***Lysinibacillus sphaericus* and *Geobacillus sp* biodegradation of petroleum hydrocarbons and biosurfactant production.**

Manchola, Lina¹ & Dussán, Jenny¹.

¹ Centro de Investigaciones Microbiológicas – CIMIC, Department of Biological Sciences, Universidad de los Andes, Bogotá, Colombia.

Correspondence to: jdussan@uniandes.edu.co

Abstract

Petroleum oil is a major driver of worldwide economic activity, but it has also created contamination problems during the storage and refining process. Also unconventional resources are natural resources, which require greater than industry-standard levels of technology or investment to exploit. In the case of unconventional hydrocarbon resources, additional technology, energy and capital have to be applied to extract the gas or oil. Bioremediation of petroleum spill is considered of great importance due to the contaminating effects on human health and the environment. For this reason it is important to reduce total petroleum hydrocarbons (TPH) in contaminated soil. In addition, biosurfactant production is a desirable property of hydrocarbon-degrading microorganisms. Seven strains belonging to *Lysinibacillus sphaericus* and *Geobacillus sp* were selected to evaluate their ability to biodegrade TPH in the presence of toxic metals, their potential to produce biosurfactants and their ability to improve the biodegradation rate. The seven bacterial strains examined in this study were able to utilize crude petroleum-oil hydrocarbons as the sole source of carbon and energy. In addition, their ability to degrade crude oil was not affected by the presence of toxic metals, such as chromium and arsenic. At the same time, the strains were able to reduce toxic metals concentration through biosorption processes. Biosurfactant production was determined using the drop-collapsed method for all strains and they were characterized as both anionic and cationic biosurfactants. Biosurfactants showed an increase in biodegradation efficiency both in liquid minimal salt medium and landfarming treatments. The

final results in field tests showed an efficiency of 93 percent reduction in crude oil concentration by the selected consortium compared to soil without consortium. The authors propose *L. sphaericus* and *Geobacillus* sp consortium as an optimum treatment for contaminated soils. In addition, production of biosurfactants could have an application in the extraction of crude oil from unconventional hydrocarbon resources.

Keywords: *Lysinibacillus sphaericus*, *Geobacillus* sp, Biodegradation, Total petroleum hydrocarbons (TPHs), Biosurfactants, Toxic metals.

Introduction

Petroleum is a major driver of worldwide economic activity, but it has also created major pollution problems due to repeated accidental spills during petroleum transport and storage during the refining process (Hamed et al., 2013). Petrochemical industries produce huge amounts of solid and liquid wastes, in the form of alkanes, cycloalkanes, aromatic compounds and toxic metals. These compounds are potentially dangerous to human health and environment. (Kaufmann et al., 2004) (Marín et al., 2006.). Crude oils are classified in light, medium and heavy oils according to the API grade, which is a measure of density compared to water. Typically, a heavy crude oil is considered more difficult to degrade than a light or medium crude oil.

Consequently, petroleum companies are typically required reduce the total petroleum hydrocarbons (TPH) present in soil as a result of crude oil spills. Many physical and chemical methods can reduce toxic petroleum constituents, such as incineration, chlorination, ozonation, and combustion, but they unfortunately require elaborate facilities and substantial amounts of energy. Moreover, these processes, especially the chemical ones, produce intermediates that may present toxicity levels similar to the original substances (Tomei et al., 2003).

The removal of heavy metals is also a significant challenge to the remediation industry worldwide, as many industrial processes such as battery manufacturing, leather tanning and the use of pesticides in agriculture systems contribute to toxic metal contamination. (Lozano, 2012)

Bioremediation is considered a low cost and environmental friendly solution to remediate petroleum and metal contamination. Studies of biodegradation processes have demonstrated that various species belonging to different genera, such as *Achromobacter*, *Alcaligenes*, *Azospirillum*, *Bacillus*, and *Pseudomonas* are capable of utilizing TPH as a sole carbon source (Hamed et al., 2013). These genera are capable of degrading crude oil in pure and mixed cultures with natural or constructed bacterial consortiums.

Biosurfactants are amphiphilic active molecules produced by microorganisms, such as bacteria, fungi, and yeast. They are complex organic molecules varying in their chemical properties, molecular size and chemical composition. (Pradhan et al., 2012). These molecules have a hydrophobic and a hydrophilic moiety that reduces the surface tension and interfacial tension between liquids, solids, and gases. Therefore, the ability of bacteria to degrade soil pollutants, such as hydrocarbons, is useful for bioremediation purposes and their ability to emulsify hydrocarbons has been studied in this respect, because emulsified petroleum is considered easier for bacteria to degrade (Ganesh & Lin, 2009). The low toxicity and high biodegradability of biosurfactants make them environmentally acceptable and good candidates for enhanced oil recovery. (Zheng et al., 2011)

The remediation of contaminated soils with toxic metals and the biodegradation of TPH have not been related, because toxic metals can inhibit TPH biodegradation. Heavy metals appear to affect organic pollutant biodegradation by interfering both with the physiology and ecology of organic degrading microorganisms, thus, imposing a double stress on the microbial populations. This

effect was demonstrated in a study where As^{3+} , Cd^{2+} , Hg^{2+} , and Pb retarded the biodegradation rate of an aromatic hydrocarbon. (Olufolahan et al, 2011)

The genus *Bacillus* is a large, diverse collection of aerobic and facultative anaerobic, gram-positive bacteria (McMullan et al., 2004). *Geobacillus* is a widely distributed thermophilic bacillus genus and has been identified in all continents. Industrial interest in *Geobacillus* species has arisen from their potential applications in biotechnological processes, for example, as sources of various thermostable enzymes, such as proteases, amylases, and lipases (Zheng, 2011). Moreover, previous studies in *Geobacillus* sp. have demonstrated the potential of strain H2OVP3 to produce anionic biosurfactants (Niño & Dussán, 2013) as well as the capacity of *Geobacillus thermodenitrificans* to remove toxic metals from wastewaters (Chatterjee et al., 2010).

On the other hand *Lysinibacillus sphaericus* (Ls), commonly isolated from soil and water, has the potential to bioremediate sites with toxic metals. Studies have demonstrated the ability of *L. sphaericus* to biosorb chromium, copper, lead, iron, and cobalt (Peña & Dussán, 2013) (Velasquez & Dussán, 2009). It has also been shown to control mosquitos from genera *Culex*, due to the ability of some strains to produce a binary toxin that causes mortality in mosquitos' larvae (Dussán et al., 2013).

The recent sequencing of *L. sphaericus* Ot4b31 genome (Peña-Montenegro & Dussán, 2013) and resequencing of *L. sphaericus* CBAM5 genome (Peña-Montenegro et al., 2014) and KEGG (Kyoto Encyclopedia of Genes and Genomes) analysis, identified proteins that might participate in peripheral pathways for degradation of benzoate, amino benzoate, toluene, and other aromatic compounds, which might be an indication of the ability of this genera to biodegrade TPH.

The region of Casanare in Colombia is an important petrochemical location with substantial petroleum contamination. The crude oils selected for this study are from: the Palmarito station (PAL) (North 5° 20' 23,9" and West 71° 14' 29,4") a light crude oil with 39,2 ° API, Trinidad (TRD) (North 5° 30' 30,4" and West 71° 45' 37,0") a medium crude oil with 33,4 ° API and La

Gloria (LGL) (North 5° 01' 41,3" and West 72° 21' 35,3") a heavy crude oil with 14,5 ° API. These stations are all located in Casanare-Colombia (Personal communication – Engineer Hugo Vladimir Ramirez Biointech E.U).

The purpose of this study was to evaluate the *in silico* results obtained by the genomes sequencing and the ability of *Lysinibacillus sphaericus* and *Geobacillus sp* to reduce TPH concentration from light (Palmarito) and medium crude oil (Trinidad) in the presence of toxic metals, as well as to evaluate the potential of both microorganisms to produce biosurfactants.

Materials and Methods

Bacterial Strains and Crude Oil Degradation Screening

Fourteen strains were previously isolated from a variety of Colombian sites (Exhibit 1). The strains were selected from the Culture Collection maintained by the Center of Microbiological Research's (CIMIC) at the Universidad de los Andes (Bogotá, Colombia), based on previous studies (Dussán et al., 2002, Lozano et al., 2002, Villegas et al., 2011, Berry & Hindley, 1987). The isolates belonged to *Lysinibacillus sphaericus* and *Geobacillus sp*. Each strain was sub-cultured in nutrient agar and incubated at 30°C for 48 hours. Subsequently, a selection pressure in liquid medium was conducted (Using crude oil as a sole carbon source). An inoculum of 10⁷ cells was incubated at 30°C in 30 ml of liquid minimal salts medium (MSM), with a composition of KH₂PO₄ (0.5 g/L), Na₂SO₄ (2.0 g/L), KNO₃ (2.0 g/L), CaCl₂ (0.001 g/L), MgSO₄ (1.0 g/L) and FeSO₄ (0.0004 g/L). Crude oils obtained from the Trinidad (API° 34.5) and Palmarito (API° 39.3) petroleum stations in Casanare-Colombia (donated by Hugo Vladimir Ramirez-Biointech E.U), were tested at a 1 percent concentration as the sole carbon source. After 25 days of incubation, a reduction of crude oil concentration was observed.

Exhibit 1. *Lysinibacillus sphaericus* and *Geobacillus sp* strains pre-selected for the study. Strains provided by the Culture Collection Center of Microbiological Research's (CIMIC) from Universidad de los Andes.

Strains	Strains
<i>Geobacillus</i> HC3H	<i>L. sphaericus</i> OT4b26
<i>L. sphaericus</i> 2362	<i>L. sphaericus</i> OT4b39
<i>L. sphaericus</i> CBAM5	<i>L. sphaericus</i> OT4b49
<i>L. sphaericus</i> NRS400	<i>L. sphaericus</i> IV(7)11
<i>L. sphaericus</i> IV (4)10	<i>L. sphaericus</i> OT4b31
<i>L. sphaericus</i> III(3) 7	<i>L. sphaericus</i> OT4b32
<i>L. sphaericus</i> ATC14577	<i>Geobacillus</i> H2OVP3

Synchronization of Cultures

To ensure that all strains were in the same metabolic state, the method reported by Ortiz et al. (2007) was used to obtain synchronized cultures, starting with a 16-h overnight Nutrient Agar culture, after 0.3 ml of culture were cultivated in acetate broth (sodium acetate 5.00 g/l, yeast extract 3 g/l, MgCl_2 1×10^{-3} M, CaCl_2 7×10^{-4} M and MnCl_2 5×10^{-5} M) and incubated on a rotary shaker at 30 °C and 150 rev/min over 72 hours. Subsequently, 1 ml of acetate culture was subjected to thermal shock (90°C for 20 min). This cycle was performed 7 times, until 90 percent of the cells were observed via microscopy to have sporulated.

Determination of Crude Oil Degradation in Liquid Pressure

Tests were carried out for Trinidad and Palmarito individually and in consortium to quantitatively evaluate the TPH reduction. Each strain 10^7 number of microorganisms (concentration) was placed in a tight-lidded glass flask with 30 ml of liquid MSM and crude oil at a concentration of 1 percent. The test for each strain had three replicate flasks. Additionally, an abiotic control was carried out in triplicate for Trinidad and Palmarito crude oil. A consortium was formulated by

inoculating equal proportions of all seven strains in a flask in the same conditions used for the individual tests. The consortium also had three replicate flasks. All flasks were incubated at 30 °C for 25 days. Samples were analyzed for TPHs using gas chromatography / mass spectrometry (GC/MS).

Determination of TPH Degradation in the Presence of Toxic Metals and Quantification of Free Toxic Metals.

To evaluate the ability of each strain and consortium to degrade crude oils and simultaneously capture toxic metals, tests were carried out for Trinidad and Palmarito crude oil individually and in consortium, as well as for two toxic metals: arsenic and chromium. Each strain was placed in a tight-lidded glass flask with 30 ml of liquid MSM with 0.1 ml of crude oil and 294,185 milligrams per liter (mg/l) of $K_2Cr_2O_7$, except in the case of 2362 strain, which is not tolerant to chromium (Lozano & Dussán, 2013). The arsenic solution used was 6,000 mg/l of KH_2AsO_4 , except for the 2362 strain, where the concentration was 4,000 mg/l (Lozano & Dussán, 2013). Each strain had three replicate flasks. Additionally, an abiotic control was carried out in triplicate for both the Trinidad and Palmarito toxic metal crude oil tests. A consortium was formulated by inoculating equal proportions of all seven strains in a flask in the same conditions as the individual tests. The consortium also had three replicate flasks. All tests were incubated at 30 °C for 25 days. Samples were analyzed for TPH reduction by gas chromatography. The free metal concentration in the medium was estimated by using the determination of specific ions in Spectroquant Nova 60 photometer with specific kits for determining chromium and arsenic. The kits used were: the 101747-arsenic test and the 114758-chromium test produced by Merck Millipore Group (Bogotá, Colombia).

Screening for Biosurfactant Production

Continuous assays were performed following the method reported by Niño & Dussan (2014) to determine the maximum growing temperature for the seven strains tested. The strains were incubated on nutrient agar under the temperatures of 30°C, 35°C, 37°, 40°C and 42°C. The strains were maintained at each temperature for 24 hours. After determining the highest growing temperature for all strains, an assay for inducing the production of biosurfactant was developed.

An inoculum of 10^7 cells was placed in 50 ml of MSM with the following composition: KH_2PO_4 (0.5 g/L), Na_2SO_4 (2.0 g/L), KNO_3 , (2.0 g/L), CaCl_2 (0.001 g/L), MgSO_4 (1.0 g/L) and FeSO_4 (0.0004 g/L). Then 0.5 ml of crude oil was added to the MSM. Trinidad and Palmarito assays were carried out individually for each strain and consortium. After 25 days of incubation at 42°C, liquid medium without crude oil was lyophilized for 2 days. The lyophilized mass of each strain was resuspended in 1 ml of distilled water with a final concentration of 50X.

Qualitative Tests

A modified drop-collapsed method was performed (Pradhan, 2012) to evaluate the biosurfactants production. 0.2 ml of water was placed on a small petri dish and 0.1 ml of heavy crude oil from the La Gloria station was placed over the water. Then, 100 ul of biosurfactant solution were dropped over this assembly. The test was carried out for each strain and consortium using heavy crude oil from La Gloria station. Water was used as a negative control and sodium dodecyl sulfate (SDS) was used as a positive control, as this is a known anionic surfactant used in many cleaning and hygiene products, as well as in molecular biology where the compound is used in preparing proteins for electrophoresis (Hilton et al., 2011).

Quantitative Tests and Biosurfactant Characterization

The biosurfactant solution produced by each strain and consortium were characterized using kits (Merck Millipore Group; Bogotá, Colombia) to determine anionic, cationic, and non-ionic biosurfactants. A quantity of 0,1 ml of biosurfactant solution, was diluted with 5 ml of distilled water and then each kit was applied. Tests were read using a Spectroquant Nova 60 to determine the composition and concentration of biosurfactants in each sample. The methods are analogous to APHA 5540 C for surfactant anionic test, DIN 38409 H2O for cationic surfactants test and APHA for 5542 C for non-ionic surfactants tests by Merck Millipore Group.

Determination of Biosurfactants Efficiency in Heavy Crude Oil Biodegradation.

The effectiveness of biosurfactants in crude oil degradation was evaluated by performing four types of tests. Test 1 involved the consortium (10^7 cells) and crude oil from La Gloria (2 percent concentration), test 2 involved the consortium, and biosurfactant produced by the consortium with oil crude from Palmarito (250 ul) and crude oil from La Gloria (2 percent concentration), test 3 involved the consortium, biosurfactant produced by the consortium with oil crude from Trinidad (250 ul) and crude oil from La Gloria (2 percent concentration) and test 4 was the positive control with consortium, SDS and crude oil from La Gloria (2 percent concentration). These treatments were carried out in liquid MMS medium (25 ml) and Landfarming method (Contaminated soils, sediments, or sludges are incorporated into the soil surface and periodically turned over or tilled to aerate the mixture) using 20 g of soil in each treatment. All treatments were carried out in triplicate and incubated at 30 °C for 15 days. Samples were analyzed for TPH reduction by gas chromatography / mass spectrometry (GC/MS).

Biodegradation of Petroleum Hydrocarbons in Soil (Open Field)

The ability of the bacterial strains to remediate the petroleum crude oil contaminated soil was investigated by carrying out a pilot-scale biodegradation experiment in an open field in Yopal, Casanare, Colombia, a region of crude oil exploitation, belonging to the French Company Perenco. The consortium was produced on nutrient agar in a quantity of 10^9 cells per cm^3 . For this experiment four treatments with crude oil were performed: the first used the bacterial consortium and was totally coated with plastic impermeable coat (IC), the second used the bacterial consortium and was left open to the atmosphere (IO), the third was a control without the bacterial consortium and was totally coated with plastic coat (WC), and the last one did not use the bacterial consortium and was left open to the atmosphere (WO). After four months, three replicates of each treatment were analyzed to know the concentration of TPH by gas chromatography / mass spectrometry (GC/MS), also the bacterial counts were performed with serial dilutions and cultivated in triplicate.

Results and Discussion

3.1 Selected Bacterial Strains

After 25 days of incubation some strains showed a reduction in crude oil concentration in contrast to the abiotic controls (Exhibit 2). The best results were obtained by seven strains that are described in Exhibit 3. The criterion for choosing the final strains for the whole study was observe the reduction of crude oil until we observed remaining fragments of crude oil. The final results were compared to abiotic controls without active microbial activity.

Exhibit 2. a) Qualitative results of crude oil degradation from Palmarito and Trinidad by III(3)7, Ot4b32 and Ot4b49 strains, after 25 days. b) Abiotic controls from Palmarito and Trinidad crude oil.

Exhibit 3. *Lysinibacillus sphaericus* and *Geobacillus sp* strains selected for the study. Center of Microbiological Research's (CIMIC) from Universidad de los Andes.

Strains	Source
<i>Geobacillus</i> HC3H	Lozano et al, 2002
<i>L. sphaericus</i> 2362	Donated by Delecluse
<i>L. sphaericus</i> CBAM5	Villegas et al, 2011
<i>L. sphaericus</i> III(3) 7	Lozano et al, 2002
<i>L. sphaericus</i> OT4b49	Dussán et al, 2002
<i>L. sphaericus</i> OT4b32	Dussán et al, 2002
<i>Geobacillus</i> H2OVP3	Lozano et al, 2002

Determination of Crude Oil Degradation in Liquid Pressure

After 25 days of incubation, samples were analyzed and showed a high efficiency of degradation by all strains (Exhibit 4). Strains showed a degradation efficiency of between 94 and 99 percent in

contrast to the efficiency levels of the abiotic control. Efficiency of degradation was calculated by the formula $E = 100 - R\%$, where $R\%$ was the remaining crude oil % in each sample.

All data were analyzed by SPSS Statistics program (SPSS, 2013). Analysis showed that there are not significant differences between strains but there is a significant difference between all strains and controls in both types of crude oils (Mann Whitney test, $p=0.001$, $\alpha=0.05$). The degradation rate was similar in Palmarito and Trinidad crude oils and there was not a significant difference between them (Mann Whitney test, $p\text{-value}=0.082$, $\alpha=0.05$), which may indicate that all strains have the same ability to degrade low and medium oil crudes. The consortium did not differ significantly from the whole set of strains, indicating that all strains contributed in same proportions to the degradation rate by pool (Mann Whitney test, $p=0.060$, $\alpha=0.05$).

Exhibit 4. Efficiency of Trinidad crude oil and Palmarito crude oil degradation, as a sole source of carbon for the seven strains and consortium.

Determination of Crude Oil Degradation in the Presence Of Toxic Metals and Quantification of Free Toxic Metals.

After 25 days of incubation, all strains showed a high efficiency of crude oil degradation in the presence of both toxic metals (Exhibit 5, 6, and 7), indicating that biodegradation of crude oils was not inhibited by chromium or arsenic in Palmarito and Trinidad crude oil in the presence of chromium. Results of Palmarito crude oil in the presence of arsenic could not be determinate, due to the microorganisms sporulated and it was not possible to separate crude oil from spore agglomeration. In terms of metal concentration in the medium, the statistical analysis showed that there are not significant differences between strains but there is a significant difference between all strains and controls in both types of crude oils with chromium and arsenic, except for strain *Geobacillus* H2OVP3 which showed a significant difference compared to other strains in removing arsenic with Palmarito crude oil ($p\text{-value} = 0.003$, $\alpha = 0.05$). This indicates that microorganisms were able to remove chromium and arsenic from the medium. (Exhibit 5, 6, and 7).

The results of this assay are consistent with other published studies in which heavy metal absorption is reported in Gram-positive cell walls and membranes (Choudhary & Sar, 2009). Heavy metal adsorption by Gram-positive bacteria such as *Bacillus* has been presumed to exhibit a large heavy metal binding capacity to environmental microbial communities and is considered an alternative method for recovering metals from wastewaters (Lin et al., 2012). In *Lysinibacillus sphaericus* this property is principally attributed to the S layer protein that is capable to capture toxic metals, as it has been demonstrated that dead cells of *L. sphaericus* show biosorption of arsenic, cobalt, copper, chromium and lead (Tuzen et al., 2007). Previous studies have determined that live and dead cells of *L. sphaericus* OT4b31 and IV(4)10 display the ability to remove chromium (Velasquez & Dussán, 2009). It has also been proposed that the biosorption of chromium is due to the S-layer protein extracted from the OT4b31 strain (Velasquez, 2009;

Lozano & Dussán, 2013). However this is the first report on crude oil degradation by *L. sphaericus*.

Exhibit 5. Palmarito crude oil degradation and free chromium concentration, for the six strains and consortium. (Solid line and dots represents free chromium concentration, whereas the bars represent crude oil degradation efficiency)

Exhibit 6. Trinidad crude oil degradation and free chromium concentration, for the six strains and consortium. (Solid line and dots represents free chromium concentration, whereas the bars represent crude oil degradation efficiency)

Exhibit 7. Palmarito crude oil degradation and free arsenic quantification for the seven strains and consortium. (Solid line and dots represents free chromium concentration, whereas the bars represent crude oil degradation efficiency)

Screening for Biosurfactant Production

Qualitative Test

The drop-collapsed method confirmed that all strains produced biosurfactants, after adding the biosurfactants in the middle of the crude oil; an instant dispersion of oil crude was observed (Exhibit 8).

Exhibit 8. Drop collapsed method with heavy crude oil. a) Before adding biosurfactants. b) After adding biosurfactants.

Quantitative Tests

The results from using the kits to analyze the biosurfactants solutions showed that all strains produced anionic and cationic biosurfactants and none produced non-ionic biosurfactants (Exhibit 9). Strain CBAM5 showed the highest concentration of anionic biosurfactants with Palmarito crude, strain 2362 showed the highest concentration of anionic biosurfactants with Trinidad crude oil, strain H2OVP3 showed the highest concentration of cationic biosurfactant with Palmarito crude oil and strain 2362 showed the highest concentration of cationic biosurfactant with Trinidad crude oil. All strains showed a higher concentration of biosurfactants with Trinidad crude oil than

biosurfactants produced with Palmarito oil. This may be because Trinidad crude oil exhibits higher pressure to microorganisms, which causes them to produce a higher biosurfactant concentration that is solubilized more rapidly than the other crude oils. This is the first instance of reported on biosurfactant production by *L. sphaericus*.

Exhibit 9. Characterization of biosurfactants produced with Palmarito (Pal) and Trinidad (Trn) crude oil in 50 ml of MMS.

Strains	Anionic mg/l		Non ionic mg/l		Cationic mg/l	
	Pal	Trn	Pal	Trn	Pal	Trn
III(3)7	565	1370	<0,1	<0,1	110	250
2362	440	1400	<0,1	<0,1	105	695
CBAM5	660	970	<0,1	<0,1	80	225
GHC3H	560	1300	<0,1	<0,1	130	640
Ot4b32	615	1305	<0,1	<0,1	125	540
Ot4b49	530	1130	<0,1	<0,1	110	625
GH2OVP3	600	1140	<0,1	<0,1	140	585
Consortium	665	1405	<0,1	<0,1	230	750

Determination of Heavy Crude Oil Biodegradation with Biosurfactants.

The analytical results indicated that there are not significant differences between treatments with the three types of surfactants produced with the two types of crude oils and the positive control (Palmarito, Trinidad and SDS). However, there is a significant difference between all these treatments and control (Consortium + Crude Oil) in landfarming and liquid MMS treatments (Kruskal Wallis test, $p=0.001$). The results indicate, that the addition of biosurfactants improve the rate of biodegradation by the selected consortium compared to the treatments which had only the inoculum (Exhibit 10).

These results were expected because surfactants have the property of increasing the solubility of hydrophobic compounds, such as crude oil (Raiger & Lopez, 2009), which indicates that biosurfactants increase the bioavailability of crude oil making it is easier for the microorganisms to degrade. In addition the biodegradation of a heavy crude oil has important significance, because it generates more environmental impact than lighter crude oils. Also in the case of unconventional hydrocarbon resources, biosurfactants might be useful, because this oil is less available due to its high density, which involve additional technology and energy. (Geoscience Australia, 2012). Suggesting biosurfactants application in oil recovery.

Exhibit 10. Evaluation of Palmarito (BIOPAL) and Trinidad (BIOTRD) biosurfactants in degradation of heavy crude oil.

Biodegradation of Petroleum Hydrocarbons in Soil

Sanples collected from the pilot-scale study were collected and analyzed. Exhibit 11 shows the results from each treatment area. As shown in the exhibit, the degradation efficiency and the count of viable cells were, higher in inoculated open soil (IO) than in the control. This might be because

in the open treatment in the field microorganisms have more aeration, which improves the rate of biodegradation. The lowest TPH degradation efficiency was in the coated without inoculum soil (WC). The results showed a higher efficiency in treatments with the selected consortium than in treatments without the selected consortium, indicating the proposed consortium as a good solution to bioremediate contaminated soils.

Exhibit 11. Results after bioremediation process in open field. Efficiency of TPH degradation and number of viable cells. Treatments in soil are IC: Coated with inoculum, IO: Opened with inoculum, WC: Coated without inoculum, WO: Opened without inoculum.

Conclusions

Based on *in silico* results from sequencing and re-sequencing genomes from *L. sphaericus* Ot4b31 and *L. sphaericus* CBAM5, respectively, that showed possible pathways to aromatic hydrocarbon metabolism, our experimental results confirmed that *Lysinibacillus sphaericus* and *Geobacillus* sp. are capable of biodegrading low and medium crude oils, even in the presence of toxic metals. Additionally, the ability produce biosurfactants was determined in all strains utilizing the drop-collapsed method and the biosurfactants produced, were characterized as a cationic and anionic biosurfactants. The bioremediation pilot-scale study utilizing the bacteria consortium prepared for

the experiments showed 93 percent degradation efficiency compared to soil without the consortium.

This work is the first report of *Lysinibacillus sphaericus* as a microorganism able to degrade petroleum hydrocarbons as well as its potential to produce biosurfactants. The pilot-scale study results in the field demonstrate the potential for biotechnological applications in contaminated soils.

Finally, the biosurfactants produced have the potential for various future process developments, as they are non-toxic and biodegradable molecules with immense industrial applications and the potential to improve environmental sustainability. In the case of the petroleum industry, they can improve the efficiency of crude oil biodegradation and they can be applied in the extraction of crude oil from unconventional hydrocarbon resources, due to the capacity to solubilize crude oil.

The authors propose *Lysinibacillus sphaericus* for future treatment of petroleum contaminated soils due to its ability to use petroleum hydrocarbons as a sole source of energy; its ability to produce biosurfactants and its ability to persist in the environment due to it is a sporulated microorganism.

Acknowledgements

This work was funded by the Research Fund from the Science Faculty of Universidad de los Andes and Microbiological Research Center (CIMIC). The authors are grateful to Biotech E.U-Engineer Hugo Vladimir Ramirez and Perenco Exploration Colombia for providing the crude oil samples used in this work.

References

- Chatterjee, S., Bhattacharjee, I. & Chandra, G. (2010). Biosorption of heavy metals from industrial waste water by *Geobacillus thermodenitrificans*. *Journal of Hazardous Materials*, 5, 117-125.
- Choudhary, S & Sar, P. (2009). Characterization of a metal resistant *Pseudomonas* sp. isolated from uranium mine for its potential in heavy metal (Ni^{2+} , Co^{2+} , Cu^{2+} , and Cd^{2+}) sequestration. *Bioresource Technology*, 100, 2482–2492.
- Dussán, J., D. Andrade., L. Lozano., & S. Vanegas. (2002). Caracterización fisiológica y genética de cepas nativas de *Bacillus sphaericus*. *Revista Colombiana de Biotecnología*, 4, 89-99.
- Dussán, J., Lozano, L., Peña, T., & Bojaca, V. (2013). Mosquitos, minería y petróleo. *Lysinibacillus sphaericus*: la versatilidad de un microorganismo en beneficio del ambiente y la salud pública. *Revista Hipotesis*, edición especial. Universidad de los Andes.
- Ganesh, A., & Lin, J. (2009). Diesel degradation and biosurfactant production by Gram-positive isolates. *African Journal of Biotechnology*, 8, 5847-5854.
- Geoscience Australia. (2012). Unconventional Petroleum Resources. Australian Government. <http://www.ga.gov.au/energy/petroleum-resources/unconventional-resources.html>
- Hamed, S., Maaroufi, A., Ghram, A., & Zouhai, B. (2013). Isolation of four hydrocarbon effluent-degrading *Bacillaceae* species and evaluation of their ability to grow under high-temperature or high-salinity conditions. *African Journal of Biotechnology*, 1636-1643.
- Hilton, B., Strader, M., Beer, A., & Roke, S. (2011). Surface Structure of Sodium Dodecyl Sulfate Surfactant and Oil at the Oil-in-Water Droplet Liquid/Liquid Interface: A Manifestation of a Nonequilibrium Surface State. *The Journal of Physical Chemistry*, 115, 2970–2978.
- Kananaviciute, R., Butaite, E., & Citavicius, D. (2013). Characterization of two novel plasmids from *Geobacillus* sp 610 and 1121 strains. *Plasmid*. 23-31.
- Kaufmann, K., Christophersen, M., Buttler, A., Harms, H., & Höhener, P. (2004). Microbial community response to petroleum hydrocarbon contamination in the unsaturated zone at the experimental field site Varlose, Denmark. *FEMS Microbial Ecology*, 48, 387-399.
- Lozano, L., & Dussán, J. (2013). Metal tolerance and larvicidal activity of *Lysinibacillus sphaericus*. *World J Microbiol Biotechnol*, 7, 1383-1389.
- Lozano, L. (2012). *Lysinibacillus sphaericus* heavy metal tolerance and mosquito biological control: from function to genome. Doctoral Thesis. Universidad de los Andes.
- Lozano, L. C., & J. Dussán. (2002). Diferenciación de aislamientos colombianos de *Bacillus sphaericus* patógenos y no patógenos para larvas de mosquitos por amplificación azarosa del ADN. *Actual Biol*, 24, 113-121.
- Lin, Y., Wang, X., Wang, B., Mohamad, O., & Wei, G. (2012). Bioaccumulation characterization of zinc and cadmium by *Streptomyces zinciresistens*, a novel actinomycete. *Ecotoxicology Environmental Safety*, 77, 7–17.
- Marín, J., Moreno, J., Hernandez, T., & García C. (2006). Bioremediation by composting of heavy oil refinery sludge in semiarid condition. *Biodegradation*, 17, 251-261.
- McMullan, G., Christie, J., Rahman, T., Banat, I., Terman, N., & Marchant, R. (2004). Habitat, applications and genomics of the aerobic, thermophilic genus *Geobacillus*. *Biochemical society*, 32, 214-217.
- Merck group: www.merckmillipore.com/.../Merck.../ViewProductDocuments-File

- Niño, J., & Dussán, J. (2013). Biosurfactant production by *Geobacillus* sp in different types of crude oil obtained from Orinoquía region, Colombia. Universidad de los Andes, Bogotá.
- Olufolahan, A., Balgobind, A., & Pillay, B. (2011). Quantitative assessment of the toxic effects of heavy metals on 1,2-dichloroethane biodegradation in co-contaminated soil under aerobic condition. *Chemosphere*, 85, 839–847.
- Ortiz, R., & Dussán, J. (2007). Evaluación de la toxicidad de célula vegetativa de *Bacillus sphaericus* en larvas de *Culex quinquefasciatus*. Universidad de los Andes, Bogotá.
- Pradhan, A., Pradhan, N., Sukla, L., Panda, P., & Mishra, B. (2012). Inhibition of pathogenic bacterial biofilm by biosurfactant produced by *Lysinibacillus fusiformis* S9. *Byoprocess Byosyst Eng*, 37, 139-149.
- Peña, T., Lozano, LC., & Dussan, J. (2014). Genome sequence and description of the mosquitocidal and heavy metal tolerant strain *Lysinibacillus sphaericus* CBAM5. (Submission) Standards in Genomic Sciences <http://www.standardsingenomics.org/index.php/sigen/author/submission/606>
- Peña-Montenegro, T., & Dussan, J. (2013) Genome sequence and description of the heavy metal tolerant bacterium *Lysinibacillus sphaericus* strain OT4b.31. *Standards in Genomic Sciences*, 9,42-56.
- Peña-Montenegro, T., Lozano. L., & Dussán, J. Submission number #606, 2014 Standards in Genomic Sciences.
- Raiger, L., & Lopez, N. (2009). Los biosurfactants y la industria petrolera. *Revista Quimica viva*, 3,146-161.
- SPSS Inc., (2013). Windows Version 22 (Chicago IL).
- Tomei, MC., Annesini, MC., Luberti, R., Cento, G., & Senia, A. (2003). Kinetics of 4-nitrophenol biodegradation in a sequencing batch reactor. *Water Res*, 37,3803-3814.
- Tuzen, M., Uluzozlu, C., Usta, A., & Soylak, M. (2007). Biosorption of copper(II), lead(II), iron(III) and cobalt(II) on *Bacillus sphaericus*-loaded Diaion SP-850 resin. *Analytical Chemistry*, 2, 241-246.
- Velasquez, L. (2009). Biosorption and bioaccumulation of heavy metals on dead biomass, living biomass and S-layer of *Bacillus sphaericus*. Universidad de los Andes, Bogotá.
- Velasquez, L., & Dussán, J. (2009). Biosorption and bioaccumulation of heavy metals on dead and living biomass of *Bacillus sphaericus*. *Journal of Hazardous Materials*, 167,713-716.
- Villegas-Torres, F., Bedoya-Reina, O., Salazar, C., Vives-Florez, M., & Dussan, J. (2011). Horizontal *arsC* gene transfer among microorganisms isolated from arsenic polluted soil. *International Biodeterioration & Biodegradation*, 65,147-152.
- Zheng, C., He, J., Wang, Y., Wang, M., & Huang, Z. (2011) Hydrocarbon degradation and bioemulsifier production by thermophilic *Geobacillus pallidus*. *Straits. Bioresource Technology*,8, 9155–9161.

Lina Manchola. MSc. Received a BS Microbiology degree from the University of Andes and MS Biological sciences from University of Andes, research assistant in environmental microbiology area.

Jenny Dussán. Director. Research Microbiological Center (CIMIC). Ph.D.- Environmental Molecular Microbiology. M.Sc Genetic and Environmental Microbiology. B.S Microbiology.