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DEPARTMENT OF MICROBIAL, BIOCHEMICAL AND FOOD BIOTECHNOLOGY

Evaluation of Remediator product.

Prof. E van Heerden {PhD.} and Elizabeth Ojo (B.Sc Hons)
Department of Microbial, Biochemical and Food Biotechnology
University of the Free State
P.O. Box 339
Bloemfontein
9300

Introduction

Remediator is a dual purpose oil/fuel absorbent and bioremediation agent for use on direct spills or on hydrocarbon contaminated soil. The product contains naturally occurring microorganisms (no genetic modification or bioengineering).

When given a hydrocarbon food source and kept moist, the microorganisms propagate rapidly to break down the hydrocarbon contaminants into their harmless constituents.

Bioremediation of hydrocarbon is effectively achieved by bioaugmentation and to an extend biostimulation with/of micro-organisms in such an environment. This requires the micro-organisms to reach the contaminants and the aerobic micro-organisms used for this purpose needs adequate supply of oxygen. Also, optimum parameters needs to be considered and the addition of nutrients to the soil and moisture could be very important.

Furthermore, soil type, moisture content, contaminant type, contaminant level and clean-up criteria all vary from project to project.

Aims of this study:

1. Evaluated the microbial diversity of the Remediator product

2. Evaluated the microbial viability of the Remediator product

3. Evaluate the optimal design for all parameters for *in situ* application and elucidate the efficacy of decomposition of oil contamination.

339 BLOEMFONTEIN 9300
(051) 401-2472
REPUBLIEK VAN SUID-AFRIKA/ REPUBLIC OF SOUTH AFRICA
FAKS/FAX: (051) 444 3219 E-mail: vheerde.sci@ufs.ac.za

Materials and Methods

Microbiology

Classical cultivation

Enrichments of microbial populations were performed in presence and absence of oil. Enrichments were incubated at 22ºC for 24 h, 48 h and 10 days.

Staining and microscopy

Each of these cultures collected at different time intervals were stained using gram staining and DAPI staining and subsequently viewed under microscopes.

Microbial Diversity

16S rDNA analysis

Phylogenetic analysis and the creation of phylogenetic trees can be performed for all microbial groups *via* the alignments for Bacterial 16S rDNA genes. The 16S rDNA analysis is standard in the scientific community, has been widely used for phylogenetic studies and done with a fair amount of ease. This technique analyzes the sequence of ribosomal RNA molecules since sequence differences in hypervariable regions reflect strain variations (Amann *et al*., 1995). This is a molecular tool used to monitor bacterial diversity in complex microbial communities and does not depend on prior culturing of organisms.

Genomic DNA extraction was performed on the collected cultural samples using standard procedure. Bacterial 16S rRNA fragment was amplified by PCR and used to analyse the diversity or microbial population of the samples.

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Denaturing gradient gel electophoresis (DGGE)

DGGE (Degrading Gradient Gel Electrophoresis) is a very useful **FAST** technique in diversity studies. DGGE is one of a family of electrophoretic methods for separation of nucleic acids like DNA that rely on a gradient of denaturant to cause changes in structure based on a single base (Muyzer and Smalla, 1998). A change in banding pattern indicates a shift in the microbial population.

Identification of Bacteria

The DGGE bands obtained were reamplified using standard protocol in available in the laboratory, these were then cleaned up and sequencing of the PCR products was performed with the ABI 377 Genetic Analyser (Applied Biosystems).

Final Evaluation and Design of optimum parameters

Test scenarios

To 5 kg of soil, 50 g of oil was mixed thoroughly and 500 g of this mixture was measured into different flasks for inoculation.

The **contro**l contained 500 g of this mixture – Designated A

The second flask 500 g of the mixture and 50 g of Remediator – Designated B

The third flask contained 500 g of the mixture as well and 250 ml of sterile water and 50 g of Remediator – Designated C

At time zero (T_0), samples were taken, followed by 12 h reading, 48 h, 96 h, 1 week and 3 weeks sampling and then after 3 weeks samples were frozen for hydrocarbon breakdown further analysis.

The hydrocarbon degradation was evaluated by the Modderfontein Laboratory Services Pty Ltd. according to their standard protocols.

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Results and Discussions

Gram and DAPI staining and microscopy

There were no significant cells present in the 24 h water, 24 h water-oil cultures but few cells in 48 h TYG and TYG-oil cultures, *this is an important aspect to consider when applying Remediator since the initial tillage and effect is only absorbance and not active breakdown.*

However after a period of 10 days the culture improved dramatically. DAPI staining was used to confirm the presence of cells in these cultures and the results showed that the 10 days culture had many cells.

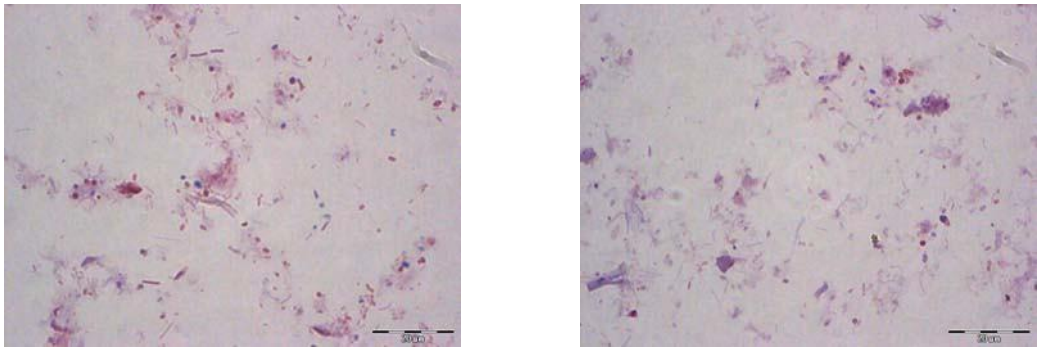

Figure 1. Gram staining from day 10 water culture.

DAPI staining

It could be important to note that the cell numbers can be stimulated immediately with the addition of an electron donor or simple carbon source, although water will help cell numbers. For example, significant biomass was obtained with the 10 day culture when stained with DAPI and viewed. Again the morphology in indicative of rods later confirmed by 16 S sequencing. However all other uses of Remediator showed active bacterial growth.

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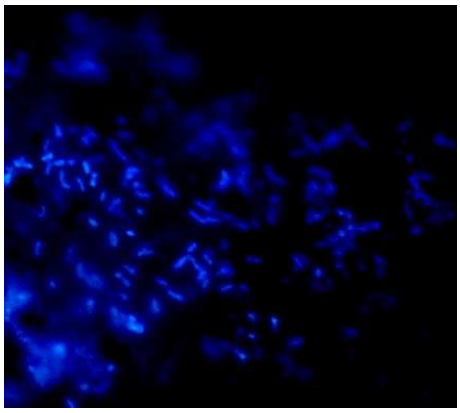

Figure 2. DAPI staining for day 10 culture.

16S rRNA analysis

The 16 S rDNA analysis is standard in the scientific community and done with a fair amount of ease. It is a molecular tools used to monitor bacterial diversity in complex microbial communities.

The basic steps in this phylogenetic approach includes (i) extraction of total DNA from a mixed community (including unculturable organisms), (ii) selective PCR-amplification of 16S rRNA gene fragments using universal primers, (iii) sequence analysis of defined fragments.

The concentrations of gDNA obtained were low in all. The gDNA extracted were amplified followed by evaluation on agarose gel and subsequent visualization by UV illumination to confirm the amplification. Figure 4 shows for example the amplification of 16S rRNA of gDNA extracted from cultures.

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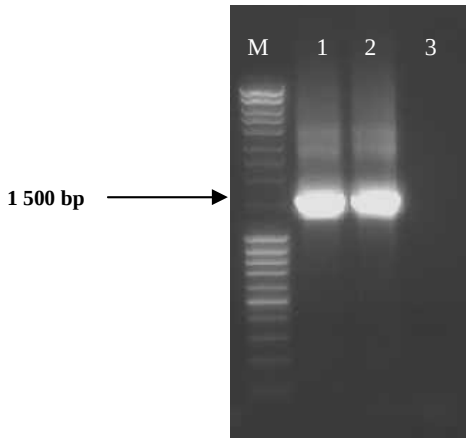

Figure 4. Amplification of the 16S rDNA fragment using genomic DNA as template. Lane M, MassRuler™ DNA ladder (Fermentas); Lane 1, gDNA extracted from day 10 water, Lane 2, gDNA extracted from day 10 TYG medium and Lane 3 non-template control.

Restriction Fragment Length polymorphism

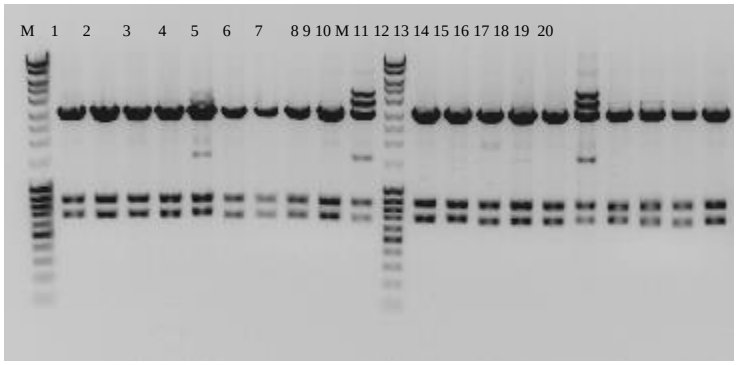

Figure 5. Shows RFLP patterns obtained using *EcoRI.* Lanes M, MassRulerTM DNA ladder (Fermentas), 1-10 (10 days water culture) and 11-20 (10 days TYG culture).

This data again confirms that the microbial population is specified rather than diverse.

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Denaturing gradient gel electophoresis (DGGE)

DGGE is one of a family of electrophoretic methods for separation of nucleic acids like DNA or RNA that rely on a gradient of denaturant to cause changes in structure. The high number of bands might indicate a high level of diversity.

The microbial population obtained from DGGE showed four bands (for 10 days water culture) and seven bands (for 10 days TYG). Hence, with enrichment medium (TYG), more species bacteria could be stimulated. However from the initial RFLP experiment and description of types of the species diversity is confirmed.

Figure 6. Shows bacteria bands obtained from DGGE. Lane 1, obtained from (10 days water culture) and Lane 2, (10 days TYG culture).

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Identification of microbes

The BLAST results obtained from the seven sequenced samples showed the presence of *Bacillus licheniformis*, Uncultured *Firmicutes* bacterium, Uncultured bacterium clone FFCH13000 16S ribosomal RNA, *Bacillus subtilis*

All microbiological experiments confirm the same results and as the product guide suggest there is a defined group associated with this product.

Viable count

This is essential to elucidate active participating communities and to identify what they are doing at any specific site.

Colonies counting were done on TYG plates after 24 h and 48 h incubation period and the viable count results were about 8×10^7 for 24 h and 8.55×10^7 for 48 h. Again looking at other products Remediator is on par if not better that other products in this market.

Appearance of colonies on Bushnell-Hass solid agar were monitored for every 24 h, significant colonies were obtained on the fifth day of incubation, these were counted and the results were about 15,000 on the fifth day, 18,000 on the sixth day. It was also noted that the bacterial population was stable for a number of days.

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Cell count and Hydrocarbon breakdown:

Parameter (hr)	A	B	C
0	5.10 x 10	2.50×10^3	4.35×10^6
12	3.00×10^3	6.00×10^5	7.00×10^5
48	5.00×10^3	1.30×10^6	9.00×10^5
96	1.75×10^4	2.45×10^6	2.00×10^6
168	5.00×10^3	6.5×10^5	4.0×10^5
504	50.50×10^3	60.50×10^3	2.1×10^4

A Control (soil + oil)
B Remediator + soil + oil
C Water + soil + oil + Remediator

The amount of bacteria increased over time in all analysis up to 96 hours, after this there is a proportional decline in the amount of active bacteria. Especially after day the first week but could be retained somewhat towards the end of the experiment (21 days).

Further analysis revealed that the Remediator product was most efficient in breakdown of the Hexane extractable hydrocarbons (1.30 [m/m] to 0.67 [m/m]) removing more than **50 %** of this fraction. The addition of water slowed the process but could over a longer period enhance the effects a future **25 %**, with slight enrichment if needed but not essential.

If there are any further queries do not hesitate to contact me directly.

Kind regards

Prof Esta van Heerden

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