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Original Research

Influence of Aromatic, Heteroaromatic, and Alkane Hydrocarbons on the Lipase Activity of *Pseudomonas* sp. in Batch Culture

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Abstract

The induction of the lipolytic system through the use of some hydrocarbons as the only carbon source for the growth of *Pseudomonas* sp. was investigated. The results showed induction of the enzymatic activity using 4 of 11 hydrocarbons used at a concentration of 3,000 mg/L (butylbenzene, 1,2,3,4-tetrahydronaphthalene, hexane and benzene), which suggests that the enzymatic expression is a function of the hydrocarbon used. Furthermore, when the concentration of these hydrocarbons was increased to 5,000 mg/L the enzymatic activity was not detected. Additionally, the effect of an external source of energy, such as sucrose, and the effect of surfactant Triton X-100 were determined. The results revealed that the presence of these may benefit the induction of the enzyme or may affect it negatively and suppress it depending of the hydrocarbon being used. Therefore, it is not feasible to consider the enzymatic activity of lipase as a biomarker in the degradation of all petroleum hydrocarbons.

Keywords: carbon source, hydrocarbons biodegradation, lipase, *Pseudomonas* sp., triton X-100

Introduction

Bioremediation technologies are recognized as excellent options for treatment of polluted sites that have to be monitored by alternative tools. Based on the fact that the analysis and monitoring of soils contaminated with hydrocarbons is often done with analytical instruments whose use generates high costs [1], the use of biological activities, such as biomarkers, to obtain relevant information to evaluate contaminated sites, has been proposed. Margesin et al. [2], proposed the evaluation of the lipase enzymatic activity to indirectly measure the degradation of petroleum derivatives in soil; they mentioned that the lipase activity in the soil increases with time and remains unaltered with the accumulation of recalcitrant and less bioavailable compounds. They also report that the lipase induction in the soil increases with an initial increase of petroleum concentrations. According to Margesin et al. [3], the lipase activity in the soil is favored at high diesel concentrations, which indicates that the induction activity is a function of soil contamination. Also, it has been detected that soils contaminated with petroleum contain important quantities of substances soluble in alcohol-

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Table 1. Description of experimental series for the evaluation of the induction of *Pseudomonas* sp. lipases.

Experimental Series					
1	2	3	4	5	6
Hydrocarbon [5000 mg/l]	Hydrocarbon [5000 mg/l] + Triton X-100 [500 mg/l]	Hydrocarbon [3000 mg/l]	Hydrocarbon [3000 mg/l] + Triton X-100 [1000 mg/l]	Hydrocarbon [3000 mg/l] + Sucrose [0.5%]	Hydrocarbon [3000 mg/l] + Sucrose [0.5%] + Triton X-100 [1000 mg/l]

benzene mixtures (bitumen), which are retained in the shallow portions of the soil, and such substances are probable stimulators of lipase activity in soil [4].

It has been previously reported in our group that *Pseudomonas* sp. is capable of inducing its lipolytic system when kerosene is used as the only carbon [5]. However, kerosene is known to be composed of an endless array of compounds so that is it not known if all of them induce the enzyme. The present work impacts the evaluation of lipase enzymatic activity response in the *Pseudomonas* sp. strain when several aromatic, heteroaromatic, and aliphatic compounds found in the kerosene fraction are used, together with other compounds with acyl ester bonds.

Materials and Methods

Strain Used

The *Pseudomonas* sp. strain previously identified by partial amplification of the 16S rRNA gene was used. The sequence was deposited in the Gene Bank Database under accession number EF191182. This strain has been studied in other research due to its ability to degrade crude oil and have a keratinolytic enzyme system [6].

Evaluation of Lipolytic System Induction of *Pseudomonas* sp.

Ten compounds derived from petroleum, both aromatic and aliphatic, were used as carbon sources; four of these are identified as being part of the kerosene fraction: hexane, 1,2,3,4-tetrahydronaphthalene, indene, and 9,10-dimethylanthracene [7]; six more were selected for being aromatic and heteroaromatic and other with acyl ester bonds: butylbenzene, butylbenzoate, benzene, benzoic acid, toluene, and pyridine, and a plasticizer with ester bond: dioctyl phthalate. All compounds were acquired from Sigma-Aldrich, Co.

Preparation of Inoculums

The inoculum was prepared in a 125 mL Erlenmeyer flask using 25 mL of mineral medium (MM): 0.30 KNO_3, 0.0332 $FeCl_3$ $6H_2O$, 0.2 $MgSO_4$ $7H_2O$, 0.1 $CaCl_2$, and 1 K_2HPO_4 (g/L), pH 6.8. Kerosene was added at a concentration of 20,000 mg/L as the only carbon source. The medium was inoculated with *Pseudomonas* sp. strain using a loop, and it was incubated at 28ºC at 180 rpm for 24 h. Thereafter, 0.1 mL aliquot of culture, was sub-culture two successive times into fresh medium, and incubated as before.

Effect of Aromatic and Alkane Hydrocarbons on the Lipase of *Pseudomonas* sp.

Six experimental series were prepared to evaluate the effect of each hydrocarbon on the *Pseudomonas* sp. lipase expression; as well as the effect due to the presence of sucrose and Triton X-100. Erlenmeyer flasks were prepared with 25 mL of MM added of the hydrocarbons as is indicated in Table 1. Each flask was inoculated with 0.1 mL of inoculum and incubated during 5 days at 28ºC and 180 rpm. The initial population in each series was 3.2×10^3 CFU/mL.

According to the results discussed below, other three additional concentrations of indene (800, 1,200, and 1,600 mg/L) and hexane (800, 1,050, and 1,300 mg/L) were evaluated using these compounds as the only carbon source. At the end of the incubation period, lipase enzymatic activity and hydrocarbon removal were evaluated. All experimental series were prepared in duplicate. Non-inoculated controls were also included. Reported values of hydrocarbon removal corresponded to net values, that is, abiotic removal values were subtracted.

Evaluation of Lipase Activity

Evaluation of the lipase activity was based on the technique reported by Margesin et al. [8] with some modifications. Two mL were taken from each culture and centrifuged on polypropylene tubes at 13,000 rpm at 4ºC for 8 min., 1.0 mL of the cell-free extract was taken and placed in a glass tube with 2.0 mL of 0.1 M KH_2PO_4/NaOH pH 7.2 regulator. The tubes were then submitted to preincubation in a thermal water bath for 10 min at 37ºC. Afterward, 20 µl of substrate [100 mMp-nitrophenol butyrate (pNPB) diluted in 2-propanol and stored at -20ºC] were added, the content was perfectly mixed, and the reaction was allowed to proceed for 15 minutes. The enzyme reaction process was stopped by introducing the tubes in a chilled bath. The liberation of p-nitrophenol (pNP) was measured spectrophotometrically at 400 nm (Thermo Scientific Genesys 10S UV-Vis). The absorbance was extrapolated in a type curve from 0 to 100 µg pNP. The lipase enzymatic activity was expressed in µg pNP/mL.

Evaluation of Microbial Growth

In order to verify that the hydrocarbons will not cause toxicity effects on *Pseudomonas* sp. growth, it was evaluated by dilution method every 24 h. Enumeration of bacteria was performed by plate-count method in nutritive agar and expressed as colony-forming units per mL (CFU/mL). The plates were incubated at 28ºC and counted on day three.

Evaluation of Hydrocarbon Removal

The residual hydrocarbons were quantified, and then the content in flasks was subjected to a liquid-liquid extraction using dichloromethane as a solvent. The content of the flask was transferred to a separation funnel (250 mL) and the hydrocarbons were extracted by five successive additions (20 mL) of dichloromethane and then concentrated in a rotary evaporator; afterwards the concentrated extract was suspended in 2 mL of dichloromethane and quantified by gas chromatography (Thermo Scientific, Trace GC Ultra, GC-FID) with an HP capillary column (30 m×320 µm×0.25 µm) operated as follows: initial temperature 80ºC, 1 min; 80-165ºC at 15ºC/min; and 165-200 at 7ºC/min; except for the case of hexane, which was 80-120ºC at 15ºC/min. Temperatures of injector and detector were 250ºC, Split was 40 mL/min. Helium was used as carrier gas (0.9 mL/min). The residual content was calculated interpolating the area of the corresponding peak in a calibration curve prepared with the corresponding hydrocarbon at concentration of 0-300 mg/L.

Results and Discussion

The first results showed that none of the hydrocarbons used at 5,000 mg/L induced the lipase activity of *Pseudomonas* sp. (Fig. 1), even though most of them supported the growth of bacteria with the exception of butyl benzoate, benzoic acid, and toluene (Table 2); therefore, the effect of the surfactant Triton X-100 was analyzed using the same hydrocarbon concentration (5,000 mg/L). The results manifested the favoring of the lipase induction only when using butylbenzene, 9,10-dimethylanthracene, and hexane, having a maximum 2.6 µg pNP/mL when using 9,10-dimethylanthracene (Figs. 1b, 1d, 1e), indicating that the surfactant can help the lipase induction; however, it was not generalized when using all the hydrocarbons. The enzymatic activity of *Pseudomonas* sp. using the surfactant as the only carbon source was evaluated in parallel, and it was found that the activity after 5 days was 14 µg pNP/mL. This indicates that the registered activity, when using hydrocarbons in conjunction with the surfactant, is not only from the Triton X-100 effect; if this was the case, the activity would only be the same to the one induced by the surfactant. The effect of Triton X-100 on lipase induction has been previously analyzed and in some cases has been reported to increase the activity up to 50 times in *Pseudomonas pseudoalcaligenes* F-111 using olive oil as a carbon source [9]; while in other cases the Triton has no effect as reported by Boekema et al. [10], who mentioned that Triton X-100 does not contain any acyl ester bonds as the Tween 80, which is an oleate that induces the lipase operon and has been reported as a substrate to evaluate lipase activity. Similar results were found to those obtained with p-nitrophenol butyrate [11]. In this research it is thought that the effect that Triton X-100 could have on *Pseudomonas* sp could coincide with findings by Jing and Bing [12], who mention that surfactants favor the solubility and dissolution of hydrocarbons to increase the contact between the microorganisms and the hydrocarbon, leading to a greater degradation of the latter.

Table 2. Population of *Pseudomonas* sp. after five days of incubation using different hydrocarbons as sole carbon source.

Hydrocarbon	Population using 5000 mg /L (CFU/mL)	Population using 3000 mg/L (CFU/mL)
Hexane	7.2×10^4	5.4×10^5
1,2,3,4-tetrahydronaphthalene	4.9×10^5	5.2×10^6
Indene	6.0×10^3	8.5×10^4
9,10-dimethylanthracene	1.7×10^5	6.4×10^6
Dioctylphthalate	7.3×10^5	8.7×10^5
Butylbenzene	8.4×10^5	2.7×10^6
Butylbenzoate	Not detected	3.9×10^4
Benzene	1.5×10^3	8.3×10^4
Benzoic acid	Not detected	Not detected
Toluene	Not detected	Not detected
Pyridine	6.6×10^4	5.7×10^4

Afterward, the same hydrocarbons were used as only carbon sources at 3,000 mg/mL, and the enzymatic activity was registered with four of them, 1,2,3,4-tetrahydronaphthalene, butylbenzene, benzene, and hexane (Fig. 1a, 1b, 1c, 1e). This suggests that the hydrocarbon concentration can be a determinant factor for enzymatic expression during the degradation process. It was also observed that not all the hydrocarbons used were inductors of *Pseudomonas* sp. lipase, at least not at 3,000 or 5,000 mg/l concentrations, suggesting the enzyme exhibits specificity for the substrate (hydrocarbon), which apparently has no relation with the presence of ester bonds. This is shown by the fact that the pure strain grew on different carbon sources (Table 2), and the enzymatic induction was shown only with some hydrocarbons and the surfactant, which have no ester bonds in their structure, such as benzene or 9,10-dimethylanthracene. On the contrary, compounds like butylbenzoate and dioctyl phthalate, which have ester bonds, did not induce enzymatic activity, although they did support strain growth and hydrocarbon degradation (Table 2), and some of them were degraded (Fig. 2).

Additionally, the results about the effect of the surfactant Triton X-100 with each hydrocarbon at 3,000 mg/L

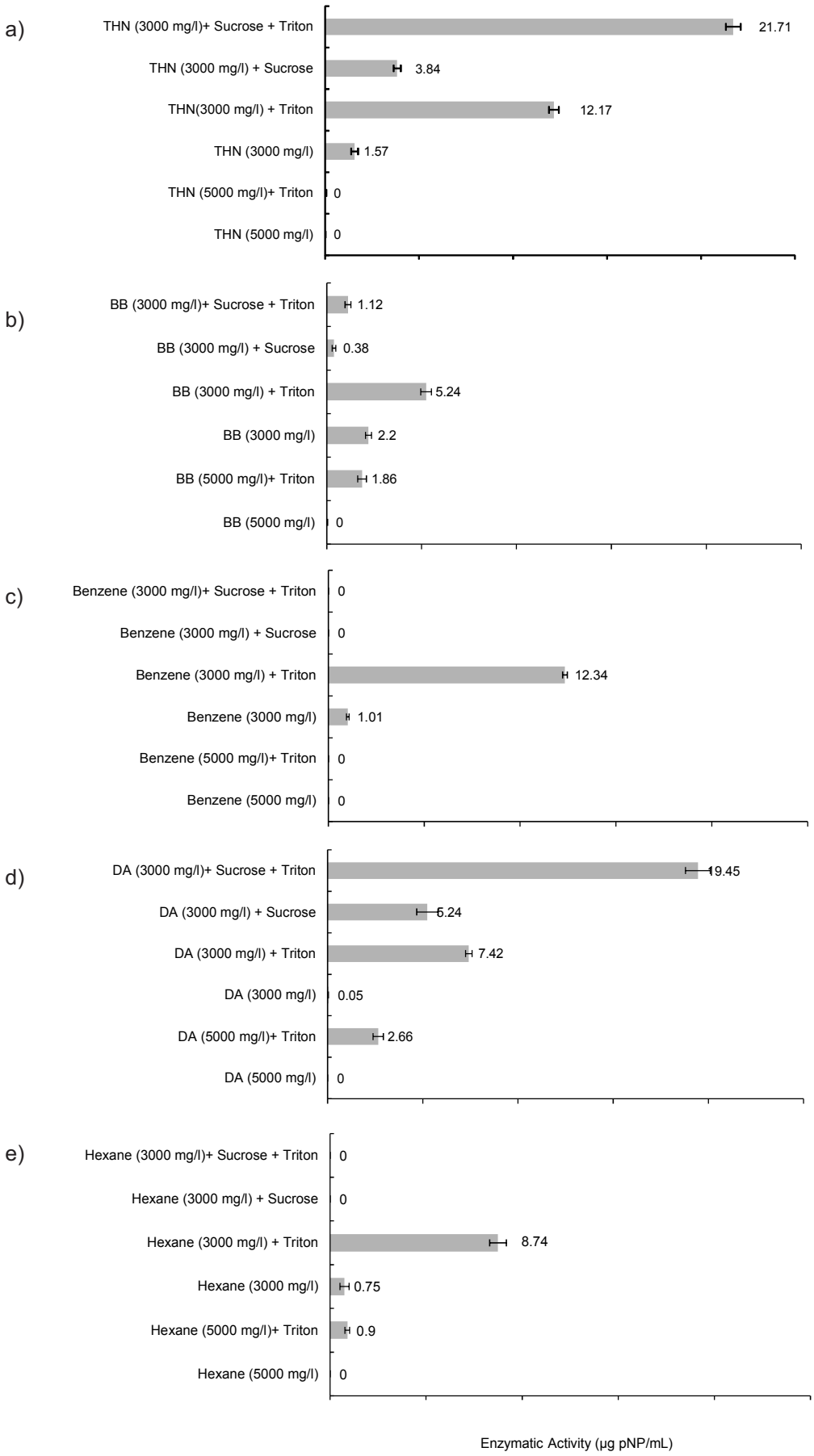

Fig. 1. Lipase activity of *Pseudomonas* sp. using: a) 1,2,3,4-tetrahydronaphthalene (THN), b) butylbenzene (BB), benzene, c) 9,10-dimethylanthracene (DA), and d) hexane, according to experimental series after five days of incubation at 180 rpm and 28ºC (error bars are standard deviation).

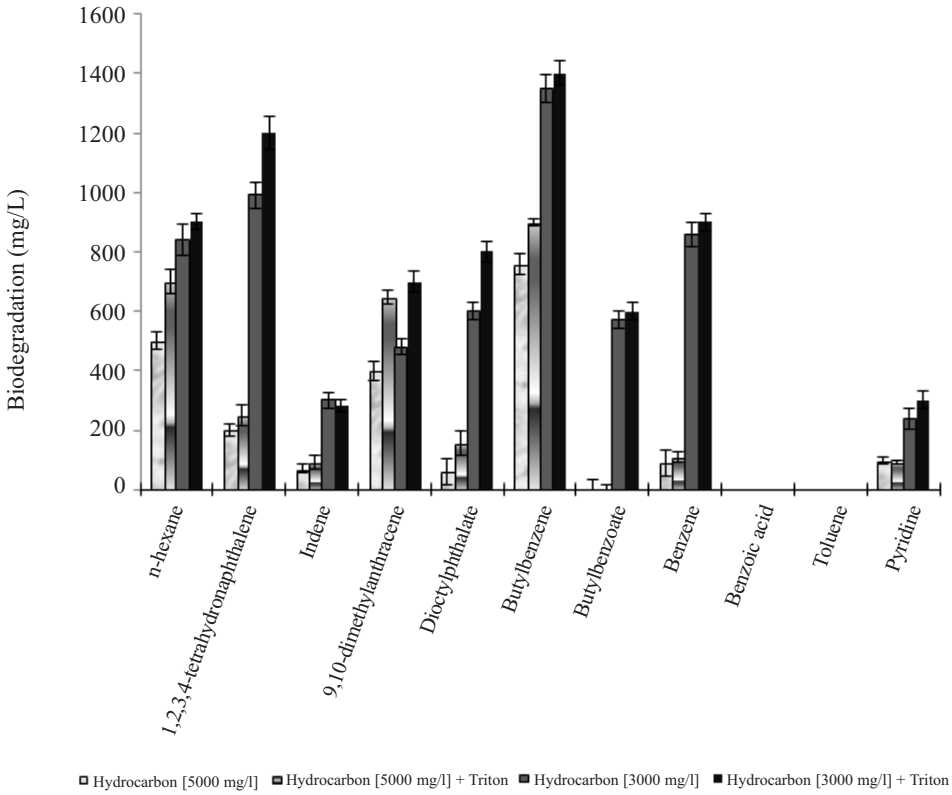

Fig. 2. Hydrocarbon biodegradation after five days of incubation at 180 rpm and 28ºC by *Pseudomonas* sp. at different concentrations and using the surfactant Triton X-100 (Error bars are standard deviation).

(experimental series 4) indicated an increase in the enzymatic activity compared to the use of hydrocarbons as the only carbon source (experimental series 3). However, the enzymatic activity was registered in the same four hydrocarbons that were able to induce activity on their own, and additionally using 9,10-dimethylanthracene (Fig. 1d). The highest activities were detected when using 1,2,3,4-tetrahydronaphthalene and benzene, 12.17 and 12.33 µg pNP/mL, respectively (Figs. 1a, 1c). These activities correspond to an increase of 7.7 and 12 times in the case of 1,2,3,4-tetrahydronaphthalene and benzene, respectively, compared to the effect of Triton X-100 alone. This did not occur in the cases with butylbenzoate , dioctyl phthalate, indene, and pyridine, where strain growth was shown with no enzymatic activity, suggesting that the degradation of these compounds is not performed by lipase action or that the 3,000 mg/l concentration is too high to induce the enzyme. It should be noted that in the case of the benzoic acid and toluene no strain growth was present, not even in the presence of Triton X-100 (Table 2). It is important to mention that most bacteria capable of metabolizing benzene also use toluene; however, it also has been reported that some cases like the toluene-metabolizing fungus *Cladophialophora* sp. strain T metabolized toluene, ethylbenzenes, and the xylenes, but biodegradation of benzene required the activity of the other indigenous soil microorganisms [13].

Furthermore, the effect of sucrose on the induction of the lipolytic system of *Pseudomonas* sp. as a carbon source of easy assimilation together with each hydrocarbon was evaluated. The results showed an increase in activity only when using 9,10-dimethylanthracene and 1,2,3,4-tetrahydronapthalene, then when compared to using the hydrocarbon as the only carbon source, the increase was from 0.04 to 5.24 µg pNP/mL and from 1.57 to 3.84 µg pNP/mL, respectively (Figs. 1a, 1d). In the case of butylbenzene, hexane, and benzene, the activity decreased from 2.22 to 0.38, from 0.74 to 0.0, and from 1.01 to 0.0 µg pNP/mL, respectively, an effect given by the presence of sucrose (Figs. 1b, 1e, 1c), suggesting that the lipase expression can be prone to catabolic repression. The remaining six hydrocarbons evaluated presented no activity even though they showed growth and biodegradation (Table 2 and Fig. 2), which corroborates that extracellular lipases do not participate in the hydrocarbon degradation metabolism. In this aspect, Boekema et al. [10] indicate that the presence of an additional carbon source such as sucrose promotes lipase activity in the olive oil degradation system through *Burkholderia glumae*, while the opposite is seen with the presence of glucose. On the contrary, Joseph et al. [14] mention that *Micrococcus roseus* produces a larger lipolytic activity when glucose is the alternative carbon source rather than sucrose, lactose, or maltose. Giti Emtiazi et al. [15] mention that glucose and glycerol supported good growth of *Pseudomonas* strain X, but repressed lipase production by 75%. This means that each microorganism presents a catabolic repression by carbon according to their carbohydrate transport system at different levels of transcription, RNA processing, translation, or protein modification.

Lastly, the combined effect of an alternative carbon source (sucrose) and the presence of surfactant (Triton X-

100) on the enzyme induction when using hydrocarbons was evaluated (experimental series 6). Results showed similar trends as experimental series 5, with the same two hydrocarbons raising the enzymatic activity up to 19.44 µg pNP/mL with 9,10-dimethylanthracene and up to 21.7 µg pNP/mL with 1,2,3,4-tetrahydronaphthalene; this was not the case with hexane and benzene, where no lipase enzymatic activity was detected, or with butylbenzene, where the detected activity was minimum. These results confirm that hydrocarbon degradation is carried out through different routes and that additional carbon sources and surfactants have different effects. As reported by Boekema et al. [10] the presence of surfactant Triton X-100 does not increase the lipase activity when sucrose or glucose are used as a carbon source for a strain of *Burkholderia glumae*, but the use of another surfactant, in this case Tween 80, does stimulate the lipase production when sucrose is used as a carbon source. This analysis concludes that the use of lipase activity as a hydrocarbon degradation marker must be limited.

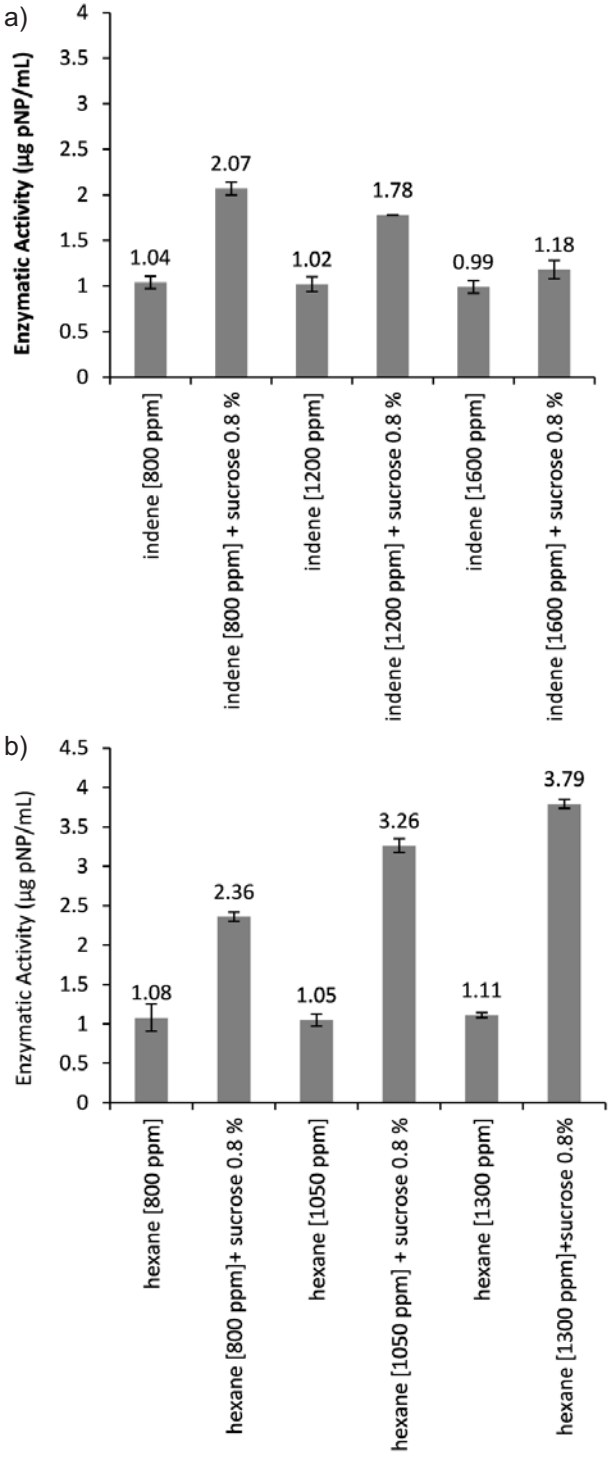

Fig. 3. Lipase activity of *Pseudomonas* sp. using: a) indene and b) hexane, after five days of incubation at 180 rpm and 28ºC (error bars are standard deviation).

As a complement, the *Pseudomonas* sp. lipase activity using hexane as a carbon source in lower concentrations as those previously evaluated (5,000 and 3,000 mg/L) was evaluated since several papers have described that some n-alkanes (such as hexadecane and octodecane) induce lipases [10, 16, 17], yet in this study lipase activity was zero when using 5,000 mg/L and 0.74 µg pNP/mL when using 3000 mg hexane /L. Therefore, concentrations of 800, 1,050, and 1,300 mg/l were evaluated; the activities obtained with this compound as sole carbon source were as low and similar in all three concentrations (1 µg pNP/mL) (Fig. 3b), which suggests that hexane concentration has no effect on lipase induction. The responses due to the sucrose effect were slightly higher, from 2.35 in the 800 mg/L concentration to 3.79 µg pNP/mL in the 1300 mg/L concentration, which indicates a positive effect in the presence of sucrose and also a dependence on the hydrocarbon concentration since the sucrose had a inhibitory effect on the enzyme at 3,000 mg/L of hexane.

Another particular case was the evaluation of indene as a carbon source for the *Pseudomonas* sp. lipase induction at lower concentrations than 3,000 mg/L, since it is a monoaromatic compound similar to 1,2,3,4-tetrahydronaphthalene with the difference of having a cycloalkene attached to the benzoic ring rather than a cycloalkane. As mentioned above, indene was one of the hydrocarbons that did not induce the enzyme at 5,000 or 3,000 mg/l concentrations, therefore concentrations of 800, 1,200, and 1,600 mg/l were also studied; a similar activity was registered in all concentrations used (1 µg pNP/mL); this is not the case for experiments where sucrose was added with activity measured was 2.07 µg pNP/mL when using the lowest hydrocarbon concentration (800 mg/L) and as 1.18 µg pNP/mL when using 1600 mg/l (Fig. 3a). As observed, the presence of sucrose promotes the enzymatic activity mostly at lower indene concentrations. Furthermore, it was observed that although the indene concentration was lowered by up to one third of the 3,000 mg/L of 1,2,3,4-tetrahydronaphthalene concentration, the lipase activity did not reach the same levels; that is, even though the compounds are so structurally similar, the lipase appears to have specificity. It should be noted that there is no reported lipase induction through indene.

Conclusion

Expression of lipase activity in the strain of *Pseudomonas* sp. is associated with the concentration and type of hydrocarbon used as well as the presence of surfac-

tant and/or an alternate carbon source. Because the presence of the surfactant TritonX-100 had a promoting effect on lipase expression using butyl-benzene, 1,2,3,4-tetrahydronaphthalene, 9,10-dimtilantraceno, hexane and benzene, the presence of another carbon source (sucrose) inhibited lipase activity in 1,2,3,4-tetrahydronaphthalene, butyl benzene, and 9,10-dimethylanthracene. Therefore the use of lipase activity as hydrocarbon degradation marker must be limited, because not all types of hydrocarbons can be inductors of activity, and not all microorganisms express the enzyme.

Acknowledgments

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