ISOLATION OF MICROORGANISMS WITH CAPABILITY TO DEGRADE POLYCYCLIC AROMATIC HYDROCARBONS (PAH´S)

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This paper summarizes a work conducted on the isolation of microorganisms from PAH's contaminated sediments. The methodology involved two selection systems called fast route and slow route in which exposure periods and contaminant concentrations are the key determinants. The microorganisms isolated through the slow route system are more likely to be successful in degrading high molecular weight PAH's. The six strains obtained through the fast route system were able to grow on low molecular weight PAH's showing preference towards the first four EPA (Environmental Protection Agency) priority PAH's.

Este estudio presenta el aislamiento microbiológico en muestras de sedimento contaminado con un alto porcentaje de hidrocarburos aromáticos polinucleares (Polynuclear Aromatic Hydorcarbons, PAHs). La metodología utilizada comprende dos sistemas de selección denominados vía rápida y vía lenta. Estas vías tienen como condiciones determinantes el tiempo de exposición y la concentración del contaminante de interés. Los microorganismos aislados mediante la vía lenta garantizan mayor probabilidad de éxito en el proceso de biodegradación de PAHs con compuestos de alto peso molecular. Seis cepas con capacidad de crecer en PAHs de bajo peso molecular obtenidos por la vía rápida, mostraron preferencia por los primeros cuatro compuestos, de los dieciséis exigidos por la EPA (Environmental Protection Agency).

Keywords: *PAH's, Microcosm, Quemostat, biotratament.*

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INTRODUCTION

Polycyclic aromatic hydrocarbons (PAH's) are frequent environment contaminants and microbial recalcitrants that accumulate easily in soils and ground waters (Cutright and Lee, 1994). PAH's are associated to carcinogenic diseases in aquatic animals by chronic exposure (Heitkamp and Cerniglia, 1988) and increases the toxicity level in sediments (Heitkamp *et al*., 1988). The PAH's include a large number of organic compounds produced by incomplete combustion of fossil fuels. Their persistence in the environment is given by the presence of three to six-ring PAH's which have been reported to have the capability of altering gene regulation (Cerniglia, 1992).

There is a growing interest in biotechnological developments aimed at the detoxification of solid wastes, and soils contaminated with PAH's; compounds wiht low water solubility and therefore are not inmediately accesible for the microorganism (Lisowska *et al*., 1994). There have been several successful investigations on indigenous microorganisms with degrading capability in sediments contaminated with pure, low molecular weight PAH's. These PAH´s are transformed into other aromatic substrates and the result suggested that biodegradation plays an important role in the degradation of mixtures in the environment (Grifoll *et al*., 1995 and Ramírez *et al*., 1996). Research on the biodegradation of PAH's higher molecular weight and their mixtures are limited by the lack of microbial isolates or consortia which can degrade these compounds (Govindaswami *et al*., 1995).

Long exposure to naturally ocurring sediments serve as repositories for PAH´s in aquatic ecosystems, where they may persist, undergo resuspension, or be degraded by complex natural communities of bacteria and fungi to envolved enzymes that degrade them (Crawford *et al*., 1989 and Heitkamp *et al*., 1988). In biological processes, it is important to demostrate that enhanced metabolic activity- the proposed treatment mechanism is actuallly responsible for the contaminant reduction. (La Belle and Hadley, 1994). Several bacteria, including members of *Pseudomonas, Arthorbacter, Mycobacterium,* and *Rhodococcus* have been reported as PAH's degraders. (Aamand *et al*., 1995).

Gorden *et al*. (1993) report that aerobic bacteria can be isolated from exposed areas. Their ability is determined through sequential and repeated inoculation in enriched salty media and by exposing the microorganisms to different concentrations of the toxic agent over a variable period i.e from few weeks up to months. In this manner, the utilization of aerobic heterotrofic bacteria is ensured the detoxification of the main polycyclic aromatic compounds reported by the EPA. This research describes and analyzes the methodologies for the isolation of bacteria having a capacity to degrade toxic organic compounds.

EXPERIMENTAL METHODOLOGY

Sediment samples.

The soil samples, required for the isolation of the existing microorganisms, were collected at eight different sites of an area contaminated with wastes from creosote production and industrial coating enamels among others.

Chemicals.

Naphthalene, acenaphthene, acenaphthilene and fluoranthene were purchased from Aldrich chemicals. Fluorene, pyrene and acetone and hexane solvents were purchased from Merck. Phenanthrene from Sigma Chemical Co. Pro Analysi.

Culture media and supplementary compounds.

R2A, nutrient, cetrimid and Noble Agar were purchased from Merck.

Oil extract.

First, the sediment samples were dried out. Then hydrocarbon extraction was carried out through the Soxhlet method according to EPA 418 standard, using Merck's tetrachloride carbon as a solvent.

Chromatographic analysis of the sediment.

Quantification of the PAH's present in the contaminated sediment was conducted in a Gas Chromatograph coupled with Mass Spectrometry (GC/MS). The oil extract obtained from the sediment was diluted in bichlorinemethane (CH_2Cl_2) and into a Hewlett Packard 5890A Series II Gas Chromatograph coupled to a HP 5972 selective detector.

Furnace temperature ranged from 373 K to 523 K. The temperatures of the injector and of the transfer line were kept at 523 K and 562 K respectively. The

analysis was conducted in the selective ion monitoring mode (SIM).

Development of the methodology for microbiological evaluation.

Two systems or isolation procedures, Fast Route and Slow Route, were developed in the methodology for microbial analysis in contaminated soils.

The fast route procedure was used to isolate microorganisms with capability to use light PAH's containing two and three rings. Samples of each spot from the contaminated area were initiallly pre-enriched in a Basal Salt Medium (SBM) (Cerniglia, 1992), containing low levels of yeast extract (250 mgm^{-3}) and (250 mgm^{-3}) bacto peptone (Difco) incubated at 303 K during a week. Serial dilutions of microcosm sediments were spread with sterile glass rods onto the surfaces of petri disches of R2A agar, Cetrimid agar and Mac.Conkey agar incubated at 303 K for 24 hours, and in SBM agar for three weeks.

The slow route procedure was applied to isolate microorganisms with capability to biodegrade PAH's containing more than three rings, using selective pressure. The pressure was exerted in a chemostat having a central reactor continuously fed with a SBM (5 drops/min) for 90 days. The assembly was made with 40 g of homogenized exposed soil taken from the different sampling sites; 400 cm^3 of SBM were added and the mixture was left in incubation at 305 K for 24 hours at 120 rpm. After this period, the assembly was allowed to settle and the supernatant was added to other 40 g of the same sample with subsequent SBM feeding (5 drops/min) and daily addition of 0,5 cm^3 of oil extract for the first four weeks. The volume of oil was increased 0,1 cm^3 every week after the fifth week with the SBM dose being decreased a weekly addition rate of 1 cm^3 was reached. This rate was maintained until the twelfth week.

The control was simultaneously conducted with clean soil from the same area with SBM. The reactor was monitored weekly, taking 1 cm^3 of tested sample and preparing a serial dilutions in R2A agar, Cetrimid agar and SBM agar + 2% pyrene incubating at 305 K for five weeks.

Microbial evaluation in the utilization of individual PAH's.

The ability of the microorganisms, isolated through the fast route, to grow and mineralize the low molecular weight PAH's was evaluated by Kirohara's *et al*., (1982) modified method. The evaluation was carried out in 2% noble agar in SBM by inoculating the 10^{-6} dilution on the plate from a preinoculum in SBM broth with 250 mgm^{-3} yeast extract at a 1,2 optic density.

As soon as the dilution was spread onto the agar surface, it was covered with a 1% (wt/vol) solution of a PAH's dissolved in acetone:hexane (1:1 vol/vol). Naphthalene, acenaphthene, fluorine, phenanthrene and fluoranthene, were evaluated for this experiment. Initially, the plates were left uncovered during one minute, in a laminar flow cabinete, for solvent evaporation.Then, the plates were placed inside plastic bags, for a better moisture conservation. Finally, incubation at 303 K for three weeks.

The selection of the colonies that had remained constant during the first eigth weeks and that showed morphologies different from those of colonies developed in the control chemostat samples, was accomplished during the slow route procedure. Once the strains were selected, they were cultured in SBM broth with 250 mgm^{-3} of yeast extract and peptone at 303 K for 18 hours.

Then, the strains were individually tested on their ability to grow on PAH's. Once an optic density of 1,2 was reached, serial dilutions were tried by culturing in each plate with noble agar and spraying them with 6% (wt/vol) phenanthrene in one case and 2% (wt/vol) pyrene in other case.

Selection of microorganisms with capability to degrade PAH's.

The bacterial colonies with capability to degrade the PAH's were evaluated after the five week incubation period in the dark at 303 K .These colonies were characterized by having "clear"zones sorrounding them.

Identification of the microorganisms selected through the fast route.

The bacterial colonies selected on their capacity to grow and utilize low molecular weight PAH's were identified with the BIOLOG system which provides 95 carbon sources for evaluation. The results of their utilization is reported in a database of more than 1.100 species/genus with their corresponding "similarity"

coefficient (SIM). The calculated value of "similarity" in the MicroLog software is used as a calling criterion to judge the reliability and confidence of the identification (Biolog, 1993).

RESULTS AND DISCUSSION

Identification of the oily extract from contaminated sediment.

The analysis of the sediment, by the GC/MS analytic technique, allowed for the determination of the concentrations of the sixteen polycyclic aromatic hydrocarbons listed by the EPA (Table 1).

It is important to remark that the concentrations, and types of hydrocarbon, are of special interest since such residue will constitute the carbon and energy source for the slow route microorganism.

Selection of microorganisms for the utilization of low molecular weight PAH's.

Thirty two isolations from the eight samples were obtained through the "fast route" system. Six of these strains (1, 8, 15, 22, 24 and 25) grew in most of the evaluated compounds, as shown in Table 2.

Table 1. Components present in the contaminated sediment.

COMPONENT	Concentration (mg/kg)
Naphthalene	2090,5
Acenaphthilene	297,4
Acenaphthene	1565,7
Fluorene	5248,2
Phenanthrene	8169,7
Anthracene	5458,1
Fluoranthene	1898,1
Pyrene	1399,5
Benzo(a)anthracene	577,3
Crisene	533,6
Benzo (k) fluoranthene	524,8
Benzo(a)pyrene	314,9
Indenol(1,2,3-cd)pyrene	61,2
Dibenzo(a,h)anthracene	262,4
Benzo(g,h,i)perylene	271,2

Strain 8, identified as *Pseudomonas stuterzi* with a SIM coefficient of 0,82, was the bacteria with the fastest growth. Colonies were observed after 24 hours; Colony counts (expressed by colony - forming units per cubic centimeter $cfucm^{-3}$) were higher in phenanthrene and fluoranthene. Strain 22, identified as *Pseudomonas aeruginosa* (0, 879 SIM), also showed a good adaptation to this type of compounds with an average count of $2,43x10^7$ $cfucm^{-3}$ (Table 3) which was higher in the last two compounds, (phenanthrene and fluoranthene).

Strains 15, 24 and 25 identified as *Pseudomonas resinovarans* (0,549 SIM) and *Pseudomonas nitroreducens* (0,619 SIM) respectively, showed a growth in the first four compounds. There was a growth decrease in the last three of the evaluated compounds and a negative one in some of the cases. Although strain 1, identified as *Pseudomonas aeruginosa* (0,995 SIM), grew only in the first four compounds, its growth was very fast, $1,3x10^7$ $cfucm^{-3}$ average counts after 48 hours.

Selection of microorganisms with capability to degrade PAH's of 3 and 4 rings.

During the selective pressure chemostat assembly experiments, through the slow route procedure, the microbial counts, indicated a decrease in growth as the oil concentration and exposure times increased, in R2A and Cetrimid agars, with respect to the control (Table 4).

Three types of colonies were observed in R2A agar and only one in Cetrimid agar; these colonies remained constant through the monitoring period . Since the strain present in agar Cetrimid corresponds to one of the strains isolated in the R2A agar, they were named A, B and C respectively. Microbial diversity was big in the control with the presence of more than six types of colonies. The growth of these three colonies (A,B,C) was evaluated in SBM, with 2% pyrene and in SBM with 6% phenanthrene. Bacteria A and C showed a positive response, with bacteria A having a larger $cfucm^{-3}$ count (as shown in Table 5). Although strain B did not grow on these compounds, it is important to conduct grow experiments with these bacteria on compounds having fewer rings.

Tabla 2. Selection of microorganisms capable of growing on low molecular weight PAH´s at 1% (wt/vol) through the fast route procedure.

STRAIN	NAPHTH	ACENAPH.	ACENPHTHI	FLUOR	ANTHRA	PHENANTHRE	FLUORANTH
1	+	+	+	-	-	-	-
2	+	+/-	-	-	-	-	-
3	+	-	-	-	-	-	-
4	-	-	-	-	-	-	
5	+	+/-	-	-	-	-	-
6	-	-	-	-	-	-	-
7	+	-	-	-	-	-	-
8	+	+	+	+	+	+	+
9	-	-	-	-	-	-	-
10	-	-	-	-	-	-	-
11	-	-	-	-	-	-	-
12	-	-	-	-	-	-	-
13	-	-	-	-	-	-	-
14	-	-	-	-	-	-	-
15	+	+	+	+	-	+/-	+/-
16	+	-	-	-	-	-	-
19	-	-	-	-	-	-	-
20	+	-	-	-	-	-	-
21	-	-	-	-	-	-	-
22	+	+	+	+	+	+	+
23	-	-	-	-	-	-	-
24	+	+	+	+	+	+/-	+/-
25	+	+	+	+	+	-	-
27	+	-	-	-	-	-	-
28	-	-	-	-	-	-	-
29	+	-	-	-	-	-	-
30	+	+	-	-	-	-	-
31	+	-	-	-	-	-	-
32	-	-	-	-	-	-	-

The recovery of heterotrophic bacteria with capability to grow in polycyclic aromatic compounds is very low. As it can be observed, the recovery through the fast route reports six microorganisms with a capability to grow in low molecular weight PAH's while only two that grow on high molecular weight PAH's.

The fast route methodology, for the recovery of microorganisms from soils exposed to PAH's, proved to be good tool for bacteria selection. It allowed the discovery of microorganisms capable of growing mostly on naphthalene, acenaphthene, ace-naphthilene and fluorene. Two of the microorganisms also showed

Table 3. Colony counts ($cfucm^{-3}$) for strains selected through the fast route

STRAIN	Naphtha	Acenaph	Acenaphthi.	Fluorene	Anthra	Phenant	Fluoranthe
1	$1,8x10^7$	$8 x10^6$	$1,3 x10^7$	Neg	Neg	Neg	Neg
8	$1,2 x10^7$	$1,9 x10^7$	$1,7 x10^7$	$2,2 x10^8$	$1,1 x10^7$	$2,8 x10^8$	$2,1 x10^8$
15	$5,2 x10^6$	$2,2 x10^7$	$1,8 x10^7$	$1,9 x10^7$	Neg	$2 x10^6$	$3 x10^6$
22	$1,6 x10^7$	$1,8 x10^7$	$1,2 x10^7$	$1,4 x10^7$	$1,0 x10^7$	$5,2 x10^7$	$4,8 x10^7$
24	$4 x10^6$	$1,0 x10^7$	$1,1 x10^7$	$9 x10^6$	$2 x10^6$	$2 x10^6$	Neg
25	$5 x10^6$	$1,2 x10^7$	$1,0 x10^7$	$1,4 x10^7$	$2 x10^6$	Neg	Neg

Table 4. Microbiological count of the slow route chemostat

WEEK	Chemostat PAH's		Chemostat Control	
	R2A ($cfucm^{-3}$)	CET ($cfucm^{-3}$)	R2A ($cfucm^{-3}$)	CET ($cfucm^{-3}$)
1	$1,3 \times 10^9$	8×10^8	$3,0 \times 10^9$	$1,7 \times 10^9$
2	$1,2 \times 10^8$	$1,5 \times 10^8$	$5,3 \times 10^8$	$3,5 \times 10^7$
3	$9,2 \times 10^7$	$4,5 \times 10^7$	$2,0 \times 10^8$	$6,7 \times 10^6$
4	$9,3 \times 10^7$	$3,3 \times 10^7$	$1,1 \times 10^8$	$1,5 \times 10^7$
5	$4,5 \times 10^7$	$1,3 \times 10^6$	$1,5 \times 10^8$	$2,2 \times 10^7$
6	$8,2 \times 10^6$	$1,8 \times 10^6$	$1,6 \times 10^8$	$2,8 \times 10^7$
7	$6,0 \times 10^6$	2×10^4	$2,0 \times 10^8$	$3,1 \times 10^7$
8	$5,7 \times 10^5$	2×10^4	$2,2 \times 10^8$	$2,3 \times 10^7$
9	$2,4 \times 10^5$	2×10^4	$2,6 \times 10^8$	$2,2 \times 10^7$

R2A: R2A Agar; **CET**: Cetrimid Agar

Table 5. Evaluation of slow route microorganis with capability to grow on mediums containing 6% phenanthrene and 2% pyrene.

COLONY	Phenanthrene 6%	Pyrene 2%
A	++	++
B	-	-
C	+	+

(++) Plenty colonies; (+) Moderate colonies; (-) No grow

a high adaptation ability to more complex substrates, such as phenanthrene and fluoranthene (Figure 1).

The slow route procedure, through the chemostat assembly with selective pressure, proved to be an excellent mechanism for the isolation of microorganisms considering the notorious decrease of native flora in the PAH's exposed sediment in relation to that of the control, as can be observed in Figure 2. This method allowed for the evaluation and selection of the optimum existing microorganisms. Two of them showed a high adaptation capacity verified by the counts (Figure 3). In this case, although strain B did not grow in the two selected parameters, it is likely to be capable of growing on other carbon sources present in the residue and can not be discarded.

The isolated strains selected by the slow and fast route, will be analized philogenetically in order to determine their capability to biodegradate using some reference strains with known molecular markers.

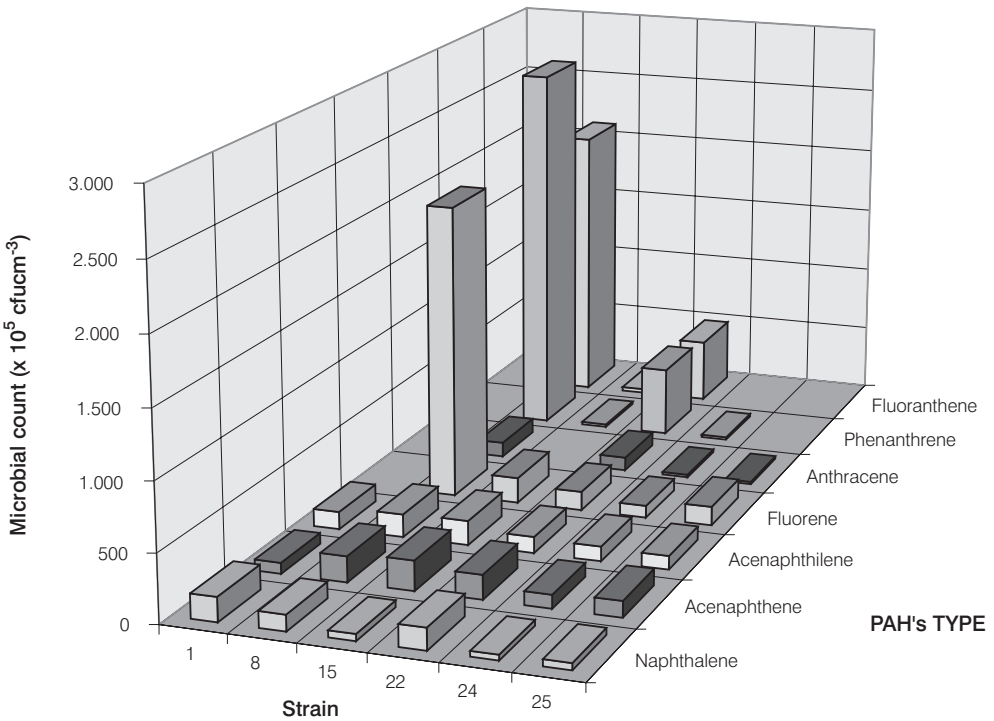

Figure 1. Evaluation of the microorganisms selected through the fast route with different low molecular weight PAH"s.

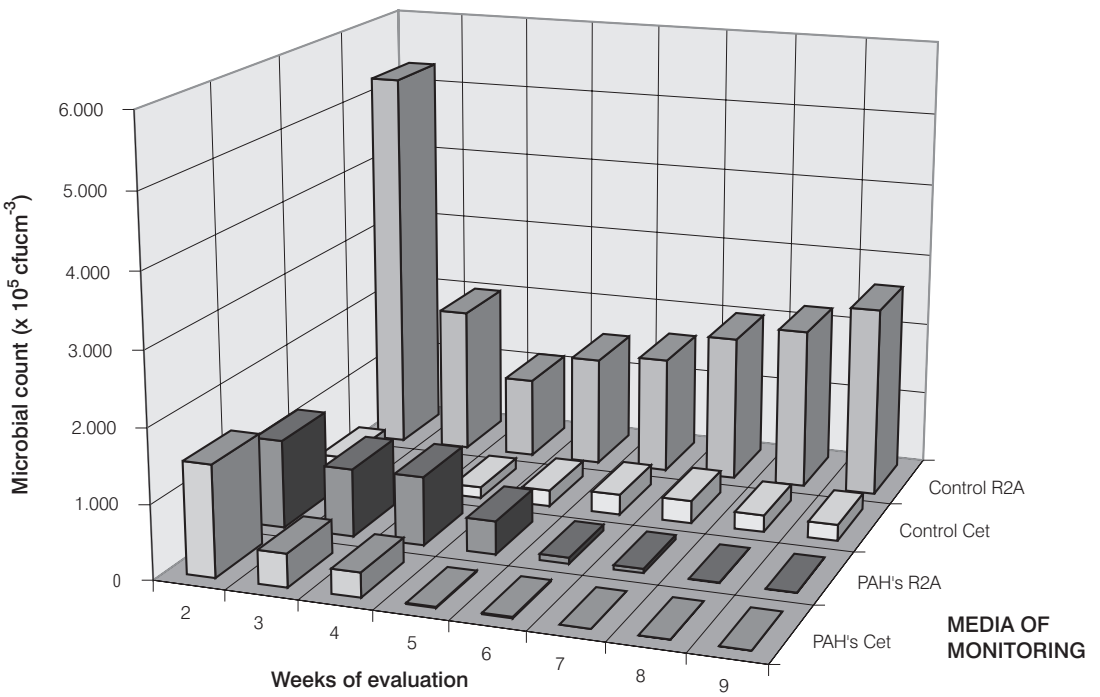

Figure 2. Microbiological monitoring of the slow route chemostat during a nine week period

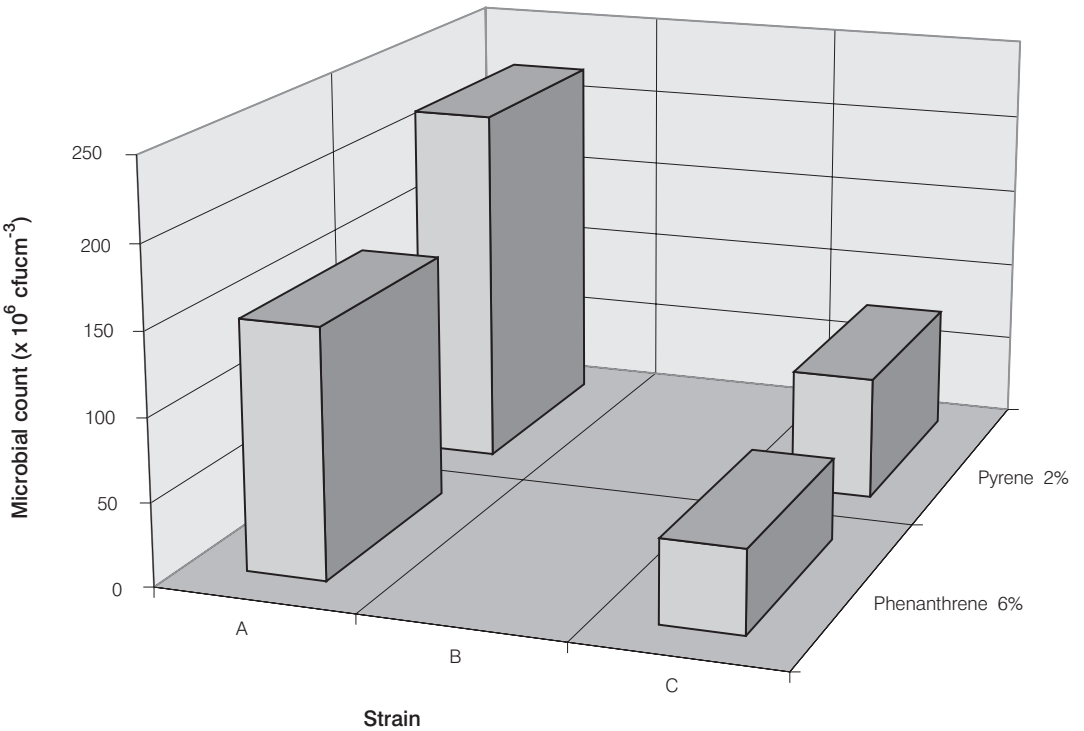

Figure 3. Evaluation of the slow route microorganisms with a capability to grow on high molecular weight PAH's

CONCLUSIONS

- The fast route method for the isolation of microorganisms allowed for the identification of microbial strains such as *Pseudomonas stuterzi, Pseudomonas aeruginosa* and *Pseudomonas resinovorans* with the ability to grow in low molecular weight PAH´s.
- The slow route method "Chemostat", showed a high selectivity for the isolation of strains for the degradation of three - and four - ringed PAH's.
- In the slow route method, the use of microorganisms constantly fed with a pressure agent is an adequate methodology for the selection and establishment of promissory microbial flora for biotreatments.
- The tests carried out in chemiostat with the presence of specific substrates demonstrate the importance of obtaining microbial consortion specialized in PAH's consumption.

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