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Biodegradation of pyrene and anthracene by *Pseudomonas aeruginosa* TGC-02 in submerged culture

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ABSTRACT
The removal of polycyclic aromatic hydrocarbons (PAH)s in contaminated sites is one of the major challenges in the petroleum industry. This work aimed to select one hydrocarbonoclastic *Pseudomonas aeruginosa* among three isolates recovered from petrol stations and to evaluate the degradation of pyrene and anthracene in submerged culture. For bacterial screening, we chose the emulsification index and the drop collapse test. The TGC-02 isolate was selected due to its emulsification index towards four fuels and oil collapse within 48h. Further, TGC-02 was inoculated into a liquid medium contaminated with 50 mg/L (PAH)s. Tests were carried out in triplicate at 5-day intervals to determine the cellular growth, pH, cell viability, protein content and enzymatic activity over 25 days of incubation. Cellular growth in the reactors with petroderivatives were not statistically different from the control. Up to 20 days, cells remained viable. Considering the abiotic losses, TGC-02 had degraded about 40 and 30% of pyrene and anthracene, respectively. pH increased from 5 to 8 and no specific activity was observed for laccase or manganese peroxidase. However, protein concentration increased during the degradation experiment. Results suggested that these enzymes are not involved in the process of anthracene and pyrene removal by *P. aeruginosa*.
Keywords – Biodegradation, Bioremediation, Hydrocarbonoclastic activity, Policyclic Aromatic Hydrocabons

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I. INTRODUCTION

Modern society is still heavily dependent on fossil fuels to perform most of its activities [1,2]. Polycyclic Aromatic Hydrocarbons (PAH)s are one of the major contaminants generated by the incomplete combustion of fossil fuels [3]. (PAH)s are very toxic, carcinogenic and teratogenic molecules for mammals [4, 5]. Their hydrophobicity allows easy absorption by contact with the skin and after ingestion or inhalation, this poses a health risk [6,7].

In a liquid medium, the physicochemical properties of (PAH)s promote the sorption of these molecules in suspended organic matter, as well as in the soil aggregates in the vadose zone, making it difficult to remove them, which is one of the major challenges of the oil industry [8]. (PAH)s sorption in the soil is especially guaranteed by its persistent nature, attributed, among other properties, to low solubility in water and the number of condensed aromatic or cyclopentane rings [9, 10]. Additionally, the number of the rings divides the (PAH)s into two groups: low molecular weight (PAH)s (LMW-PAH) with two and three rings and high molecular weight (PAH)s (HMW-PAH), formed by four or more rings [11].

Bioremediation stands out as one of the best strategies for removing (PAH)s from contaminated water [12]. Several bacterial groups can convert these compounds to CO_2, such as Actinobacteria [13] and Cyanobacteria [14]. Additionally, Proteobacteria stand out for their metabolic versatility, and can be used as (PAH)s removal agents. However, little is known about the enzymatic mechanism involved in the process, and it is believed that oxidoreductases participate in the process [15].

Pseudomonas aeruginosa is the main representative of the fluorescent pseudomonads group (Proteobacteria). Its metabolic versatility and persistence in environments that exert high selective pressure, making the species one of the best

candidates as a bioremediation agent for recalcitrant and persistent molecules [16,17]. Additionally, *P. aeruginosa* is one of the most prevalent bacteria found in sites contaminated by petroleum hydrocarbons [18]. The literature reports the use of more than 90 organic compounds as sources of carbon and energy for the bacterium [19], among them aromatic hydrocarbons [20] and asphaltenes [21]. Thus, the aim of the present study was to identify hydrocarbonoclastic isolates of *P. aeruginosa* and to evaluate the degradation capacity towards anthracene and pyrene in water.

II. MATERIAL AND METHODS

2.1 (PAH)S

96% pure anthracene and 98% pure pyrene were used (Merk KGaA, Darmstadt, Germany). (PAH)s solutions were prepared in pure acetone (Sigma-Aldrich, Darmstadt, Germany).

2.2. Microbes and acclimation

Three isolates of *P. aeruginosa* recovered from soil samples surrounding petrol station areas in the city of João Pessoa, Brazil, TGC-02, TGC-03 and TGC-07 [3] were used in this study. We assumed that the exerted selective pressures represented by the concentration of the Total Petroleum Hydrocarbons (TPH) in these soils, which ranged 10,000-12,000 mg/Kg [22], allowed these isolates as potential candidates for this study. The isolates are registered in the National Genetic Heritage and Associated Traditional Knowledge Management System (SisGen) under number A6B80BD, and kept in the Laboratory of Environmental Microbiology.

The isolates were acclimated to anthracene and pyrene for 96h at 30°C in flasks containing Minimal Mineral Medium, MM (0.5 g/L K_2HPO_4, 0.5 g/L $(NH_4)_2SO_4$, 0.5 g/L $MgSO_4$, 0.01 g/L $FeCl_2$, 0.01 g/L $CaCl_2$, 0.001 g/L $MnCl_2$, 0.001 mg/L ZnSO, to which had been added 0.1 g/L of yeast extract and increasing concentrations of (PAH)s (1, 5, 10, 25 and 50 mg/L). Every 24h, an aliquot (10% v/v) was transferred to a new flask, evaluating the growth of the cells by observing the turbidity by visual inspection [23].

2.3 Determination of hydrocarbonoclastic activity

The tests, carried out in triplicate, had the objective of selecting, among the three isolates of *P. aeruginosa*, the one with the highest petroleum hydrocarbon degrading potential. The screening consisted of two tests: calculation of the emulsification index and a drop-collapse assay. In the first, the technique described by Cooper and Goldenberg [24] was used, using a 1:1 ratio of the aqueous phase and the oil phase, represented by gasoline, diesel oil, kerosene and lubricating oil, purchased at gas stations. The mixture was stirred vigorously for two minutes every 24h interval over 2 days. The emulsification indexes of 24h (E_{24}) and 48h (E_{48}) were measured by dividing the emulsion height and the total height of the phase mixture, multiplied by 100. A 1% SDS solution was used as the control.

The drop-collapse assay was performed according to Hanson and Desai [25]. A 12-well cell culture plate (working volume/well 2 mL) was filled with 1.5 mL of MM, 10 µL of oil phase (gasoline, diesel, lubricating oil, benzene, toluene and Xylene), 1.5 µL of the 1% 2,6-Dichlorophenolindophenol indicator solution (DCPIP) and 2.5µL of the bacterial suspension with turbidity standardized by tube #1 on the MacFarland scale. The plates were incubated at 30°C for 120h. Changes in the colour or appearance of the oil drop were visually recorded every 24 hours. The cell viability was verified every 24h by transferring 10µL to 96-well plates containing nutrient broth, followed by incubation at 30°C for 24-48h and visual inspection of the turbidity [26].

2.4 (PAH)s biodegradation assay

The assay was performed in triplicate for 25 days under static incubation at room temperature in amber glass microcosms containing 30mL of mineral medium to which 0.1 g/L of yeast extract and 50 mg/L of pyrene or anthracene had been added. The inoculum corresponded to 10% (v/v) and was prepared in 0.85% NaCl, from a recent TGC-02 culture, with turbidity standardized using tube #1 on the MacFarland scale. Aliquots were withdrawn at 0, 3, 5, 10, 15, 20 and 25 days for enzyme activity and protein concentration assays. A cell viability test was performed for each sample collected. The assay was performed in microdilution plates, adding 1mL of the sample and equal volume of 1% (v/v) resazurin solution. Cell viability was determined by observing the change of the colour from blue to pink or colourless [27]. Five experimental groups were formed, including controls (two abiotic controls and one with TGC-02 inoculated in the medium without (PAH)s. Room temperature and pH were monitored during the experiment.

2.5 Determination of laccase and manganese-peroxidase (MnP) activity

Samples were centrifuged at 10.000 rpm for 10 minutes (Mini-Spin Eppendorf Rotor F-45-12-11). The assays were performed with the supernatant and with the pellet, resuspended in 2 mL of 0.85% NaCl and sonicated for 10 minutes (Unique USC 1800).

For the determination of laccase activity, we adapted the method described by Arora et al. [28]. Samples of 13 µl from the amber glass microcosms were transferred into a tube containing 288 µl of a solution (224 µl of 200 mM sodium acetate buffer pH 4.5 and 64 µl of 5mM guaiacol – extinction coefficient of 26,60 $M^{-1}cm^{-1}$). Then, the absorbance (λ = 450 nm) was determined at 5, 10, 15 and 30 minutes (MindRay MR-96A). A positive control of the assay was performed with laccase from *Trametes versicolor* at 0.01mg/mL (Sigma-Aldrich 38429) to verify that the assay was properly carried out.

For the determination of MnP activity, we adapted the methodologies described by Wariish et al. [29] and Kuan et al. [30]. Samples of 50µL from the amber glass microcosms were mixed with 450µL of Milli-Q water, 250µL of sodium acetate buffer (200 mM, pH 4.5), 100µL of manganese sulphate (20 mM), and 100µL of sodium lactate (250 mM). The reaction was started by adding 50 µL of H_2O_2 (50 µM) and the production of the lactate-Mn complex (extinction coefficient of 65,00 $M^{-1}cm^{-1}$) was monitored at 270 nm for 10 min (Even IL0082-Y-BI). A positive control of the assay was conducted with MnP from *Bjerkandera adjusta* at 0.01mg/mL (Sigma-Aldrich 68528) using the same reasoning as for laccase positive assay.

2.6 Quantification of the protein

The method described by Sedmark and Grossberg [31] was used for protein quantification. Samples of 150µL of the supernatant were mixed with 150 µL of Coomassie Brilliant Blue G250 and absorbance was determined at λ = 630 and 450 nm. The ratio of the means of each absorbance was replaced in the equation $y = 0.0496x + 0.224$ (r^2=0.9907), obtained in the calibration curve with bovine serum albumin (Sigma-Aldrich A9418).

2.7 (PAH)s quantification

The (PAH)s concentration was determined by gas chromatography (HP 5880, column 30 m x 0,25 mm) coupled to mass spectrometry (EM 5987), using USEPA methods [32, 33]. The extracts were obtained by Soxhlet extraction [34], using dichloromethane. The pre-concentration of the samples was carried out in nitrogen atmosphere.

2.8 Cell growth determination

The cell growth was quantified by the spectrophotometric method [35]. An aliquot of each experimental group was subjected to absorbance analysis at 600 nm (Even IL0082-Y-BI). The blank corresponded to the minimum mineral medium, with and without the (PAH)s.

2.9 Statistical data analysis

All the experiments were carried out in triplicate and the results are presented as the mean and standard deviation of three independent observations. After confirmation that the data followed a normal distribution, statistically significant differences at 95% of confidence were checked by using One-way ANOVA with Tukey post-hoc test (GraphPad Prism 7, GraphPad Software Inc).

III. RESULTS AND DISCUSSION

3.1. Screening of *Pseudomonas aeruginosa* isolates

The three isolates reached emulsification indexes higher than 40 and 15% in 48h, respectively for the hydrocarbons with higher carbon chains, i.e., lubricating oil and kerosene (Fig. 1). Specifically, TGC-02 emulsified all petroderivatives except diesel oil. Its hydrocarbonoclastic activity was expected because all the strains were recovered from soils impacted by petroderivatives [3]. The emulsification of the petroleum hydrocarbons by *P. aeruginosa* may occur through the synthesis of biosurfactants, particularly rhamnolipids [36].

Diesel and gasoline are more toxic petroderivatives because they have shorter carbon chains and thus, are more volatile [22]. However, Lebonguy et al. [37], evaluated the emulsifying activity of two *P. aeruginosa* isolates using diesel oil, gasoline and hexane. They observed an E_{24} of 53% to diesel oil. Considering the fact that TGC-07 was the only isolate to emulsify the diesel, even at a lower percentage, suggests that there was a possible contamination by this fuel in its isolation site.

With respect to longer carbon chain hydrocarbons (Fig. 1), the three isolates emulsified kerosene (C14-C20), especially TGC-02, about 25%, as well as exhibiting a higher E_{48} of the lubricating oil (C20-C40). The emulsification of the lubricating oil was expected since the isolates were already acclimated to the compound [3], indicating that the presence of kerosene particularly promoted greater cellular stress to TGC-02. This hypothesis is supported by the study of Shahaliyan et al. [19], who performed the same test with a strain of *Pseudomonas* sp. and observed an E_{24} of 46% to kerosene.

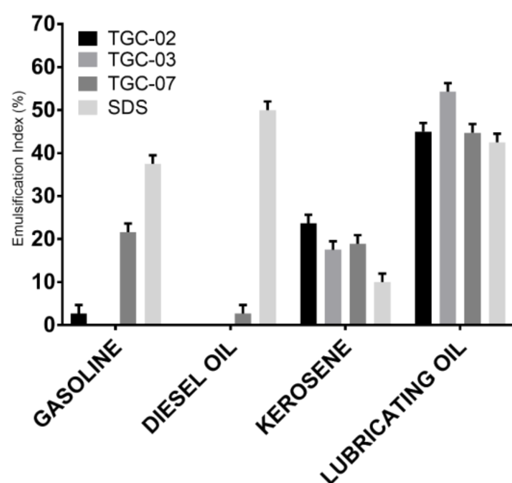

Fig. 1. Emulsification index of petroderivatives by *Pseudomonas aeruginosa* isolates

In the drop-collapse test (Table 1), TGC-07 and TGC-03 presented hydrocarbonoclastic activity for fuels over a longer time than TGC-02, but did not demonstrate interaction with the benzene, toluene and xylene (BTX). Meanwhile, TGC-02 consumed 4 of the 6 hydrocarbons tested within 48 hours (except toluene and xylene), remaining viable up to 196h. The viability of the cells was much higher than that determined by Pirrôlo et al. [38]. The authors checked the oil drop collapse after 196h with *P. aeruginosa* LBI. In addition, BTX is more volatile at room temperature and therefore more toxic to bacterial cells [39], as demonstrated by Thenmozhi et al. [40] when investigating *P. aeruginosa* strains in the presence of toluene.

Table 1. Time (h) when the hydrocarbon drop has collapsed

	Hydrocarbons					
Isolates	LO	D	G	B	T	X
TGC-02	48	120	48	96	-	-
TGC-03	48	48	48	48	-	-
TGC-07	96	96	96	-	-	-

LO – lubricating oil, D – diesel, G – gasoline, B – benzene, T – toluene, X - xylene

Although all three isolates showed hydrocarboclastic activity, TGC-02 was chosen for the (PAH)s biodegradation test, considering the number of emulsified hydrocarbons and those used as the carbon source in the drop-collapse test.

3.2 Cell growth and degradation of (PAH)s

With respect to abiotic losses, TGC-02 reduced 37.83±0.12% of pyrene and 33.01±0.10% of anthracene (Table 2). Our results were higher than those observed by Xia et al. [41], 21.93%, using *P. aeruginosa* grown in solid state for 20 days. Under controlled conditions of agitation and temperature, higher rates of biodegradation may be reached. This was the case of a previous 25-days study where a strain of *P. aeruginosa* degraded more than 50% of pyrene in a submerged culture [42]. However, it is important to note that our study aimed to measure the potential of TGC-02 using static incubation at room temperature.

Table 2. PAH degradation by *Pseudomonas aeruginosa* TGC-02*

PAH	Degradation (%)
Pyrene	37.83±0.12
Anthracene	33.01±0.10

*Excluding abiotic losses

The degradation of (PAH)s, especially HMW-PAH, by *P. aeruginosa* are widely reported and showed good results. Lin et al. [43] observed the consumption of 40% of pyrene using *Pseudomonas* sp. in 30 days. The degradation kinetics of this (PAH)s in the same period of 30 days was studied by Gosh et al [44], who observed that the rate of degradation was inversely proportional to the concentration initially present.

Cellular growth in the reactors with petroderivatives were not statistically different from the control (reactors with culture medium only). By the 10th day, the cell concentration had reached the stationary phase, with a reduction in cell density on the 15th day and regrowth by the 20th day when the cells were no longer viable (Fig 2). In addition, the bioprocesses favoured an establishment of an alkaline medium with the average room temperature around 25ºC, a value within the range considered ideal for the degradation of hydrocarbons at room temperature, mimicking real conditions [45].

The concentration of organic matter present in the microcosms containing pyrene and anthracene may justify the fact that the TGC-02 had started the stationary phase by the 5th. On the other hand, in the microcosms without (PAH)s, the log phase was longer, suggesting some degree of toxicity of the pyrene and anthracene. Possibly, after biomass growth up the 10th day, the main carbon source had been depleted, reflecting the reduction of cell density. However, cell debris itself may have been used as a carbon source for the maintenance of the remaining cells, which remained viable up to the 20th day. After this, cell viability was extinguished, suggesting the accumulation of toxic metabolites or depletion of nutrients necessary for cell metabolism.

The culture conditions in the (PAH)s-contaminated liquid medium may favour the establishment of a medium saturated in metabolites, leading to intoxication of the cells. This was

observed in a previous study [46]. When the authors compared the characteristics of submerged and solid cultures, they observed that the destabilization of enzymes is more common in the first case, with excessive cell aggregation occurring, resulting in the death of nearby cells. In addition, other factors such as the formation of peroxides and the limited bioavailability of the molecules participated in the cell viability process [47].

There was a slightly higher percentage of pyrene consumption compared to anthracene. Although pyrene (four rings) is more stable and complex than anthracene (three rings), *P. aeruginosa* preferentially degrades pyrene [3]. In brief, it may be explained by the fact that HMW-PAH biodegradation may occur through cometabolic pathways and when LMW-PAH are present, they are used as cosubstrates [48].

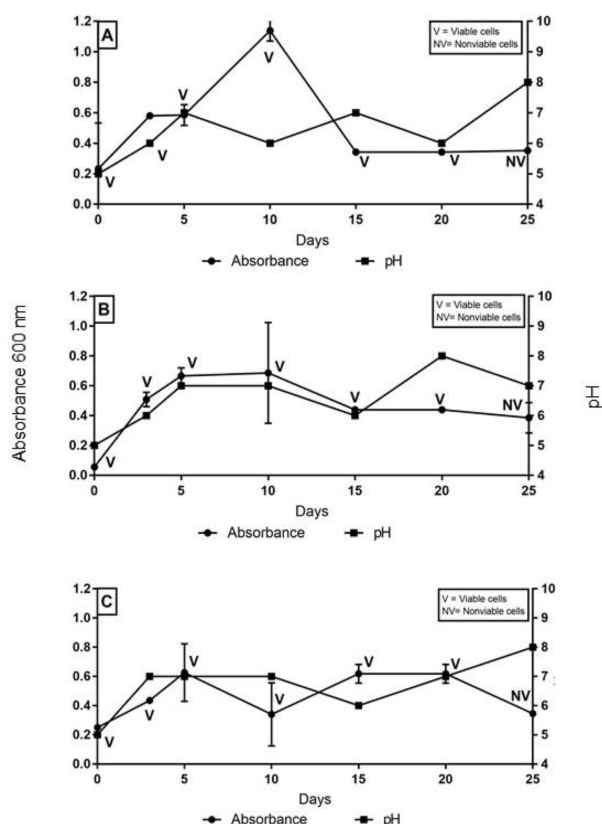

Fig. 2. Growth kinetics and cell viability of *Pseudomonas aeruginosa* TGC-02: control (A), in the presence of pyrene (B) and anthracene (C).

Vaidya et al. [49] proposed that the metabolism of pyrene in *P. aeruginosa* may be related to the metabolic pathway of phthalic acid. Because of the metabolization of pyrene to phthalic acid, this compound may be converted to pyruvate and citrate. Other metabolites may be formed, such as dihydroxypyrene, 4-oxa-pyrene-5-one, 1,2-dimethoxypyrene [42, 44]. While 1,2-dimethoxypyrene is a result of the oxidation of the C4 and C5 of the pyrene, the others are produced by fission and decarboxylation of the intermediates used to form 4-phenolic acid, followed the path of phthalic acid.

In the presence of anthracene, TGC-02 demonstrated a cellular stress behaviour. Anthracene is an LMW-PAH, containing three condensed rings that may form different toxic compounds that accumulate when the molecule is destabilized, for example, anthraquinone, anthrone and phthalic anhydride [50].

The pH increased from approximately 6 to 8 indicating the formation of alkaline metabolites during the hydrocarbon degradation process [51]. The pH, as a chemical factor, directly affects the microbial activity due to the H^+ concentrations, which can make feasible or prevent enzymatic activities. The variation of this indicator could be observed throughout the process. The pH increase may have a positive influence on the availability of macro and micronutrients in the medium, allowing the isolates in cultivation to possess catabolic activity for assimilation and biomass growth [52].

3.3 Determination of the enzymatic activity and the protein content

TGC-02 surprisingly did not express MnP and laccase, but an increase in protein concentration was observed throughout the process of degradation, corroborating the increase in biomass and substrate consumption (Fig. 3).

We hypothesized two reasons for the absence of laccase and MnP activities: the first refers to the low concentrations of both enzymes in the medium, lower than the levels detectable by the methodologies used; the second reason is that these enzymes are not directly related to the degradation of anthracene and pyrene and that other oxidoreductases assume the role of MnP and laccase in the oil hydrocarbons metabolism in *P. aeruginosa*. Yan and Wu [53] evaluated 46 genes involved in the degradation of (PAH)s by *P. aeruginosa* PAO1. The authors observed that the expression of these genes varied according to growth conditions and that some of them were activated by the oxygen concentrations such as NADH dehydrogenase I, 3-oxoadipate enol lactonase, 4-hydroxybenzoate-3-monoxygenase, quercetin- 2,3-dioxygenase and 1,2-dioxygenase homogentisate. In addition, the expression of enzymes responsible for the degradation of (PAH)s depends on multiple factors, such as tolerance, resistance, production of exopolysaccharides and quorum sensing [54, 55].

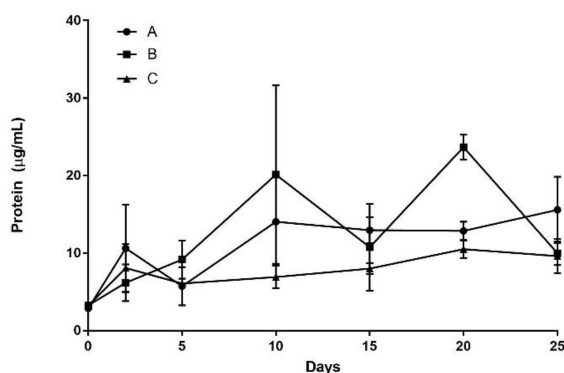

Fig. 3. Range of the total protein in *Pseudomonas aeruginosa* TGC-02: control (A), in presence of pyrene (B) and anthracene (C).

IV. CONCLUSIONS

P. aeruginosa TGC-02 presented the highest hydrocarbonoclastic activity among the three evaluated isolates, reducing by approximately one third the amount of pyrene and anthracene contaminants of the aqueous medium used in the degradation tests. Laccase and manganese peroxidase activities were not detected. However, there was an increase in the protein concentration, coinciding with the cellular growth increase, suggesting that the enzymes investigated are not involved in the process of anthracene and pyrene removal by *P. aeruginosa* in water.

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