Biosurfactants Produced by *Lysinibacillus sphaericus* and Entomotoxigenic Activity against *Aedes aegypti* Larvae

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Abstract

Lysinibacillus sphaericus is a Gram positive, spore forming bacterium used in bioremediation of water and soils contaminated with hydrocarbons and in the biological control of mosquitoes. This contamination problems are linked to the increase of oil spills worldwide over the years, occurring mostly in tropical and subtropical areas where *Aedes aegypti*, known vector of dengue, chikungunya, yellow fever, and Zika diseases lives. Previous studies with *L. sphaericus* as a biological control agent have determined that the vegetative cell has larvicidal activity against A. aegypti larvae. In bioremediation studies it has been established that *L. sphaericus* degrade hydrocarbons under stress conditions as it is capable of producing biosurfactant compounds, both anionic and cationic, that reduce the surface tension between water and hydrocarbons. These are then emulsified and used as a carbon source by the bacteria. The three bacterial strains of *L. sphaericus* (III(3)7, 2362, OT4b25) examined in this study were able to produce biosurfactants and use crude oil as a carbon source in three crude samples with different density (API gravity). Biosurfactant production was determined using the drop collapsed method for all the strains and then the compound was tested against instar *3 A. aegypti* larvae. The results shown that the biosurfactants produced by the strains III(3)7 and OT4b25 in heavy crude (11° API) showed the best mortality against the larvae. Our results evidence the larvicidal capacity of the biosurfactants produced *by L. sphaericus* against the larvae of *A. aegypti* and allow us to suggest that there may be more alternatives for biological control other than the direct use of the microorganism on the contaminated site.

Introduction

The increasing demand of petroleum and carbohydrates fuels in the worldwide economy nowadays comes with an increment on the oil spills cases around the world due to the petroleum transport. Most of the gravest oil spills occurred on tropical and sub-tropical waters including the Gulf of Mexico oil spill on 2010 (Kostka et al., 2011). The damage of oil pipelines is also a considerable factor of polluting waters carbohydrates near coast zones, this pipes damage due to different factors such as: bad or inefficient maintenance of hydrocarbon extraction infrastructure, environmental accidents (earthquakes, tsunamis, landslides and floods) and terrorist attacks. All this factors seem to be common on tropical zones where the ecosystem is very sensitive to this kind of pollutants (Agudelo et al., 2015), (Nansingh & Jurawan, 1999), (Sowmya & Jayappa, 2016).

The hydrocarbon problematic overlaps in the same zones with another environmental topic that is also a healthy human risk issue, the mosquito *Aedes aegypti* that transmit tropical diseases such al zika, malaria, filariasis, dengue, west nile virus and yellow fever (He et al., 2011). The abundance of *A. aegypti* on tropical zones is linked with the increase of the already mentioned diseases and nowadays the mosquito developed multiple resistances to insecticides (Marcombe et al., 2012), making it a serious public health emergency. The mosquito has possible resistance genes to some pullulates (David et al., 2010) that combined with the anthropogenic environment pollution such as oil spills and the action of phenotypic plasticity on the mosquitoes population it will drive selection mechanisms in favor of *A. aegypti* resistant strains (David et al., 2010), additionally contaminated water with hydrocarbons affects a variety of coast fishes (Cavalcanti et al., 2007) and odonates (Fincke et al., 1997) that are the natural predator of *A. aegypti*.

Bioremediation brings new opportunities to solve specific pollution problems on a determined zone, instead of using conventional chemical compounds it uses plants, microorganisms, fungus or biological produced enzymes to reestablish a biome ecological functions and populations for a low-cost. A bacteria capable of remediating contaminated soils and waters with heavy metal compounds and hydrocarbons is *L. sphaericus,* a gram positive, aerobic, spore forming bacterium (Lozano & Dussán 2013). It degrades petroleum by producing biosurfactants, an amphiphilic complex organic molecule. These molecules have a hydrophobic and a hydrophilic moiety that reduces the surface tension and interfacial tension between liquids, solids, and gases (Manchola & Dussán, 2014). Biosurfactant molecule can emulsify or solubilize petroleum, depending on the electric charge of the molecule it can be anionic, cationic or non-ionic, in these order of ideas the nature of the biosurfactant will determinate if the bacteria solubilize or emulsify the hydrocarbon so the bacteria can obtain the carbon source in the petroleum from the water.

L. sphaericus can also be used as a biological larvicide of *A. aegypti* larvae (Silva & DUssán, 2016). Most strains produce a binary parasporal inclusion body toxin during sporulation, composed of BinA and BinB proteins that binds to receptors in epithelial midgut cells and then causing cellular lysis (Lozano & Dussán 2013). It also produce larvicide toxins on vegetative cells (Mtx1, Mtx2 and Mtx3) that can form pores and kill the larvae. However there are reports of different bacteria biosurfactants of being capable to act as larvicidal molecules (Geetha et al., 2014), (Deeplani et al., 2014), (Ghribi, et al.,2012). There are three well known strains that share the capability of degrade crude oil and act as biological control: III(3)7, 2362 and OT4b25.

The purpose of this study is to produce, characterize and test the biosurfactant as a possible biological larvicidal compound of *A. aegypti* third instar larvae.

Materials and methods

Synchronization of Cultures

To ensure that all the tested strains were in the same metabolic state the strains were cultured overnight for 16 hours on nutrient broth, then 1 mL of the grown bacteria was transferred to a sporulation broth and incubated for 48 hours at 28°C. After 48 hours 1 mL of the formed pellet of sporulated bacteria was subjected to thermal shock at 90°C for 20 minutes to ensure that the vegetative cells die and have a pure culture of spores. This was subjected for three cycles to ensure that most of the culture was synchronized (Ortiz & Dussán 2007).

Biosurfactant production

50mL of MSM (Minimal salts medium) were poured on a glass bottle of a maximum capacity of 50mL, then 10^9 synchronized colony forming units of bacteria were added. Finally 1mL of crude oil was pipetted. For each strain (III(3)7, 2362 and OT4b25) this was done on 15 bottles by triplicate, meaning that by each strain there was a total of 45 bottles with MSM, oil and bacteria. Three different density or API gravity crudes were tested, two medium API gravity (°23 and °32) and one heavy API gravity (°11). The MSM was used to force the bacteria to use the hydrocarbon as a carbon source. In summary 45 bottles assays were tested per strain and each strain were tested individually with each of the three API gravity crudes (figure 1).

After 15 to 20 days of incubation of the bottles at 28°C the MSM medium without crude oil was lyophilized for 48 hours. The lyophilized mass of each strain was resuspended in 1 mL of distilled water with a final concentration of 50X (Manchola & Dussán, 2014).

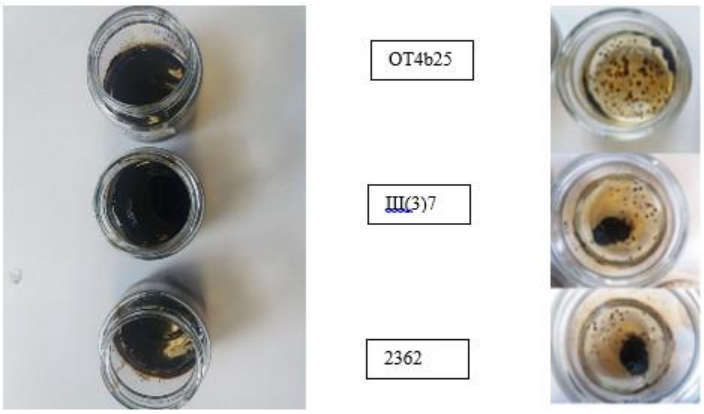

Figure 1. Crude oil degradation after 25 days on API gravity 11 for each strain tested

Characterization of biosurfactant

Qualitative test

A modified method of the drop collapsed method by Pradhan (2012) was made by Manchola and Dussán (2014) to evaluate the surfactant presence; 01 mL of crude oil was placed on a small petri dish and then 100µl of biosurfactant solution was dropped over the crude, The test was carried out for each strain on each API gravity crude oil.

Quantitative test

The biosurfactant solution produced by each strain in each API gravity were characterized using kits (Merck Millipore Group) to stablish if there is anionic, cationic and nonionic biosurfactants. 0,1 mL of biosurfactant was diluted with 5 mL of distilled water and then each kit was applied. The results were read using a Spectroquant Nova 60A. Distilled water was used as a negative control and SDS (sodium dodecyl sulfate) as a positive control for the presence of surfactant, as SDS is known as anionic surfactant

Bioassays of larvicidal activity

The larvicidal activity was tested by adding 30 mL of diluted biosurfactant on a glass bottle of a maximum capacity of 50 mL and then 20 larvae of third instar were placed and incubated at a temperature of 32°C by triplicate. There were two controls, the first one was 30mL of tap water free of chlorine with 20 larvae and without biosurfactant, the second control was tap water free of chlorine with a lyophilized MSM to make sure that none of the compounds in the MSM were causing larvae mortality (Silva & DUssán, 2016). Larval mortality was scored after 24 and 48 hours for each strain and each API gravity lyophilized assay (Lozano & Dussán 2013).

Statistical analysis

The statistical analysis was determinated in the R software (R Core Team, 2014). The Shapiro-Wilcoxon test was used to stablish the normality of the data distribution and then an ANOVA (Analysis of variance) test and a Tukey's HSD (Honest significant difference) test provided the statistical support for significant differences between the treatments with the different strains and API gravity biosurfactant samples with the controls at 24 and 48 hours. A paired sample t-test was made to analyze differences between the mortality at the two scored times (24 and 48 hours). The alpha used for all the analysis was α =0,05. The data that does not showed a normal distribuion was analyzed with the Kruskal-Wallis test to validate significant differences between treatments and control.

Results

Characterization of biosurfactant

Qualitative test

The results from the drop collapsed method confirmed that all strains produced biosurfactants in all API gravity by a dispersion of oil crude at the moment the drop is added (figure 2).

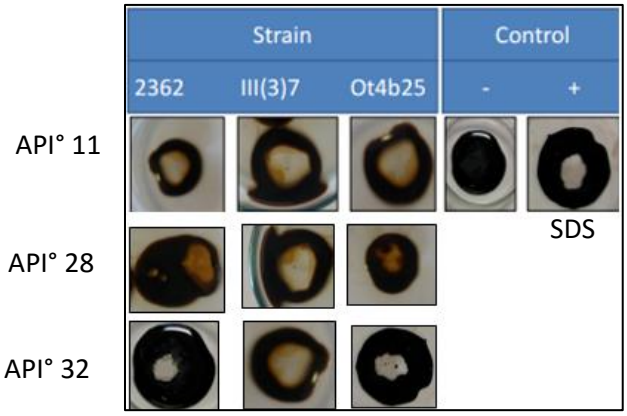

Figure 2. Qualitative test showing the pressence of biosurfactant. Dispersed oil confirms the presence of biosurfactant. For the Strains each line represents the API gravity. On the control side the negative contros was tap water and the ositive control was SDS.

Quantitative test

The table 1 shows the results from using the Millipore group kits to analyze the biosurfactant showed that all the strains produced anionic an cationic biosurfactants and small or not detectable amounts of nonionic

Strain	Anionic mg/l			Nonionic mg/l			Cationic mg/l		
	API°11	API°28	API°32	API°11	API°28	API°32	API°11	API°28	API°32
III(3)7	2540	1000	1020	<0,1	<0,1	<0,1	420	380	340
2362	1500	2500	2440	<0,1	<0,1	<0,1	460	420	320
Ot4b25	2640	1740	1740	0,15	<0,1	<0,1	1960	1560	1080

Table 1. Quantitative test showing the concentration and the type of biosurfactant in the different assays. The kits detection limits where: 0,05 to 2 mg/l anionic, 0,1 to 7,65 mg/l non-ionic and 0,05 to 5mg/l cationic

Bioassays of larvicidal activity

The figure 3. Shows the comparassion between the mortality of the larvae at 24 hours with 48 hours of being in contact with the biosurfactant. Tha Shapiro-Wilk test showed a P-value of 0.3785 for the graph 1.A, 0.5544

for the graph 1.B and 0.6643 for the graph 1.C. Meaning the normality of the data and then a paired sample t-test was performed with resulting p value of 0.02286 meaning that the means of the mortality at 24 and 48 hours are not equal on the assays of API gravity °11.

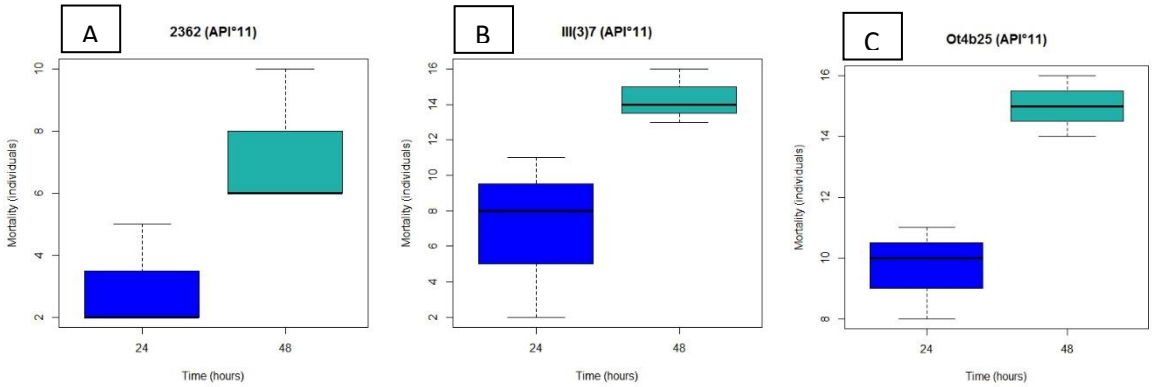

Figure 3. Comparassion of the larvae mortality between 24 and 48 hours for the biosurfactant produced by the three strains at API gravity °11 (1.A Strain 2362, 1.B III(3)7, 1.C Ot4b25).

The Figure 4 show similar results in the statistical analysis as the graph 1. The Shapiro Wilk test P-Value = 0.7393 for 2.A, 0.4844 for 2.B and 0.415 for 2C. With normal data the paired sample t-student was applied. The means are not equeal in none of the three strains for the API gravity 28 mortality by biosurfactant, the respective P-values = 0.0202 for 2.A, 0.01324 for 2.B and 0.04941 for 3.C.

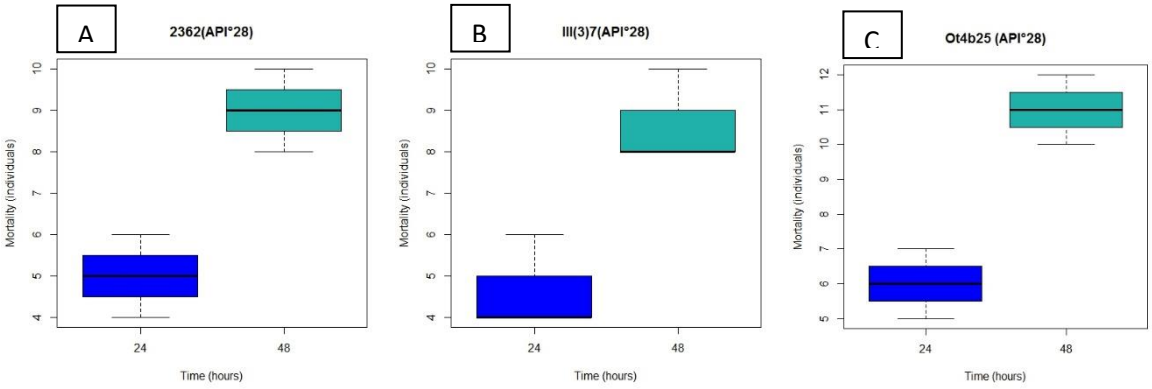

Figure 4. Comparassion of the larvae mortality between 24 and 48 hours for the biosurfactant produced by the three strains at API gravity °28 (1.A Strain 2362, 1.B III(3)7, 1.C Ot4b25).

The analysis for the API gravity °32 on the graph 3 shows the same tendency as the previous graphics. The P values for the Shapiro Wilk test are: 0.1525 for 3.A, 0.06534 for 3.B and 0.3308 for 3.C. The data is normal for each treatment. The paired sample t-student showed the next P values= 0.02286 for 2.A, 0.002192 for 2.B and 0.04941 for 3.C.

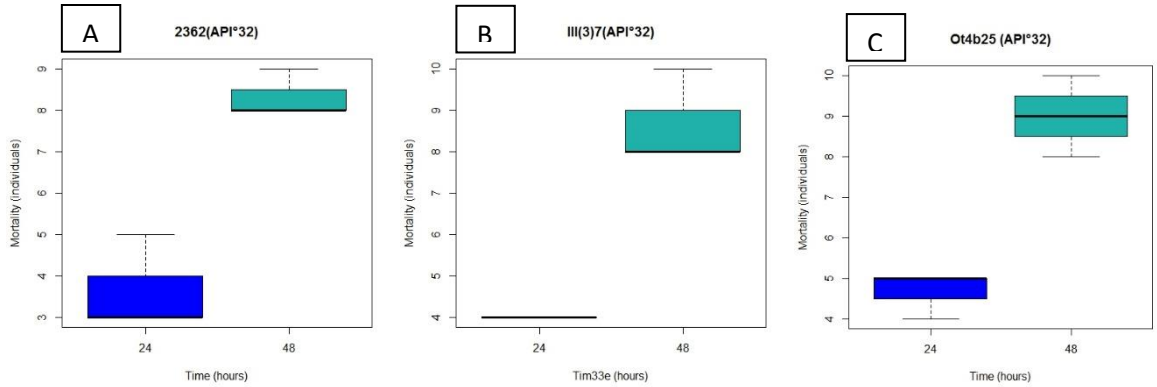

Figure 5. Comparassion of the larvae mortality between 24 and 48 hours for the biosurfactant produced by the three strains at API gravity °32 (1.A Strain 2362, 1.B III(3)7, 1.C Ot4b25).

In order to compare and identify the most effective biosurfactant produced, a global analysis of the three strains and controls was made for each API gravity crude oil (Figure 6-8). The graph 4 shows the larvae mortality on the bioassays made with the biosurfactant produced by the API 11 hydrocarbon degradation. The statistical analysis showed that the data follow a normal distribution (Shapiro-Wilk P-value= 0.08329). The ANOVA test stablished that at least one of the treatments mean showed a signifficant difference between the other treatments including the controls. The Tukey HSD test showed that the two of the strains of *L. sphaericus* biosurfactant treatmnets were signiffcatively different to the controls (III(3)7-Control = 0.0020 < α, Ot4b25-Control= 0.0015 < α, III(3)7-Control_MMS 0.0044 < α, 2362-Control_MMS=0.16 < α). The 2362 strain showed small values of signifficance with the control of tap-water and none differences with the MSM control (2362-Control = 0.051 > α, 2362- Control_MMS = 0.162 > α). The controls showed no siggnificatevly differences between each other (Control_MMS-Control = 0.91 > α)

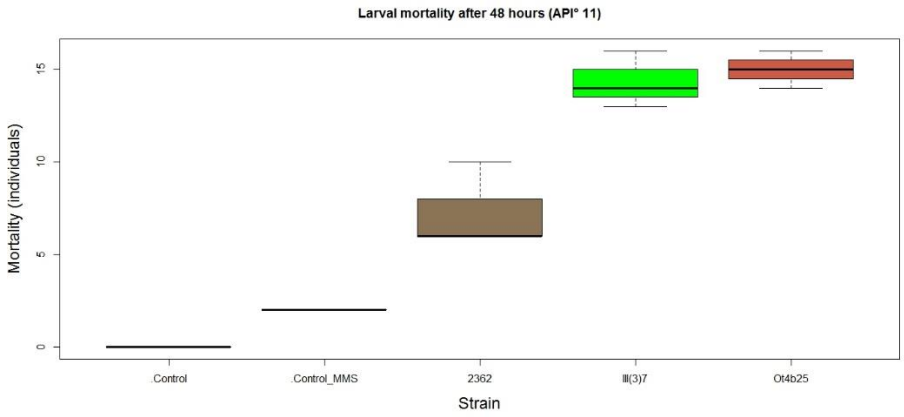

Figure 6. Mortality results of the three strains at 48 hours of being exposed to the biosurfactant produced by the degradation of API gravity 11 crude oil.

The Figure 7 shows the mortality on API gravity 28. The statistical test used for this graph was the Kruskal-Wallis test because the data was not normal (Shapiro-Wilk P-value=0.0031), and it showed that at least one mean is different (Kruskal-Wallis P-value= 0.010). Dispite the not normality of the data an ANOVA test was performed due to the number of individuals per assay (60), so the tukey test showed that the three strains not differ signfically from each other (2362-III(3)7= 0.99 > α, Ot4b25-III(3)7= 0.16 > α, Ot4b25-2362= 0.25 > α). They differ significantlyfrom the control assays because they showed that none indivudual larvea died

during the bioassay (III(3)7-Control= 0.0023 < α, 2362-Control= 0.0019 < α, Ot4b25-.Control= 0.00062 < α, III(3)7-.Control_MMS= 0.0023 < α, 2362-.Control_MMS 0.0019 < α, Ot4b25-.Control_MMS= 0.00062< α).

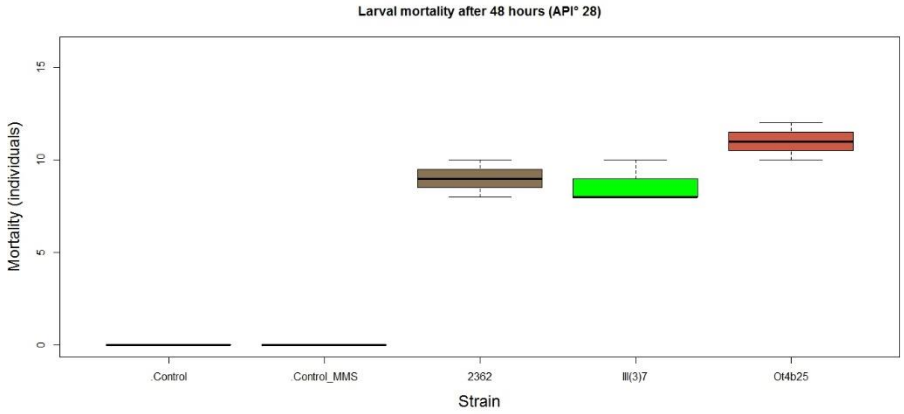

Figure 7. Mortality results of the three strains at 48 hours of being exposed to the biosurfactant produced by the degradation of API gravity 28 crude oil

The Figure 8 shows a statitical tendency in the mortality similar to those in the graph 5. The data don't follow a normal distribution (Shapiro-Wilk P-value= 0.0019), the test erformed was a Kruskal-Wallis (Kruskal-Wallis P-value= 0.010) test and an aditional Tukey HSD test was performed after an ANOVA due to the number of samples. In this order of ideas all the analysisi made showed that the three strains don't differ significantlyeach other (Tukey HSD: 2362-III(3)7= 0.99 > α, Ot4b25-III(3)7= 0.16 > α, Ot4b25-2362= 0.25 > α). And they differ from the control showing a signifficant grade of mortality by the biosirfactants (III(3)7-Control= 0.0023 < α, 2362-Control= 0.0019 < α, Ot4b25-.Control= 0.00062 < α, III(3)7-.Control_MMS= 0.0023 < α, 2362-.Control_MMS 0.0019 < α, Ot4b25-.Control_MMS= 0.00062< α).

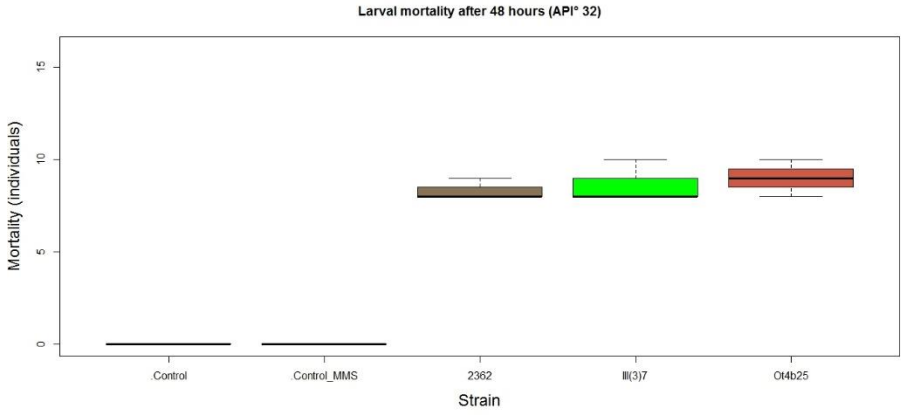

Figure 8. Mortality results of the three strains at 48 hours of being exposed to the biosurfactant produced by the degradation of API gravity 32 crude oil

Discussion

The results showed that biosurfactant compounds were successfully produced and the drop collapsed method on Figure 2 give evidence of that. The characterization of the biosurfactant (Table 1) indicates that most of

the biosurfactants produced by *L. sphaericus* are anionic, followed by cationic and nearly none of nonionic, this corresponds to the characterization made by Manchola and Dussán (2014) where the most produced biosurfactant for the strains III(3)7 and 2362 was the anionic type. The biosurfactant of the strain Ot4b25 was not characterized on previous studies, but it follows the concept that *L. sphaericus* produces in the majority the anionic type biosurfactant and the amount it produces follows the type of API gravity of the crude oil (Manchola & Dussán, 2014), where the crude oil with heavy API gravity (°11) causes the bacteria to produce more amounts of biosurfactant, in contrast lower API gravity causes less quantities of biosurfactant. The amount of biosurfactant produced may be due to the fact that high API gravity hydrocarbons are denser and are charged by more amounts of carbon bonds required by the bacteria metabolic sustain.

The Figure 3-5 shows a possible accumulative factor through the time, causing that the rate mortality of the individuals through the time duplicates. This is an interesting fact that deserves to be investigates on future studies because at 48 hours the biosurfactant showed the optimal mortality, this pose the question of what is the possible mechanism that is followed for the biosurfactant to be absorbed by the larvae and how it affects the metabolic functions to the continuous contact with biosurfactant over time.

Comparing the Figure 6 with the 7 and 8 it shows that the biosurfactant produced on API 11 crude oil represents the highest mortality of the three API gravity crudes. One hypothesis proposed in this study is because of the carbon chains on the hydrocarbons, being the API gravity 11 crude the denser (Rodríguez M et al., 1999) as stated previously in the characterization (Table 1, means that a high density crude oil has more carbon chains able to the bacteria to use by breaking carbon bond and solubilizing more crude. To achieve this the bacteria produce more biosurfactant on crude with higher amounts of carbon chains. Finally it is stated that more biosurfactant means on increase in the mortality.

It is important to mention that the results obtained showed mortality also in pupae's. An interesting fact because the pupae do not feed and are less metabolic active that the larvae

Compared to the controls, the mortality per individual by all the strains was significantly different, this indicates that the biosurfactants produced by strains of *L. sphaericus* capable to degrade crude oil and produce larvae killing toxins can serve as a possible biocontroller compound to *A. aegypti* instar 3 larvae

Conclusion

The strains III(3)7 and Ot4b25 on API° 11 crude, showed the highest larvicide effect against A. aegypti larvae.

Future research could evaluate the biosurfactant efficiency given field conditions.

The biosurfactant is a promissory alternative to chemical compounds without releasing microorganisms into the environment.

Acknowledgments

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Recommendations

Future approaches could address the biosurfactant effect against pupae survival.

It is important to do additional assays testing the toxicity of the biosurfactants on other organisms.

It is important to study the physicochemical mechanism of the biosurfactant on the larvae tissue.

It is important to stablish the minimum quantity of diluted biosurfactant to kill one larvae or a pack of 20 larvae to standardize it for future studies.

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