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Isolation and adaptation of hydrocarbonoclastic bacteria from *Tenebrio molitor* gut

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RESUMEN

En este trabajo, el objetivo fue aislar e inducir a la adaptación bacterias hidrocarbonoclastas, del intestino del *Tenebrio molitor*, para usar diésel como fuente de carbono. Para monitorear la resistencia de las larvas a los hidrocarburos, las larvas en grupos de diez fueron sometidas a cuatro concentraciones de diésel, donde la más alta fue de 20000 mg/kg, la prueba duró 30 días y el diésel no afectó el crecimiento de las larvas. La mayoría de las larvas (38) utilizadas en la prueba evolucionaron a pupas y luego a escarabajos produciendo la segunda generación de larvas, de las cuales algunas fueron diseccionadas, para aislar las bacterias hidrocarbonoclastas que podrían existir en sus intestinos. Utilizando el extracto líquido del intestino de las larvas y un medio selectivo (carbón combinado) se preparó un caldo que tenía una concentración de diésel de 1210. mg/L. Después de un período de 12 días, había $1.87x10^9$ UFC /mL.

ABSTRACT

In this work, we aimed to isolate and induce the adapt of hydrocarbonoclastic bacteria, from *Tenebrio molitor* gut, to use Diesel as carbon source. In order to monitor the resistance of the larvae to hydrocarbon, the specimens in groups of ten were grown in matrices subjected to four Diesel concentrations, the highest was 20000 mg/kg, the test lasted 30 days and Diesel did not affect the larvae growth. Most larvae (38) used in the test evolved into pupae and then into beetles producing the second generation of larvae, some of those were dissected to isolate hydrocarbonoclastic bacteria that could exist in their gut. We used the liquid extract from larvae gut and a selective medium (Combined carbon) to make a broth which had Diesel concentration of 1210.37 mg/L. After a period of 12 days there were $1.87x10^9$ CFU/mL.

Introduction

Different experiments with polymer-eating larvae have been recently carried out (Yang et al., 2014; Tang et al., 2017; Yang et al., 2015a). One of these larvae are mealworms (*Tenebrio molitor*). *Tenebrio molitor* is an organism capable of degrading polymers (hydrocarbon) such as polystyrene, polyethylene, and even polyvinyl chloride (Tang et al., 2017; Yang et al., 2015a; Yang et al., 2018a; Yang et al., 2018b; Brandon 2018; Wu et al., 2019). Some authors (Yang et al., 2018a; Yang et al., 2018b) suggest that the hydrocarbon degradation capacity of larvae can be improved if its hydrocarbon diet is supplemented with larvae´s natural sources of nutrition, like grains and cereals (Ramos-Elorduy et al., 2002). This process is called co-diet.

Results reported by Yang *et al.* (2018b) suggest that the hydrocarbon-degrading capacity of larvae is likely widespread among *Tenebrio molitor* species, and larvae fed with 10% w/w polymer (polystyrene) and 90% bran had nearly doubled rates of degradation of polymers with respect to those of polymers-fed larvae. Also, their results indicated that the second larvae generation, produced by the co-diet fed larvae, had favorable capabilities for hydrocarbon degradation, thus, demonstrating the selective breeding of hydrocarbon-degrading larvae. Furthermore, there was evidence that hydrocarbon degradation occurred through depolymerization and oxidation (Yang *et al.,* 2018b; Yang et al., 2015a; Yang et al., 2015b).

The larvae of *Tenebrio molitor* have the capacity of degrading polymer because of the consortia of bacteria present in their gut that can use hydrocarbons as carbon source and play an essential role in degradation and mineralization of them (Yang et al., 2015b). The role is symbiotic, that is, it benefits both the microbiota and the host, in a similar way as the biodegradation of cellulose in ruminant mammals and wood in termites (Yang et al., 2015a). Also, Yang et al. (2015b) isolated and identified 13 bacterial strains from larvae gut, including *Exiguobacterium sp*., *Chryseobacterium sp.*, *Klebsiella pneumonia*, *Bacillus cereus*, *Morganella morganii*, *Enterobacter hormaechei*, *Proteus vulgaris*, *Enterobacter cancerogenus*, *Enterococcus viikkiensis*, *Enterococcus faecalis*, *Bacillus stratosphericus*, *Citrobacter freundii*, and *Enterococcus gallinarum*. The degradation of hydrocarbons, such as maltene fraction (oily sludge), total PAHs, anthracene, kerosene, phenanthrene, etc., has been assed using some strain of these genes of bacteria (Jasmine & Mukherji, 2015; Faiq Ali et al., 2018; Patel et al., 2013; Wu et al., 2013).

Fernández et al., (2006) proposed the selection and isolation of hydrocarbonoclastic bacteria through a selective culture medium, Combined carbon (Rennie, 1981), and a specific hydrocarbon. For example, Rivera et al. (2002) and Hernández-Rivera et al., (2011) isolated strains of hydrocarbonoclastic bacteria from the soil applying this last method and using crude oil as a carbon source. Another work reported the isolation from the soil of at least three bacteria that can degrade Diesel (Njoki Mwaura et al., 2018). On the other hand, the dissection and extraction method of bacteria from larvae gut that Galvis et al. (2016) described in their work, can be replicated with some modification with the *Tenebrio molitor* larvae. If these two-last methods are combined, it will be possible to screen hydrocarbonoclastic bacteria from larvae gut that can degrade hydrocarbons.

In this work we aimed to isolate and induce the adaptation of hydrocarbonoclastic bacteria from the gut of the second generation of *Tenebrio molitor,* that used Diesel as a carbon source. We first observed the effect of Diesel, more toxic and complex hydrocarbons, on the *Tenebrio molitor* larvae through co-diet of Diesel and cornflour and, on second instances, we bred the second generation of specimens through selective breeding. Also, we report the CFU/mL of hydrocarbonoclastic bacteria in the first and second generation.

Methods

The effect of hydrocarbons on larvae

The test of the hydrocarbon effect on *Tenebrio molitor* larvae was carried out by placing sets of 10 larvae in four containers with different Diesel concentrations (1300, 6000, 13000, and 2300 mg/kg), each container was prepared with 10 g of cornflour mixed with Diesel. The test lasted 36 days. We chose these concentrations using as reference the maximum limit of middle petroleum fraction (MPF) or Diesel in soils established by the Mexican official regulation NOM-138-SEMARNAT / SSA-2012" (DOF, 2012).

We monitored the containers every seven days to detect whether some larvae survived to the matrix conditions or evolved into pupae. Whichever case, we removed them and placed them in a different container.

Selective breeding larvae

After the last test, we put the surviving pupae in a different container. Once, they evolved into beetles, we again moved the beetles into another container where they were kept with surviving beetles. We kept beetles isolated and fed them with cornflour mixed with Diesel to produce the second generation of larvae. Yang et al. (2018b) suggested that feeding *Tenebrio Molitor* larvae with a mix-diet of larvae´s natural source of nutrition and a contaminant (in this case, Diesel) could allow

reproducing a second generation with favorable properties for the degradation of the used contaminant.

Selective isolation of hydrocarbonoclastic bacteria from *Tenebrio molitor* gut

In this phase, we selected larvae (a specimen of each generation) and carried out dissection to isolate hydrocarbonoclastic bacteria from their gut. The dissection was prepared according to the method reported by Galvis et al. (2016) for larvae dissection, but we modified some characteristics for the *Tenebrio molitor* case. We removed the larval guts carefully using dissection forceps, and we placed them in Eppendorf microtubes that contained 0.6 mL of 0.9% saline solution (we marked this solution as liquid extract from gut). Then we closed the microtubes and shook them in a Vortex. All this was performed under sterile conditions.

For each generation of larvae, we had a liquid extract from gut. We homogenized the solutions and inoculated 0.025 mL triplicates in Petri dishes with Combined carbon solid medium (CCs) (Rennie, 1981), and we afterwards performed the count plate method for hydrocarbonoclastic bacteria proposed by Fernández et al. (2006).

We only inoculated the second-generation solution in a tube with 10 mL of Nutrient Broth and incubated it for 48 hours. After this period, we poured 600 µL of broth solution into a tube that contained 9.8 mL of Combined carbon liquid medium (CCl) with 90 µL of Diesel, in duplicates. For 280 hours, we monitored the growth of hydrocarbonoclastic bacteria through McFarland standard turbidity method (OMS, 2003).

Afterwards, we inoculated the solution of the last test (600 µL), in triplicates, in tubes that contained 9.8 mL of CCl and 10 µL of Diesel and monitored the growth of bacteria using the McFarland method for 290 hours.

Results and discussion

The effect of hydrocarbons on larvae

Diesel did not affect the larvae since most of them survived the test in the first stage. The number of specimens of *Tenebrio molitor* at the beginning of the test (all of them were larvae) and at the end (almost all of them were pupae) are presented in table 1. The rates of growth and decay of larvae and pupae can be observed in figure 1; the larvae curve decreased while the pupae curve grew during the test. It should be noted that the larvae used in the test were in the adult phase, so they evolved into pupae throughout the test.

Only two larvae died, which were in the matrices contaminated with 20,000 and 13,000 mg/ kg MPF, one in each matrix. The rest of the larvae continued the life cycle. They were unaffected by the contamination and evolved into beetles. The results are similar to those reported by Yang et al. (2018), who fed larvae with polymer (polyethylene) plus bran, and this did not affect their life cycle.

The results were positive. However, it would be advisable to carry out the same test by changing the matrix to a non-food component, since the corn flour, being a food component, could cause the pollutant not to generate ecological stress; so, the larva grew without major problems.

Table 1. Number of specimens of *Tenebrio molitor* at the beginning and the end of the test

Diesel concentration, mg/kg	the beginning		the end	
	Larvae	Pupae	Larvae	Pupae
20000	10	0	1	8
13000	10	0	2	7
6000	10	0	2	8
1300	10	0	0	10

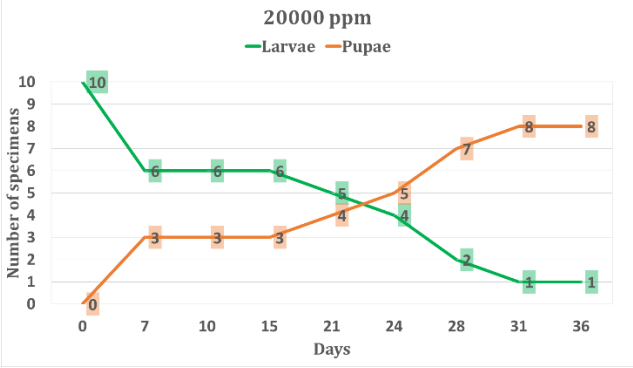

Figure 1. Accumulative number of specimens (*Tenebrio molitor*) in the matrix of 20000 ppm Diesel .

Selective breeding larvae

About 30 days after the test, the young larvae of the second generation appeared in the container where the survivor beetles were kept (Figure 2).

Figure 2. Young larvae of the second generation.

Selective isolation of hydrocarbonoclastic bacteria from *Tenebrio molitor* gut

The results found through the count plate method for hydrocarbonoclastic bacteria (Fernández et al., 2006) are summarized in table 2. The population of hydrocarbonoclastic bacteria from the second-generation larvae gut was almost double than that of the first generation, this characteristic may be the reason why the second generation has better properties to degrade hydrocarbons according to what Yang et al. (2018b) suggest in their work.

Table 2. CFU/mL hydrocarbonoclastic bacteria from *Tenebrio molitor* gut.

	CFU/mL	SD
The first generation	$3.58x10^3$	$5.4x10^2$
The second generation	$6.19x10^3$	$7.8x10^2$

During the first adaptation process of hydrocarbonoclastic bacteria, they reached a population of $3.03x10^9$ CFU in 280 hours (11 days). This first adaptation was in the medium with a Diesel concentration of 10893 mg/L. The growth curve per hour of the second adaptation process (the medium with Diesel concentration of 1210 mg/L), where we used the previously adapted bacteria, can be observed in figure 3. This growth curve is a smoothed curve of data (points), which was fitted through the Hold model. The curve has 8% average relative error and $9.94x10^7$ standard deviation (root-mean-square deviation, RMSD) regarding real data.

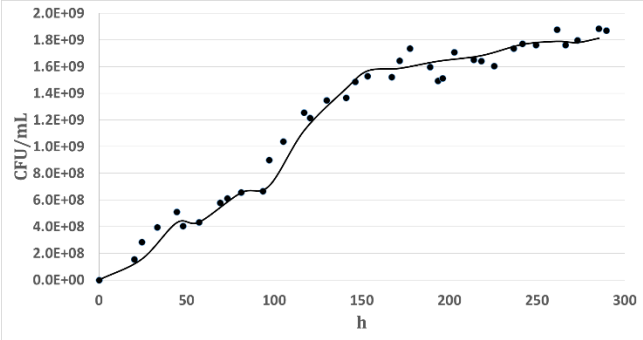

Figure 3. Growth of hydrocarbonoclastic bacteria (CFU) in Combined carbon selective medium.

Comparing the results found in the first and the second adaptation, we can state that the amount of carbon source in the medium affects the growth of hydrocarbonoclastic bacteria. In the first process, where the concentration of diesel was higher, they reached a population of $2.29x10^9$ CFU / mL in 24 hours, while, in the second one, the bacterial count was $2.83x10^8$ CFU / mL in the same period. Also, based on data observed in Figure 3, we can infer that the accelerated growth occurs after 100 hours (about 4 days).

Conclusions

We isolated and induced the adaptation of hydrocarbonoclastic bacteria, from the gut of the second generation larvae of *Tenebrio molitor,* to use Diesel as a carbon source. Our results revealed that the population numbers of hydrocarbonoclastic bacteria depended on the amount of Diesel in the selective medium. The higher concentration of diesel (10893 mg / L), the higher the population ($2.29x10^9$ CFU/mL), while the lower concentration of diesel (1210 mg / L), the lower the population ($2.83x10^8$ CFU/mL), in 24 hours. Also, we observed that the accelerated growth of hydrocarbonoclastic bacteria occurs after 4 days of incubation.

Finally, this work revealed that a co-diet of diesel and cornflour did not affect the life cycle of the *Tenebrio molitor* and the second generation of larvae had more hydrocarbonoclastic bacteria in their gut.

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