 (0.01g); $Na_2MO_4 \cdot 2H_2O$ (0.01g); $AlK(SO_4)_2$ (0.01g). ME_2 also contained 3% Proteose peptone #2. Cystein hydrochloride was added in the concentration of 0.025% (wt/vol).

Various concentrations of glucose and other sources of carbohydrate were prepared at concentration ten times the intended final concentration. To create anaerobic conditions, the sugar solution was boiled under a stream of nitrogen gas and poured into a serum bottle, which was also flushed with nitrogen gas. The headspace was composed of nitrogen. In the case of the corn syrup derivatives (Maltrin), an average molecular weight (MW) of glucose was assumed to determine molarity.

Inoculation Protocol

A serum bottle with 100 ml of anaerobic ME_1 (ME without any additions) was inoculated directly from a freshly grown (24h) plate of *B. mojavensis* strain JF-2. The serum bottle was allowed to incubate for 24 hours at 40^{o}C. From this culture a 5ml inoculum (1%) was used for each serum bottle. A 1% inoculum was used.

Growth

All tubes and serum bottles were incubated at 40^{o}C in a stationary incubator. Growth was measured using a spectrophotometer with the wavelength set at 600 nm. Growth was also measured by dry weight analysis. The sample was centrifuged at 8000 xg. The pellet was re-suspended in 10 ml of 10 mM phosphate buffer and re-centrifuged to remove medium components from the cell pellet. The pellet was then re-suspended in 2 ml of 10mM phosphate buffer and poured into a pre-weighed aluminum pan. An uninoculated sample was treated similarly and the final dry weight of the uninoculated residue was subtracted from the dry weight obtained with the cell pellet. The pans were dried for 6 hrs in a drying oven at 70^{o}C.

Analytical measurements

The surface tension was measured using a De Nuoy ring tensiometer. Three ml of the culture supernatant was allowed to equilibrate at room temperature in small plastic weigh pans before the surface tension was recorded. Nanopure water was used as the high surface tension standard (~ 73 dynes/cm) and Micro-90 detergent was used as the low standard (~ 27 dynes/cm).

The concentration of glucose was measured by phenol sulfuric method (8).

The extraction efficiency was used to determine the degree to which biosurfactant is recovered from the growth medium. This value was used as a correction factor to determine the actual amount present in the medium. Medium was prepared as described above. Surfactin was added in the concentration of 0.5 mg/ml to 60 ml and an identical amount of medium without surfactin served as the control. In each case, the medium was autoclaved and allowed to cool before the addition of surfactin. After 20 min centrifugation at 6000 xg to remove any particulate matter, three 20 ml samples with surfactin were each acidified to a pH of 2.0. Three samples of medium without surfactin were treated similarly. All samples were refrigerated overnight after acidification and then centrifuged as above to collect the precipitate. Each precipitate was extracted by adding two ml of methanol to the precipitate and hand shaking for one minute. A 1.0 ml sample of the methanol solution was centrifuged for 3 min on a micro-centrifuge at 13,000 xg. The remaining volume was stored. Each sample was then analyzed on the HPLC (described below) for the presence of surfactin.

The JF-2 biosurfactant was quantified by high performance liquid chromatography (HPLC). A C18 column was used with a mobile phase of 70% methanol and 30% 10 mM phosphate buffer. The HPLC was run at a flow rate of 1 ml/min and the injection volume was 20 µl. An ultra violet detector was used with the wavelength set at 210 nm. Samples for HPLC analysis were prepared as described above.

Results and Discussion

Growth Experiments

Table 5 shows the growth rates of *B. mojavensis* JF-2 obtained with the addition of various carbon sources to the medium. This data indicate that the use of glucose gave the fastest growth rate under anaerobic conditions. The fastest growth rate occurred when the glucose concentration was 77 mM. However, the growth rate did not change dramatically over a range of 11 to 99 mM glucose. Glucose may not be practical for MEOR fieldwork so other sources of sugar were tested to determine what commercial sources might be useful. Corn syrup derivatives were potential candidates for this purpose and previous experiments have shown that JF-2 would grow anaerobically on corn syrup; Maltrin was the corn syrup derivative used. The use of all three Maltrin types, M180, M200, M250, resulted in slower growth rates than that obtained using glucose as the carbon source. However, two, M180 and M200 gave very slow growth rates, about 10 to 12 times slower than glucose. Maltrin M250 did support a growth rate similar to that of glucose (0.10 opposed to a range of 0.10 to 0.17) M250 has a higher solubility and a higher percentage of monosaccharides than the other two Maltrins. Although all three corn syrups have a similar disaccharide composition (M180 5.8%; M200 7.4%; M250 6.9%) they do differ significantly in the percentage of monosaccharides (M180 1.3%; M200 2.3%; M250 7.6%). The Maltrin experiment suggests a preference for monosaccharide carbon sources over disaccharides or polysaccharides. This might suggest that corn syrup derivative containing a high percentage of monosaccharides would be a good substrate for field applications (M250).

Table 6 shows the relative growth yield (dry weight) for JF-2 when grown on four different sugars under anaerobic conditions. Growth with fructose produced the best growth yield at 73.7 g dry weight/mole substrate. Growth with

glucose was next highest, while growth with sucrose and lactose produced similar growth yields near 31 g dry weight/mole substrate.

Percent Recovery of Biosurfactant

In order to quantify biosurfactant production accurately, it is necessary to concentrate the biosurfactant with an extraction procedure as described in the methods section. However, it is not clear how efficient this procedure is in recovering biosurfactant. This was tested using a known amount of biosurfactant as the standard. A test of extraction efficiency was performed to determine the degree to which biosurfactant is recovered from the growth medium. As can be seen in Table 7, the average percent recovery of surfactin from medium was about 70%. The recovery was very similar in all three samples containing surfactin. Thus this value can be used as a correction factor to quantify the amount of biosurfactant in the medium. (The corrected concentration of biosurfactant will equal the measured concentration divided by 0.70.) This was used as a correction factor to determine the actual amount present in the medium.

The effect of medium additions on biosurfactant production

Along with enhancing growth under anaerobic conditions, the presence of Proteose peptone #2 (PP2) in the medium also led to increased biosurfactant production. The biosurfactant was present after extended incubation suggesting either that the production of biosurfactant is not limited to the exponential phase of growth or that biosurfactant produced during exponential phase was stable for a long period of time. We have been unable as yet to determine exactly when biosurfactant is maximally produced. However, we do know that within the first 48 hours of incubation, growth is extensive but little biosurfactant appears to be produced. Both surface tension data and HPLC analysis failed to detect significant amounts of biosurfactant during this period. However, after 18 days, a significant amount was detected in the culture supernatant of medium containing

PP2 (Table 8). Interestingly, the culture grown without PP2 used almost the same amount of sucrose that the culture growing with PP2, but did not produce a detectable amount of biosurfactant (Table 8).

Conclusions

Growth with glucose gave the fastest growth rate for *Bacillus mojavensis* JF-2 (JF-2) and monosaccharides were preferred over disaccharides as substrates for growth. The fastest growth rate occurred with a glucose concentration of 77 mM. Although glucose gave the fastest growth rate, fructose gave the highest growth yield. The reason for this is not known at this time. The sample preparation steps used for high performance liquid chromatography (HPLC) analysis resulted in a biosurfactant recovery (from culture supernatant) of about 70%. This provides us with a correction factor to quantify the biosurfactant concentration in the media. The presence of Proteose peptone #2 (PP2) in the medium not only allows for better growth under anaerobic conditions but also led to increased biosurfactant production under anaerobic conditions. At this time, a medium containing PP2 and a monosaccharides source of sugars is best for anaerobic biosurfactant production

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Table 5. The effect of different carbohydrates on the growth rate of JF-2 in ME_2. The molarity of the Maltrin sugars was calculated assuming an average molecular weight equal to glucose.

Substrate	Substrate Concentration (mM)	Growth Rate (hr^{-1})
Glucose	0	0.03
Glucose	11	0.13
Glucose	22	0.13
Glucose	33	0.14
Glucose	44	0.12
Glucose	55	0.12
Glucose	66	0.10
Glucose	77	0.17
Glucose	88	0.16
Glucose	99	0.15
Maltrin M180	55	0.01
Maltrin M200	55	0.01
Maltrin M250	55	0.10
Sorbose	55	-0.08
Starch	55	0.05
Raffinose	55	0.07

Table 6. Molar growth yields of JF-2 with different carbohydrates.

Substrate	Substrate Concentration (mM)	Growth Yield (g dry weight/mole substrate)
Glucose	20	36.8
Sucrose	20	31.5
Fructose	20	73.7
Lactose	20	30.5

Table 7. The amount of surfactin recovered by acid precipitation and methanol extraction in three replicate twenty-ml samples

Sample	Initial amount (grams)	Amount recovered (grams)	Percent Recovery
1	1.09	0.71	65
2	1.09	0.77	71
3	1.09	0.73	68

Table 8. Biosurfactant production by JF-2 with and without Proteose peptone 2 added to the medium after 18 days of incubation under anaerobic conditions.

Proteose Peptone Added	Biosurfactant Sample 1 (mg/ml)	Surface Tension (mN/M)	Yield (mg biosurfactant/ mmol of sucrose)	Amount of sucrose consumed (mmol)
Yes	0.064	44	0.00372	15
No	ND[a]	58	ND	17
Yes uninoculated	ND	66	ND	ND

[a] ND, not determined