Oil Sludge Degradation by *Lysinibacillus sphaericus* and *Geobacillus spp.* Strains

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Abstract: A study of oil sludge degradative capacity of *Geobacillus spp.* and *Lysinibacillus sphaericus* consortium was undertaken. Greenhouse experiments were undertaken using four different treatments-consortium inoculation or non inoculation- in closed or open containers. The bioremediation process was evaluated in terms of bacterial growth, total petroleum hydrocarbon (TPH) removal and volatile organic compound (VOC) concentration. The number of bacteria remained almost constant throughout the experiment, but presented a small decrease in the closed inoculated containers by the end due to recalcitrance of available compounds. The highest percentage of TPH removal was obtained in the open containers where the consortium had been added. Although the closed inoculated containers presented TPH removal to a lesser extent than the open inoculated containers, the percentage of TPH removal in closed inoculated containers was significantly different from the percentage of TPH removal in closed containers without inoculation, making the degradative capacity of the strains under study evident. Furthermore, important concentrations of volatile organic compounds were found in the headspace of closed containers in the beginning of the experiment, which drastically reduced by the end, especially in those containers where consortium had been added. Therefore, differences in removal efficiency between closed and open containers may have been caused by the possibility of volatilization of some of the VOCs in containers that are open to the atmosphere. This means that, the closed container processes is cleaner and safer.

Keywords: *Landfarming, bioremediation, L.sphaericus, Geobacillus, oil sludge*.

Introduction

Petroleum is a naturally occurring mix composed of hydrocarbons of high economic importance and other organic compounds. However its exploitation has brought with it ecological and social disasters due to the high toxicity, carcinogenicity and recalcitrance in the environment of the process byproducts (Zhang et al., 2012).

Over the past few decades, several technologies for the removal of pollutants from petrochemical industry processes have been developed; among them, *Landfarming* has frequently been chosen because of its low cost, high efficiency and because it has been considered environmentally friendly (Hejazi et al, 2003). Landfarming technology involves the use of natural biological, chemical and physical processes in the petroleum contaminated soil to transform the organic contaminants (Marin et al.,2005), but it has been proven that biological activity is responsible for most of the transformation of organic contaminants, which is why the process may sometimes be improved by the addition of microorganisms which may use hydrocarbons and organic compounds as a carbon source. Members of more than 60 genera of aerobic bacteria and 5 genera of anaerobic bacteria which have this capability have been described, including bacteria of the genus *Acinetobacter*, *Pseudomonas*, *Bacillus*, *Rhodococcus* and *Alcalinovorax* (Zhang et al., 2012).

Some *Geobacillus spp.* and *Lysinibacillus spp.* strains have been isolated from hydrocarbon contaminated soil (Zhang et al., 2012; Zheng et al., 2011; Bahuguna et al., 2011). However, their degradative capacity has not yet been evaluated. Besides, genome analysis of *Lysinibacillus sphaericus* strain OT4b.31 revealed the presence of heavy metal resistance clusters as well as some hydrocarbon degradation genes (Peña & Dussan, 2013).

Most bioremediation processes are conducted in aerobic conditions, which leads to the volatilization of certain organic compounds such as aromatic hydrocarbons (Hawthorne et al., 2000); and frequently, petroleum components with potential mutagenicity and carcinogenicity (Mao et al., 2012).

Hawthorne e*t al.*(2000) found that volatilization rather than degradation has a key role in soil remediation; therefore, volatile organic compounds (VOCs) emissions present a risk not only for the environment, but also for workers and nearby populations.

Some studies focusing on bioremediation and VOCs emissions have been undertaken using gram negative bacteria (Barbosa et al., 2012), but none using gram positive bacteria of the genus *Geobacillus spp.* or *Lysinibacillus spp.* The aim of this study was to evaluate the oil sludge degradative capacity of a consortium composed of *Lysinibacillus sphaericus* and *Geobacillus spp.* strains as well as VOC emissions in a landfarming process.

Materials and Methods

Oil sludge and soil:

Oil sludge and soil samples were collected from Palmarito, Departamento Casanare, Colombia (N. 5º 20´ 23,9" W 5º 20´ 23,9") The API grade of the oil sludge was 39.3.

Microorganisms and culture conditions:

The consortium was composed of five *Lysinibacillus sphaericus* strains and two *Geobacillus spp.* strains, detailed in Table 1.0, and was grown on Standard Plate Count agar (SPC) at 30°C for 24 h.

Table 1.0 Strains in the consortium

Strain	Isolated by
Geobacillus HC3H	Lozano et al., 2002
Geobacillus H_2VP_3	Lozano et al., 2002
L. sphaericus 2362	Delecluse et al., 1996
L.sphaericus CBAM5	Villegas et al., 2011
L.sphaericus III(3) 7	Lozano et al., 2002
L.sphaericus OT4b32	Dussan et al., 2002
L.sphaericus OT4b49	Dussan et al., 2002

Greenhouse experiments:

Twelve impermeable membrane coated containers with 1000 g of soil and 500 ml of oil sludge each, were placed in a greenhouse with an average temperature of 30 ± 5°C (Annexes: Figure 1.0-4.0). Four different treatments were evaluated. In the first and third, bacterial consortium was added to the soil, but the third was totally coated with impermeable membrane, whereas the first was left open to the atmosphere. In the second and fourth, no inoculation was made, but again, the fourth was totally coated with the impermeable membrane, whereas the second was left open to the atmosphere. All the variations of the experiments were replicated three times. Samples from all experiments and headspace samples from closed containers were taken on day 8, 15 and 50 to evaluate hydrocarbon degradation, bacterial growth and concentration of volatile organic compounds.

Bacterial Growth:

Bacterial growth was monitored by SPC agar of 10^{-2} to 10^{-7} dilutions. The count was undertaken after incubation at 30°C for 24 h.

Hydrocarbon degradation:

Determination of hydrocarbon was made by gas chromatography with a flame ionization detector (GC-FID). Concentrations were calculated by area under peaks.

Determination of volatile organic compounds in containers:

Samples were taken by injecting a needle and extracting the headspace of the containers in a syringe. These were then analyzed using High Performance Liquid Chromatography with a Diode Array Detector (HPLC-DAD). Concentrations were calculated by area under peaks.

Statistical analysis:

A significance value of 0.05 was chosen in all analyses performed. Statistical significance was evaluated by analysis of variance (ANOVA) and Tukey-Kramer test using R project 3.0.2.

Results and Discussion

Bacterial growth throughout the experiment is shown in Figure 1.0. As shown on the graph, the number of bacteria remained almost constant over the first 50 days of the experiment in all four variations. A significant decrease is seen in treatment III (closed container and bacterial consortium) on day 50, which

can be explained by the fact that by the end of the experiment more recalcitrant compounds become available, so microorganisms need more time to degrade them and, therefore, growth is not so stimulated as much.

Although containers under treatment II and IV were not inoculated, they presented a bacterial title of around 10^4, which can be due to oil sludge resistant microorganisms that were either in the soil before mixing it with oil sludge or in the oil sludge itself, given that they were no sterilized prior to the experiment.

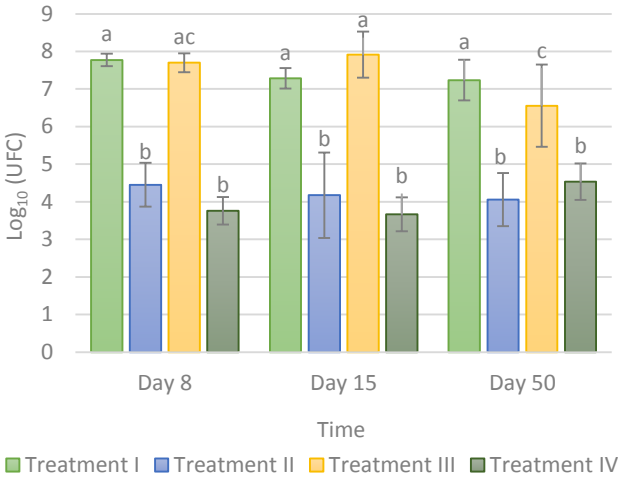

Figure 1.0. Bacterial growth on day 8, 15 and 50. The data are the means of three replicates. Error bars represent standard deviation. Means with the same letter are no significantly different.

Total petroleum hydrocarbon (TPH) concentrations at Day 8, 15 and 50, as well as the percentage of degradation- calculated using initial and final concentration-are shown in Table 2.0. A significant difference is observed in TPH concentration between treatments I and II, treatments I and III and treatments I and IV, but no difference is observed between treatment II and treatment III, i.e between experiments in open non- inoculated containers and experiments in closed inoculated containers, which suggests that removal due to biodegradation occurs to a comparable extent than removal due to volatilization.

Besides, closed inoculated containers (treatment III) do not show a greater decrease in TPH concentration than open inoculated containers (treatment I). This strongly suggests that volatilization losses may be playing a key role in the bioremediation process. Furthermore, it can easily be shown that added microorganisms improve the process efficiency because treatment I and treatment III present a greater decrease than treatment II and treatment IV, respectively, which were no subjected to the addition of the different *Lysinibacillus sphaericus* and *Geobacillus spp.* strains (Table 1.0).

By the end of the experiment, 98.72% and 26.40% of TPH removal were detected in treatment I and III, respectively, while removal percentage for treatments II and IV were 30.06% and 7.02% respectively, which makes the degradative capacity of applied bacterial consortium evident. Net removal percentages due to biodegradation by consortium were calculated by subtracting the removal percentage of open containers without inoculation from that of open inoculated containers. Similarly, net removal percentages due to biodegradation in closed containers were calculated by subtracting the removal percentage of closed containers without inoculation from the values of the removal percentage of closed inoculated containers.

Table 2.0 Total Petroleum Hydrocarbons (TPH) concentration on day 8, 15 and 30 for each treatment.

Treatment	TPH Concentration (ppm)			Removal (%)
	Day 8	Day 15	Day 50	
I	36,667 ± 2081[a]	666.67 ± 208.2[a]	466.67 ± 115.5 [a]	98.72
II	54,333 ± 3511 [b]	48,667 ± 1527 [b]	38,000 ± 6,082 [b]	30.06
III	59,333 ± 4163 [b]	54,000 ± 4359 [b]	43,666 ± 2,309 [b]	26.40
IV	76,000 ± 2000 [c]	72,000 ± 2646 [c]	70,666 ± 2,516 [c]	7.02

Data are presented as means ±SD. Means in the same column with a letter in common are not significantly different among treatments (significance level= 0.05)

Table 3.0. Benzene, toluene, ethylbenzene and phenol concentration in parts per million (ppm) in headspace of containers under treatments III and IV.

	Treatment	Concentration (ppm)			
		Toluene	Ethylbenzene	Benzene	Phenol
Day 8	III	12,297 ± 543.0 [a]	9,121 ± 368.5 [a]	8,779 ± 202.0 [a]	23,546 ± 1000.8 [a]
	IV	8,629 ± 67.00 [b]	5,050 ± 129.6 [b]	5,374 ± 273.5 [b]	28,012 ± 235.5 [b]
Day 15	III	2,679 ± 330.1 [a]	1,474 ± 221.1 [a]	0.86 ± 0.15 [a]	2.38 ± 0.11 [a]
	IV	7,522 ± 317.0 [b]	4,125 ± 182.2 [b]	5,219 ± 35.57 [b]	20,362 ± 466.53 [b]
Day 50	III	430 ± 110 [a]	728 ± 212 [a]	0.283 ± 0.148 [a]	2.17 ± 0.204 [a]
	IV	4,276 ± 390 [b]	3735 ± 181 [b]	4,686 ± 244 [b]	19,503 ± 1,140 [b]

Data are presented as means ±SD. Means in the same column on the same day, with a letter in common are not significantly different among treatments (significance level= 0.05).

Thus, net degradation percentages due to biodegradation by consortium are 68.66% and 19.38% for processes in open containers and closed containers, respectively. Differences in available oxygen can be responsible for differences in hydrocarbon degradation rate in open and closed containers.

Toluene, ethylbenzene, benzene and phenol concentrations in the headspace of the totally closed containers, i.e. treatment III and IV, are shown in Table 3.0, which shows that toluene, ethylbenzene, benzene and phenol concentrations on day 8 are higher in biocontainers with treatment III than in biocontainers with treatment IV; and on day 15 the opposite situation is observed. This can be explained by the higher degradative rate when treatment III is applied. On day 50, concentrations of VOCs in containers under both treatments are the lowest of the entire experiment.

Initial and final concentrations of each organic compound shown in Table 3.0 are significantly different in both treatments; however, containers under treatment III present the smallest concentrations by the end of the experiment. Removal percentages of each compound listed on Table 3.0 are shown in Figure 2.0. It is easily seen that highest removal percentages are in those containers under treatment III. Therefore, it is possible to infer that the consortium under study is also able to properly degrade confined volatile organic compounds. Degradation of volatile organic compounds in closed containers under treatment IV, where no inoculation occurred, may be caused by the presence of soil or oil sludge native microorganisms since containers were not sterilized.

As predicted, volatile organic compound concentrations in the headspace of containers is comparable to that of TPHs in soil. This is why open containers had the greatest removal efficiency, but this removal does not necessarily imply bacterial degradation, given that part of the petroleum hydrocarbons are being released into the atmosphere. Hence, in spite of the fact that bioremediation processes in closed containers are not as efficient as those conducted under aerobic conditions, most of the removal is by means of degradation, meaning that processes in closed containers are cleaner, safer and environmental friendly.

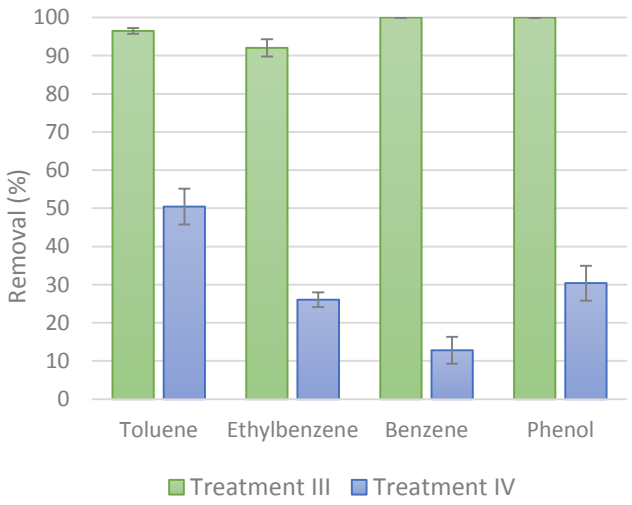

Figure 2.0. Removal percentage of VOCs in containers under treatment III and IV. The data are the means of three replicates. Error bars represent standard deviation.

Conclusions

The oil sludge degradative capacity of a consortium composed of two *Geobacillus spp.* and five *Lysinibacillus sphaericus* strains (Table 1.0) was studied. Bioremediation processes in closed and open containers with or without inoculation were performed. Both inoculated open and closed containers presented a TPH removal percentage that was higher than non-inoculated open and closed containers respectively, which make the oil sludge degradative capacity of the strains under study evident.

The highest total petroleum hydrocarbon removal efficiency was obtained in inoculated containers that were open to the atmosphere. However, high concentrations of volatile organic compounds were found in the headspace of closed containers, where TPH removal was observed to a lesser extent, strongly suggesting that most of the TPH removal in open containers happens by means of volatilization and releasing into atmosphere, rather than degradation.

Concentration of organic volatile compounds decreases in the headspace of closed containers throughout the experiment, especially in containers where inoculation was applied. It is therefore possible to infer that the consortium is also able to degrade confined VOC, which prevents their emission into the atmosphere. Thus, despite being slower, bioremediation processes conducted in inoculated closed containers are safer and cleaner than those conducted under aerobic conditions.

Further research

The consortium here studied shows outstanding TPH removal percentages in a very short time. However, greenhouse conditions differs from conditions in the field, where process are typically conducted. So, it is necessary to evaluate the applicability of this consortium in the field by studying its behavior in field processes. It would also be valuable to have a better understanding of the changes in the microbial structure, in order to try to optimize the process.

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Annexes

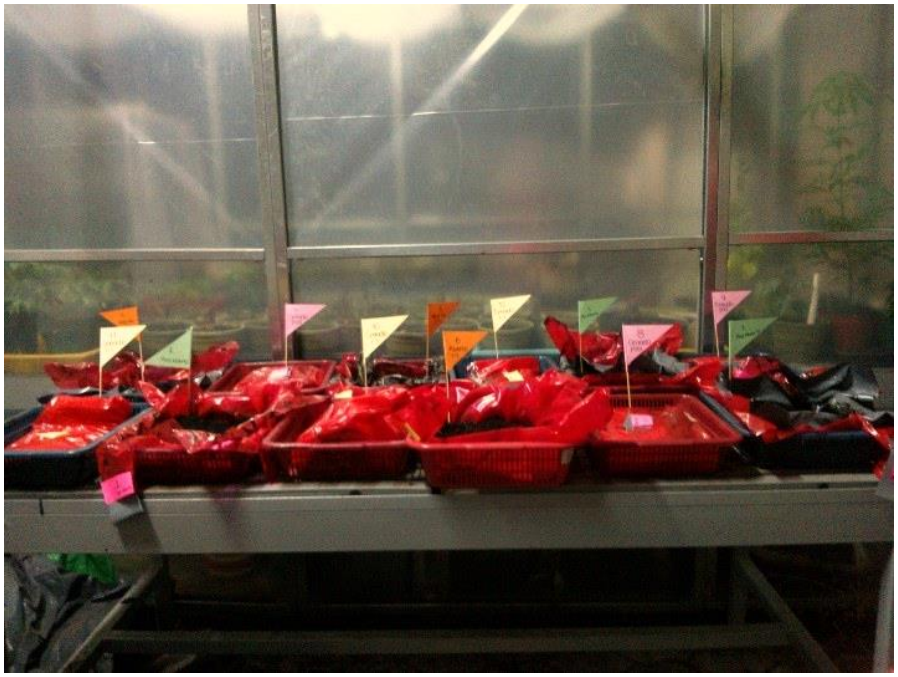
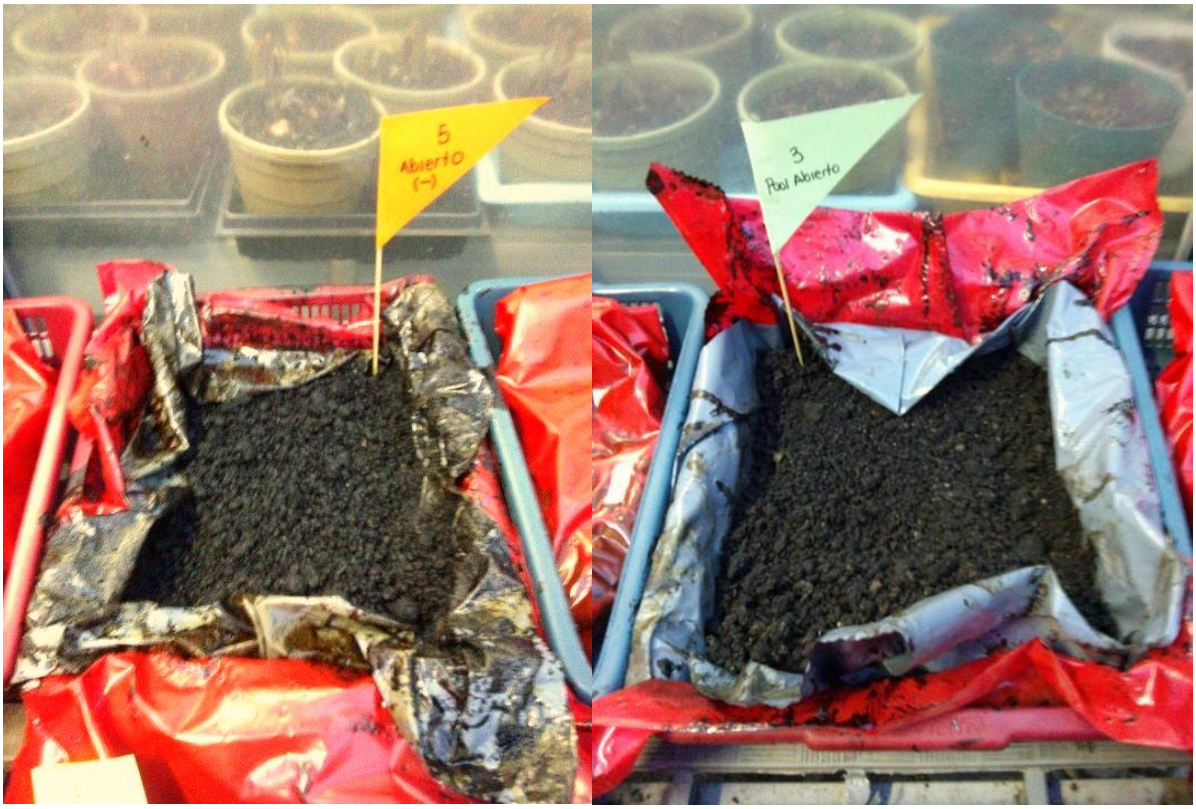


Figure 1.0 Closed and open containers under treatment in greenhouse (Frontal view)

Figure 2.0 Closed and open containers under treatment in greenhouse (Side view)

Figure 3.0 Closed container



a) b)

Figure 4.0 a) Open non inoculated container and b) open inoculated container on day 8.