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ASSESSMENT OF ENRICHMENT OF JET FUEL BIOREMEDIATION PROCESSES WITH A HEAVY HYDROCARBON-DEGRADING BACTERIUM

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ABSTRACT

Experiments were performed to determine if an isolated Arthrobacter globiformis SBI-5 could enhance degradation of JP-5 fuel added to natural waters (i.e., pond water) and to two types of soil, sand and potting soil). When JP-5 fuel was added to the pond water (2.5%, v/v), the natural flora possessed the ability to degrade the JP-5 fuel with an efficiency nearly equalling that displayed by SBI-5 under specialized culture conditions (addition of yeast extract and other growth promoting materials). When SBI-5 was added to pond water containing its natural viable flora, degradation of JP-5 fuel was depressed. The addition of yeast extract to a similar culture of pond water partially promoted the growth of SBI-5. The loss of JP-5 fuel from the soil samples over time was similar regardless of the presence or absence of the jet fuel degrading microbe (e.g., SBI-5) or its biosurfactant.

INTRODUCTION

The bioremediation of soils contaminated with toxic materials with different microorganisms has recently attracted a considerable attention compared with the conventional technologies such as incineration (1). At sites where the appropriate indigenous strains are present, remediation may consist only of optimizing the soil conditions for microbial growth. An alternative technique would be inoculation of soils with exogenous degrading microorganisms in conjunction with manipulation of the soil parameters to enhance the proliferation of the inoculant strains (2,3), but this tendency faces a great contradictions. Whereas several investigators have reported the failure of microbial inocula to stimulate rates of biodegradation in soil(4), others have observed significantly enhanced rates of biodegradation directly attributed to soil inoculation (5,7). Environmental factors, such as soil pH, aeration, nutrients and moisture content are well known to determine the survival, desirable metabolic and physiological characteristics of any inoculated strain (2).

Arthrobacter globiformis SBI-5 was isolated from a spill dumping site in Cheshire CT., USA. It is able to degrade several hydrocarbons, alcohols and fatty acids and use them as a sole carbon and energy source. The growth of SBI-5 on these carbon sources was associated with production of a lipopolysaccharide bioemlusifier (8,9).

The purpose of our study was to investigate the degradation of JP-5 fuel in natural water and in two types of soil inoculated with a strong hydrcarbon-degrading isolate. Effect of addition of nutrients to the polluted natural environment soil components on the bioremedia-

tion of the heavy hydrocarbons was also examined.

MATERIALS AND METHODS

1. Microorganism and culture conditions :

Arthrobacter globiformis SBI-5 was used in this study. A chemically defined medium S-MSB-1-Y was used as a control medium. MSB-1 was prepared as described by Pendrys[10]. The medium was supplemented with 3.5 ml of a salt solution contained the following salts (g/L) calculated as the anhydrous salts) : $CaCl_2$, 0.389; $Cu(NO_3)_2$, 0.64; ammonium sulfate, 0.6; $ZnSO_4$, 0.718; $FeSO_4$, 0.696 to 1 liter of MSB-1 medium. A 0.1 g yeast extract (Difco) was added to 1 liter of S-MSB-1 medium to give S-MSB-1-Y.

2. Preparation of the cell suspension :

The microbial isolate was cultured in Tryptic-Soy-Broth (TSB) and incubated at 30°C in a gyrating water bath (150 rpm) for 24 hours in the presence of 2.5% JP-5 fuel (v/v). The grown, preconditioned cells were harvested by centrifugation, washed twice, then resuspended in sterile MSB-1 buffer or demineralized water. The cell concentration was then adjusted spectrophotometrically to OD_{546} = 0.1 and used for further investigation.

3. Experimental design :

3.1. Bioremediation of JP-5 fuel in pond water :

Water was collected from a local pond in Wallingford, Connecticut, which collects the run off from adjacent farm land and a heavily traveled road. The water was employed as sterile and unsterilized culture medium for growing SBI-5 in presence of JP-5 fuel.

A volume of 250 ml of either sterile or unsterilized pond water containing JP-5 fuel (2.5% v/v) in a 500-ml Erlenmeyer flask was inoculated with the above described suspension of SBI-5 (1% v/v). Then, the culture media were incubated in a shaker water bath at 150 rpm at 30°C, while sterile, humidified air was sparged directly through the culture broth (1 vol/vol/min). Every 24 hours, samples were withdrawn from the culture broth for analysis. As a control culture medium, sterile, chemically defined fermentation broth, S-MSB-1-Y, was used.

3.1.1. Improving the culture conditions of SBI-5 in pond water:

Nutrients, metal ions and buffering salts were added to improve the potential of cu turing media for supporting the growth of SBI-5. In short, test experiments inoculated with 1% (v/v) SBI-5 cell suspension (OD_{540} = 0.1) and containing 2.5% JP-5 fuel (v/v) were performed using a control of sterile S-MSB-1-Y medium (Culture #1). Four other test experiments were performed using unsterilized pond water containing 2.5% JP-5 fuel (v/v) supplemented with one of the following : S-MSB-1-Y broth constituents (Culture #2), yeast extract (0.1 g/L) (Culture #3), mineral metals salt solution (Culture #4), or potassium phosphate salts (Culture #5).

3.2. Bioremediation of jet fuel in sand :

White playground sand was used. Eight Petri dishes (Pyrex, 120 mm) were filled with 100 g of non-sterile sand per Petri dish. Duplicate Petri dishes were divided into 4 groups. To each Petri dish was added 2.5 ml JP-5 fuel (2.5%, v/w of sand) and 25 ml aqueous medium and cells which were prepared as follows :

a- Culture A : Heat-treated SBI-5 culture broth from A 120-old culture. Whole SBI-5 culture broth with suspended cells was heated to 80°C and held at that temperature for 30 minutes. The total protein concentration was adjusted to 290 mg/L.

b- Culture B : Fresh SBI-5 culture broth from A 120 hrs-old culture with final total protein content of 290 mg/L.

c- Culture C : Freshly prepared, precon-

ditioned SBI-5 cells suspended in demineralized water (OD_{546} = 0.1) grown in presence of 2.5% JP-5 fuel (v/v).

d- **Culture D :** Sterile growth medium (S-MSB-1-Y) without the addition of exogenous microorganism.

Cultures (A-D) were incubated at room temperature (about 22°C) for 28 days. At times zero, 2,7,11,17 and 28 days, 0.5 g sand was withdrawn from each culture and used for determination of residual JP-5 fuel concentration. Sterile, demineralized water (5 ml) was added to each culture after sampling on days 7, 11 and 17 to keep the sand wet during the period of incubation.

3.3. Biodegradation of JP-5 fuel in cactus potting soil :

Cactus potting soil prepared for succulents is rich in organic materials. Five different groups of duplicate Petri dishes (Pyrex with 120 mm outer diameter) were used. Each Petri dish contains 25 g dry soil blended with 312 µl of JP-5 fuel (0.6%, v/w). Then, 12.5 ml aqueous culture medium A,B,C,D or E was added to duplicate Petri dishs with soil-containing JP-5 fuel. The cultures were prepared as follows :

a- **Culture A :** Demineralized water (control).

b- **Culture B :** Preconditioned SBI-5 in demineralized water.

c- **Culture C :** Non-sterile S-MSB-1-Y.

d- **Culture D :** Preconditioned, SBI-5 in sterile S-MSB-1-Y.

e- **Culture E :** Heat-treated, 120 hrs-old SBI-5 culture broth (as described above).

All of the five cultures were incubated at room temperature (about 22°C) for 10 days. Periodically, 0.5 g soil samples were withdrawn and the JP-5 fuel content was determined by extracting with n-heptane and analyzed as described below.

4. Analysis :

Samples were assayed for total protein, OD_{546} and pH. To determine the concentration of the jet fuel in the culture media, soil or pond water, liquid samples were extracted with equal volumes (v/v) of n-heptane and soil samples were extracted with volumes equal to their weighing (v/w) of n-heptane. After phase separation by centrifugation at 13,000 xg for 5 minutes, an aliquot of 10 µl was analyzed by capillary gas chromatography. A Shimadzu model GC-8AX gas chromatograph equipped with a hydrogen flame ionization detector and split/splitless injector SPL-G9, SPL-G9M (Supelco) were used. The sample was assayed using a 100 meters of fused silica capillary column with inner diameter 0.25 mm and film thickness 0.5 microns (Petrocol DH, Supelco). The sample was split 100:1 using nitrogen with a pressure of 3.6 kg/cm^2. Nitrogen carrier gas flowed through the column at 60 ml/min. The starting column temperature of 50°C was maintained for 10 minutes; the temperature was then increased 10°C/min. to a final temperature of 250°C, and was held constantly for 30 min. Both injector and detector were held constantly at 280°C. The relative concentration of total petroleum hydrocarbons (TPH) was determined by measuring the peak heights of the major 6 peaks of the jet fuel at time of sampling with the sample withdrawn at time = 0.

RESULTS

1. Biodegrading JP-5 fuel in pond water :

Table (1) shows the pH, OD_{546}, total protein and residual JP-5 fuel (%) of the culture broth from control and pond water-based cultures (Cultures #1-4). The pH was changed little with time. The results show that only in the control culture (i.e., the S-MSB-1-Y culture broth) did optical density and protein concentration increase significantly over the course of several days. The table shows also the

Table (1) : Biodegradation of JP-5 fuel in pond water

Parameter measured	Culture No.(*)	Sampling time (hours)					
		Zero	48	72	96	120	168
pH	#1	6.7	6.4	6.3	6.3	6.3	6.4
	#2	7.25	7.3	7.3	7.4	7.3	7.3
	#3	7.1	6.83	6.9	7.2	7.2	7.2
	#4	7.2	7.2	7.0	7.1	7.1	7.1
OD_{546}	#1	0.55	8.7	13.2	13.0	12.4	12.2
	#2	0.27	0.85	0.64	0.43	0.43	0.45
	#3	0.56	1.25	1.34	1.0	1.0	0.5
	#4	0.58	0.6	0.7	0.7	0.6	0.5
Total protein (mg/L)	#1	7	200	350	390	360	310
	#2	8	8	8	10	18	16
	#3	8	16	16	17	15	14
	#4	16	15	15	15	14	14
Residual JP-5 (%),(**)	#1	100	37	20	15	10	13
	#2	100	100	100	100	100	100
	#3	100	42	27	16	8	2
	#4	100	100	100	100	100	100

(*) Culture definition :
Culture #1 : Control (Sterile S-MSB-1-Y medium).
Culture #2 : Unsterilized pond water with SBI-5 cells.
Culture #3 : Unsterilized pond water.
Culture #4 : Sterile pond water with SBI-5 cells.

(**) Relative percent is the average of at least two determinations taken from two equivalent experiments

change in the relative concentration (%) of JP-5 fuel in culture broth over time. Both the control Culture (#1) and the unsterilized pond water which was not inoculated with SBI-5 (#3), degraded JP-5 fuel over time. Surprisingly, unsterilized pond water did not degrade JP-5 fuel when it had been pre-inoculated with SBI-5 (#2 and #4, respectively).

1.1. Improving the culture conditions of SBI-5 in pond water :

Since pond water failed to support the growth of SBI-5 (Cultures #2 and #4 described above), nutrients, metal ions and buffering salts were added to promote the growth of SBI-5. Table 2 shows the pH, OD_{546}, total protein and residual JP-5 fuel profiles of the control and supplemented culture broth inoculated with SBI-5 in presence of JP-5 fuel. Culture 1 (control culture) and Culture 2 (unsterilized pond water supplemented with constituents of S-MSB-1-Y), show the most substantial drop in pH. Culture 3 (unsterilized pond water supplemented with yeast extract) and Culture 5 (unsterilized pond water supplemented

Table (2) : Improving culture conditions of SBI-5 in pond water.

Parameter measured	Culture No.(*)	Sampling time (hours)				
		Zero	48	72	120	168
pH	#1	6.78	6.6	6.5	6.5	6.48
	#2	6.78	6.688	6.6	6.4	6.37
	#3	6.8	6.8	6.8	6.7	6.6
	#4	6.9	7.0	7.1	7.0	7.0
	#5	7.2	7.16	7.14	7.1	7.12
OD_{546}	#1	0.2	8.7	13	13	12
	#2	0.28	1.1	1.5	12.3	14
	#3	0.29	0.7	1.1	1.1	1.3
	#4	0.17	0.2	0.3	0.2	0.25
	#5	0.2	0.22	0.17	0.42	0.34
Total protein (mg/L)	#1	7	200	350	360	310
	#2	7	19	37	290	370
	#3	6	15	18	23	25
	#4	7	9	9	10	12
	#5	8	9	10	10	15
Residual JP-5 (%)(**)	#1	100	87	78	30	12
	#2	100	100	100	100	42
	#3	100	95	90	85	75
	#4	100	75	64	59	35
	#5	100	89	82	63	47

(*) Culture definition :
Culture #1 : Control (Sterile S-MSB-1-Y medium).
Culture #2 : Unsterilized pond water + constituents of S-MSB-1-Y medium.
Culture #3 : Unsterilized pond water + 0.1 g/L yeast extract.
Culture #4 : Unsterilized pond water + mineral salts solution.
Culture #5 : Unsterilized pond water + potassium phosphate salts.

(**) Relative percent is the average of at least two determinations taken from two equivalent experiments.

with potassium phosphate salts) show only small decreases in pH over the course of fermentation. In contrast, the pH of the culture broth of Culture 4 (unsterilized pond water supplemented with mineral salts) slightly increased during the course of fermentation.

The results show also that when there is a drop in the pH of the culture broth there is a corresponding increase in protein concentration and optical density. The 72 hour lag period observed for protein concentration and optical density in the broth of Culture #2 is noteworthy. Once the inhibitory factors associated with the unsterilized, S-MSB-1-Y supplemented pond water were overcome in Culture #2, both protein levels and optical density start to increase at the same rate as that observed for Culture #1. The

other three Cultures (# 3,4,5) show no significant increase in protein and optical density. Moreover, Table 2 shows the residual JP-5 fuel in the culture broths where Culture #1 shows the greatest biodegradation of JP-5 fuel (88% of the JP-5 fuel added to the culture was degraded after 168 hours of fermentation). The other supplemented cultures of pond water showed steady decreases in the concentrations of JP-5 fuel.

Despite the promising increases in the optical density and protein concentration in the culture broth of Culture #2, JP-5 fuel did not start to be degraded until after nearly 120 hours of fermentation. Apparently, SBI-5 has initially utilized carbon and energy sources in pond water other than JP-5 fuel since significant growth was observed in the culture before JP-5 began to be degraded.

2. Effect of SBI-5 on biodegradation of JP-5 fuel in soil :

2.1. Organic-poor sand :

The specific aims of this experiment are to investigate the potential of using SBI-5 culture broth with its surfactant to increase the rate of biodegradation of JP-5 fuel in sand and to determine if the natural flora of the sand possessed the capacity to biodegrade JP-5 fuel. Table 3 shows the relative amount (%) of JP-5 fuel recovered from the soil samples. The results suggest that JP-5 fuel was degraded faster in culture C (containing freshly prepared, preconditioned SBI-5 cells); however, the difference in the rate JP-5 fuel lost from the four cultures (Cultures A,B,C and D) is not great. It is not clear if the JP-5 fuel loss from the cultures was due to biodegradation or to the effect of some physical factors such as evaporation or adsorption.

2.2. Organic-rich potting soil :

The specific aims of this experiment were to determine if the natural flora in an organic-rich soil could degrade JP-5 fuel with and without heat-treated, 120 hrs-old SBI-5 culture broth and to determine if preconditioned, viable SBI-5 cells in fresh S-MSB-1-Y could biodegrade JP-5 fuel in the organic-rich soil. Table 4 shows the relative residual JP-5 fuel (%) in the organic-rich soil over time. There is approximately, in average, 70% reduction in the residual JP-5 fuel in organic-rich soil by day four while with sand (organic-poor) culture experiments, residual JP-5 fuel decreased by about 10-15% by day four.

DISCUSSION

When JP-5 was added to the pond water, it was quickly degraded by a plethora of organisms observed in the water. In fact, the degradation of the jet fuel by the microbes in the pond water was nearly as great as that observed for highly selected microbes (SBI-5) grown under highly favorable conditions (i.e., pH, nutrient supplements, temperature, mixing, etc.). Apparently, the algae and fungi in the pond water which were observed microscopically in the pond water, were able to degrade JP-5.

Addition of mineral salts solution and potassium phosphate salts to improve the bioremediation of toxic compound (JP-5 fuel) do not result in any enhancement of SBI-5 growth in pond water. Many investigators(**11-13**) found that addition of phosphorous to natural waters has been reported to increase, decrease, or have no effect on the mineralization of organic chemicals. It was also found that low concentrations of N and P in seawater limit the growth of bacteria that degrade the hydrocarbons (**14**).

SBI-5, which has shown tendency for degrading JP-5 fuel in optimized culture, was then added to the pond water in which JP-5 fuel was added to simulate a spill. To our surprise the JP-5 fuel was not degraded. Possibly sterile pond water lacks sufficient nutrients to support the growth of SBI-5. In unsterilized pond water, SBI-5 appears to inhibit the degradation of JP-5 fuel by the natural flora of the pond water. The fuel was only poorly degraded if yeast extract was also added to the pond water. Analysis of the microbes in the pond water test solution showed that the natural flora was lost

Table (3): Relative percentage of JP-5 fuel in organic-poor soils after different incubation periods(*).

Time (days)	Cultures(**)			
	A	B	C	D
Zero	100%	100%	100%	100%
2	94	106	74	102
7	95	67	78	85
11	81	83	62	80
17	18	18	7	9
28	18	15	7	11

(*) Relative percent is the average of at least two determinations taken from two equivalent experiments.

(**) Culture definition :
Culture A : Heat-treated, 120 hrs-old SBI-5 culture broth.
Culture B : Non-treated, 120 hrs-old SBI-5 culture broth.
Culture C : Freshly prepared preconditioned SBI-5 in S-MSB-1-Y.
Culture D : S-MSB-1-Y without adding microorganism.

Table (4): Relative percentage of JP-5 fuel remaining in organic-rich potting soil(*).

Time (days)	Cultures(**)				
	A	B	C	D	E
Zero	100	100	100	100	100
2	44	29	30	29	42
7	23	24	20	21	33
10	22	16	20	19	28

(*) Relative percent is the average of at least two determinations taken from two equivalent experiments.

(**) Culture definition :
Culture A : Demineralized water (control).
Culture B : Preconditioned, SBI-5 in demineralized water.
Culture C : Non-sterile S-MSB-1-Y.
Culture D : Preconditioned, SBI-5 in sterile S-MSB-1-Y.
Culture E : Heat-treated, 120 hrs-old SBI-5 culture broth.

and that only SBI-5 (and perhaps other strains with similar morphology) was present at the end of the experiment. These results suggest that SBI-5 is unable to grow in the low nutrient pond water, but, to our surprise, SBI-5 did possess the ability to inactivate (and perhaps destroy) the natural flora of the pond water. Once yeast extract was added, SBI-5 was slightly able to grow and to partially degrade the JP-5 fuel. It has been shown previously that microbial nutritional requirements appear to be site specific(15).

JP-5 fuel was also added to soil samples inoculated with SBI-5 to determine if the addition of jet fuel consuming microbes could utilize this hydrocarbon as a source of energy and carbon. The relatively small losses of JP-5 fuel from sand cultures suggest that evaporation is probably not a significant factor contributing to the loss of JP-5 fuel from the organic-rich soil experiments. Possibly, the reduction in the concentration of JP-5 fuel observed after only 4 days of culturing in organic-rich cactus soil may be due to the absorbance of JP-5 fuel by the organic material in the soil. This seems unlikely, however, because the soil was extracted with n-heptane that should remove JP-5 components from all parts of the soil. Prediction of the fate of hydrophobic organic contaminants in soils is complicated by the competing process of sorption and biodegradation. From a practical standpoint, rendering organic contaminants unavailable to potential microbial degraders by sorption to soil could seriously impede efforts to bioremediate contaminated sites(16).

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تقييم عمليات المعالجة البيولوجية لوقود الطائرات باستخدام البكتريا المحلله للهيدروكربونات الثقيله

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تقوم هذه التجارب على دراسة أهمية إضافة العزلة أرثروباكتر جلوبيفورفمس (SBI-5) للمياه الطبيعية (مياه البحيرات) ولنوعين مختلفين من التربة (الرملية والتربة الصناعية لإنبات الزهور) لزيادة تحلل وقود الطائرات عند تواجده فى هذه الأماكن. عند إضافة ٥ر٢٪ من الوقود (JP-5) لمياه البحيرات، وجد أن الفلورا الطبيعية الموجوده بها تملك القدره على تحلل الوقود بكفاءة تساوى كفاءة العزلة (SBI-5) عند تنميتها معملياً تحت ظروف مثالية. كذلك وجد أن إضافة العزله (SBI-5) لمياه البحيره المحتوية على الفلورا الطبيعية تتسبب فى الاقلال من تحلل الوقود كما أدت إضافة مستخلص الخميره لمياه البحيره الى زيادة طفيفة فى نمو العزله (SBI-5) فى مياه البحيره. كما وجد أن آختفاء وقود الطائرات من عينات التربة على مدى زمن التجربة لم يعزى إلى وجود أو عدم وجود العزلة (SBI-5) أو وسيط الإستحلاب المنتج بواسطتها.