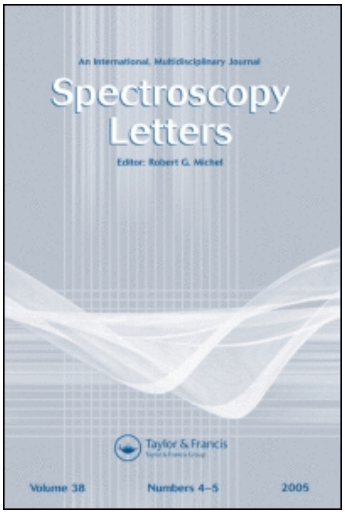

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Bioenhancement of Soil Microorganisms in Natural Surfactant Solutions: II - Anaerobic

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BIOENHANCEMENT OF SOIL MICROORGANISMS IN NATURAL SURFACTANT SOLUTIONS: II - ANAEROBIC

Key Words: Natural Surfactant, Ritha, Sapindus mukorossi, Bioenhancement, Anaerobic, Nutrients, Biodegradation

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ABSTRACT

Natural surfactant solutions obtained from fruit pericarps of *Sapindus mukorossi* are shown to solubilize significant quantities of hydrophobic organic compounds and mobilize them from soil matrix. It is very crucial to determine the fate of surfactants employed for soil flushing in the subsurface and also in the effluent recovered. This paper appraises the bioenhancement of soil microorganisms in natural surfactant solutions under anaerobic conditions. Sealed 125 ml serum bottles are used for developing the anaerobic cultures. The cultures are maintained in anoxic conditions by degassing and filling the bottles with nitrogen. Three concentrations of natural surfactant 0.1, 1 and 2% and two different nutrient media, basal salt media (BSM) and heterotrophic media are used in the studies. Natural surfactant solutions can serve as both carbon and energy source for

anaerobic microorganisms and also degrade to considerable extent. The growth curves for anaerobic cultures followed similar trends as those for aerobic cultures reported in an earlier paper. Addition of BSM to cultures increased the growth significantly. However, heterotrophic media amended cultures showed only an increased initial growth rate. The microbial growth increased significantly when the surfactant concentration was increased from 0.1 to 2% by weight. The results suggest that natural surfactant can readily degrade under anaerobic conditions and needs to be studied further in the presence of soils and sediments.

INTRODUCTION

Surfactant solutions are being proposed for remediation of contaminated soils at abandoned hazardous waste sites. Laboratory studies show a great potential for surfactants mainly as an agent to mobilize contaminants from soils (1, 2). Surfactants can solubilize hydrophobic organic compounds (HOCs) and thus make them accessible for removal by either flushing or degradation by microorganisms. However, the effect of surfactants on the contaminant biodegradation has not been studied fully. It is crucial to determine the fate of surfactant employed for remediation of subsurface soils and its effect on the degradation of contaminants. Rouse et al. (1994) made an excellent review on the role of surfactants on the biodegradation of HOCs (3). The literature suggests that surfactants may enhance, not have any effect or inhibit biodegradation of contaminants (4-6). Most of these studies are conducted under aerobic conditions and no information is available on the biodegradation of surfactants under anoxic or anaerobic conditions. The subsurface soils are typically cutoff from the atmosphere and hence are under anoxic conditions. Hence, it is important to appraise the bioenhancement of soil microorganisms under anaerobic conditions.

Kommalapati (1995) employed natural surfactant solutions prepared from fruit pericarps of *Sapindus mukorossi* commonly known as Ritha or soapnut as an alternative to synthetic or commercial surfactants for remediation of contaminated soils (7). These solutions are shown to be comparable to commercial surfactants in solubilizing and desorbing HOCs such as hexachlorobenzene (HCB) and naphthalene from soil (8, 9). Soil flushing of one-dimensional columns contaminated with HCB using natural surfactant solutions recovered about 100 times more HCB than a simple water flood in 12 pore volumes (10). Other possible applications and properties of natural surfactant solutions are reported elsewhere (7). In our first paper in the series, we presented the results of aerobic bioenhancement of soil microorganisms in natural surfactant solutions (11). The results indicated that the natural surfactant solutions can support aerobic microbial populations and readily degrade. In this paper, the results of bioenhancement studies under anaerobic conditions in the presence of natural surfactant solutions are evaluated. The effect of nutrients on the microbial populations is also appraised. The effect of a HOC, hexachlorobenzene on the bioenhancement of soil microorganisms is investigated and presented elsewhere (12).

MATERIALS AND METHODS

Sapindus mukorossi (Ritha)

Dry fruits of *Sapindus mukorossi* were procured from India. After removing the seed, pericarps were dried in the oven for 2 days at 50°C and stored. The pericarps were ground in small batches and sieved through US Standard Sieve No. 20 (840 μm). The fine powder thus obtained was used for preparing natural surfactant solutions. Some of the properties of natural surfactant solutions are reported in our earlier paper (11).

Nutrient Media

Nutrients for the bacterial cultures were added in the form of either basal salt medium (BSM) or heterotrophic media (HM). Concentrated BSM (10X) was prepared by dissolving 58.0 g K_2HPO_4 or 65.52 g K_2HPO_4 $3H_2O$, 45.0 g KH_2PO_4, 20.0 g $(NH_4)_2SO_4$, 1.6 g $MgCl_2$, 200 mg $CaCl_2$, 20 mg $NaMoO_4$, and 10 mg $MnCl_2$ in one liter of deionized (DI) water (13). Heterotrophic media which is typically used for anaerobic cultures, was prepared by dissolving 300 mg KCl, 900 mg NH_4Cl, 90 mg $CaCl_2$ $2H_2O$, 250 mg K_2HPO_4, 250 mg KH_2PO_4, 35 mg NaCl, 20 mg $MgCl_2$ $6H_2O$, 159 mg Na_2CO_3 and 240 mg Na_2S in one liter of DI water (14).

Preparation of Natural Surfactant Solutions

Appropriate quantity of dry fruit pericarp powder (10 g per 100 ml water) was added to DI water and stirred for 3 hours at room temperature. The mixture was centrifuged at 10,000 rpm for 45 minutes and the supernatant was filtered through a 44 mm pre-filter (Corning Costar Corp. Oneonta, NY) and a Metricel 0.45 μm membrane filter (Gelman Scientific, Ann Arbor, MI) in sequence (7, 8).

Anaerobic Bioenhancement Studies

The anaerobic soil microorganisms from a local Superfund site north of Baton Rouge, LA. were collected and added to a sterilized 125 ml serum bottles along with 100 ml of 1% filter sterilized natural surfactant solution to develop a culture for anaerobic study. The bottles were sealed degassed and filled with nitrogen and the culture was kept on a mechanical shaker at room temperature. The microorganisms reached log growth in about two weeks. This inoculum was used as a seed for all the anaerobic bioenhancement studies.

Anaerobic studies were conducted very similarly to aerobic experiments reported elsewhere (11). Serum bottles of 125 ml capacity were filled with DI water or appropriate nutrient solution before sterilizing. An appropriate amount of filter sterilized natural surfactant solution was transferred to the culture bottles. The acclimatized seed from the preliminary experiment was added to these bottles anaerobically. All the bottles were degassed and filled with nitrogen and maintained under anaerobic conditions by repeating the procedure periodically (14). Three concentrations of natural surfactant 0.1, 1 and 2% and two different nutrient media, BSM and heterotrophic media were used in the study. The bottles were kept on a mechanical shaker at room temperature (23 $\pm$ 2°C) and stirred gently. Samples were taken anaerobically at appropriate intervals and monitored for the growth of microorganisms by determining the absorbance of the samples at a wavelength of 540 nm. Samples were also analyzed for total organic carbon (TOC) at regular intervals using a Model TOC-500 fixed with ASI 502 autosampler (Shimadzu Corporation, Kyoto, Japan).

RESULTS AND DISCUSSION

Anaerobic bioenhancement studies were conducted with seed inoculum acclimated to natural surfactant solutions for about 4 weeks. Figure 1 shows the growth curves for soil microorganisms under anaerobic conditions with natural surfactant as the only carbon source. The X axis shows the incubation time in days and the Y axis shows the growth of microorganisms as measured by the absorbance at 540 nm in a completely mixed batch reactor. Natural surfactant concentration used was 1%, as this was found to be the optimum concentration from soil flushing studies (10). The cultures had a lag phase of about one day. The growth beyond the lag phase was relatively rapid and reached a stationary phase

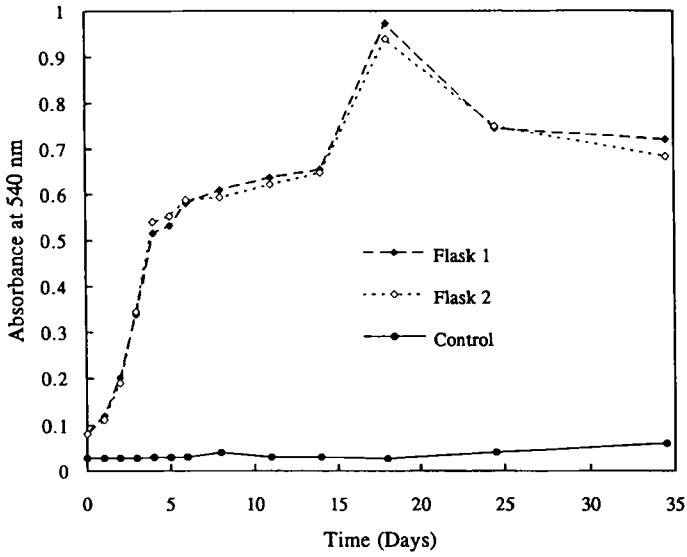

Figure 1: Bioenhancement of Soil Microorganisms in Natural Surfactant Solutions under Anaerobic Conditions

by the end of the week. The anaerobic microorganisms showed an increase in growth again after about 15 days, which continued for about a week and by the fourth week the microorganisms were in the stationary phase again. This increased growth rates after the stationary phase were also noted for aerobic cultures (11). The exponential growth following the first stationary phase was believed to be due to the degradation of natural surfactant components that were resistant for degradation during the initial log phase. However, due to the lack of analytical techniques to identify and quantify the individual components of the fruit pericarp, it was not feasible to verify this hypothesis. However, TOC was used to monitor the disappearance of organic carbon. The TOC was reduced by about 41% and 36% by the end of 25 days in the two bottles where natural surfactant was

the only carbon source. The TOC dropped by 32% by the end of 5 days and beyond 5 days the degradation rate was slow.

Anaerobic degradation does not yield as much energy as the aerobic process and is generally a very slow process. Under aerobic conditions oxidation of 1 mole of glucose will yield 686 Kcal as opposed to less than 100 Kcal by anaerobic microorganisms. The cell yield for anaerobic process, 0.06 mg per mg of organic matter is much less than that of aerobic process, 0.5 mg/mg (15). In our study the bio-growth as recorded by the absorbance for anaerobic cultures (0.9) was about one third of that for aerobic cultures (2.8) (11). Similar to aerobic cultures, it is evident that the interactions of natural surfactant with cell walls are not interfering with the ability of bacteria to degrade the surfactants under anaerobic conditions also. Surfactant - cell interactions are reported to be crucial for some surfactants in determining the biodegradation capabilities of the microorganisms (3, 16). It is clear that natural surfactant solutions at 1% concentration can support anaerobic soil microorganisms and can readily serve as both carbon and energy sources. This concentration of natural surfactant can solubilize about 20 times more HCB than water. It was shown in our earlier paper that natural surfactant can support biogrowth and degrade to a considerable extent under aerobic cultures (11).

Effect of Nutrients

Nutrients in the form of basal salt media (BSM) and heterotrophic media (HM) were added to cultures to determine whether the cultures are nutrient limited. BSM is typically used for aerobic cultures and the heterotrophic media is a common media which can support all the anaerobic heterotrophic microorganisms (14). Figure 2 shows the effect of nutrient media on the cultures growing on natural surfactant. The systems amended with basal salt

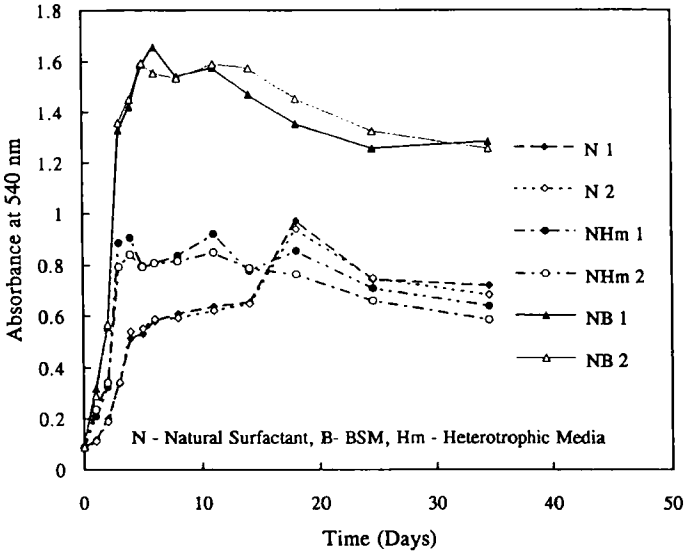

Figure 2: Effect of Nutrients (Basal salt media and Heterotrophic media) on Anaerobic Bioenhancement of Soil Microorganisms

media doubled the growth of microorganisms in the first week. The absorbance, which was used to monitor the biogrowth was 1.6 for cultures amended with BSM compared to 0.8 for cultures without nutrients. These results are in agreement with those reported for aerobic cultures and indicate that the cultures are nutrient limited.

Addition of heterotrophic media to cultures also showed a faster and higher growth than the corresponding culture without the nutrients in the first 10 days but lower than that observed with basal salt media. However, the difference in the biogrowth of cultures with and without heterotrophic media became insignificant by the end of 5 weeks. The cultures with BSM on the other hand had a significantly higher growth than the systems with natural surfactant and natural surfactant and heterotrophic media at the end of 5 weeks. Aerobic

cultures had a slow but a consistent increase in growth beyond the stationary phase indicating that the cultures are utilizing the nutrients released during the endogenous phase. This consistent growth was not observed for anaerobic cultures beyond the stationary phase, which occurred by the end of first week. The cultures amended with nutrients reached a stationary phase about two days before the nutrient limited cultures.

Percent TOC for all the cultures at the end of 25 days is shown in Figure 3. As can be seen from the figure TOC reduction is about the same for all the cultures. The percent of TOC reduced was in the range of 35 to 45% for all the cultures. It should be noted that the initial concentration of natural surfactant used in cultures with heterotrophic media was higher than the other cultures. The cultures with BSM degraded TOC by about 45 % and the cultures with heterotrophic media by about 40% as against 38% by cultures without any nutrients. The degradation of natural surfactant as indicated by TOC is lower for anaerobic cultures than that observed for aerobic cultures. In aerobic studies the addition of nutrients improved the degradation of natural surfactant by more than 10% however, the increase in degradation is considerably lower for anaerobic cultures.

Effect of Natural Surfactant Concentration

Natural surfactant concentrations of 0.1, 1 and 2% were used to study the effect of concentration on the anaerobic bioenhancement of soil microorganisms. The cultures were also amended with nutrient media, BSM. Figure 4 shows the growth curves for the three concentrations both without and with BSM. This growth curves clearly support our earlier conclusion that the nutrient media is significantly increasing the growth of microorganisms. The cultures with 1% natural surfactant and nutrients had higher growth than 2% natural surfactant cultures without nutrients. The cultures with 2% natural surfactant seem to have

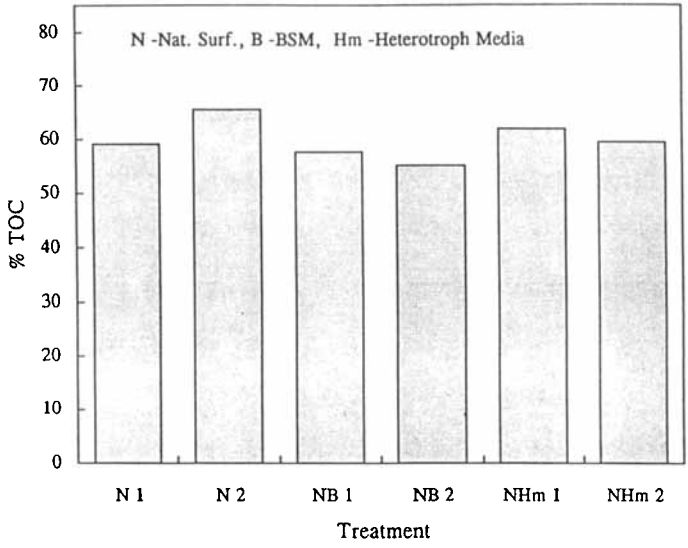

Figure 3: Percent Total Organic Carbon Remaining in Anaerobic Cultures after 25 days for Different Treatments

a lower growth than the 1% natural surfactant, however by about 25 days the cultures growing on 2% natural surfactant are able to catchup with the 1% natural surfactant cultures. This probably is due to the limitation of nutrients and also possible toxic effects of higher natural surfactant concentrations. But the cultures of same natural surfactant concentration when amended with BSM showed no inhibition and had a significantly higher growth. The cultures with 2% natural surfactant utilized about 67% natural surfactant as against 38% and 35% for 1% and 0.1% natural surfactant solutions respectively. The higher natural surfactant concentrations can provide higher amounts of degradable carbon for the microbial populations and thus the increased growth. From the TOC values, it is evident that the presence of nutrients did not significantly affect the amount of TOC

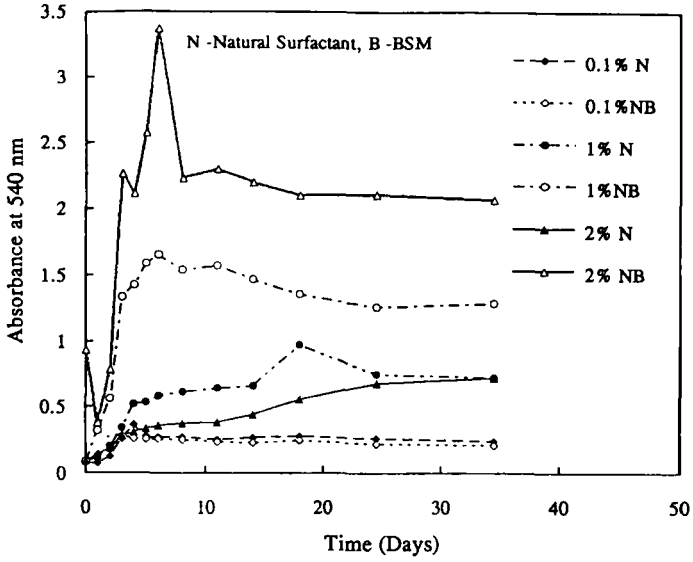

Figure 4: Effect of Natural Surfactant Concentration on the Anaerobic Growth of Microorganisms

degraded, even though the growth showed significant increase. This is in contrast to the observations made in aerobic studies.

CONCLUSIONS

From the anaerobic bioenhancement studies using soil microorganisms in the presence of natural surfactant solutions, the following conclusions can be made:

- Natural surfactant solutions can support biological growth and serve readily as both carbon and energy sources under anoxic conditions. The results suggest that the natural surfactant-bacterial cell interactions are not inhibiting the microbial growth as was reported for some surfactants.

- Addition of basal salt media (BSM) increased the bio-growth significantly suggesting that the cultures are nutrient limited.
- Addition of heterotrophic media increased the initial growth rate significantly, however the ultimate degradation was same as the cultures without nutrients.
- The increase in natural surfactant concentration from 0.1 to 2.0% increased the microbial growth significantly. The percent TOC degradation was significantly higher at higher surfactant concentration.

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