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Bioreactor treatment of aromatic hydrocarbons by indigenous micoflora and *Gliocladium viride*

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The PAH remediation depend on the bioavailability, aqueous solubility and high soil–water ratios that promote their accumulation in terrestrial and aquatic environments. Pollutant biodetoxification can be enhanced by bioaugmentation of degraders microorganism that produced biosurfactants. Therefore, the aims of this study were to assess the degradation potential of fluoranthene, pyrene and chrysene by filamentous fungi and to compare the detoxification ability of the indigenous fungi with isolated strains. A bioslurry reactors was implemented to evaluate the wild micoflora, *Gliocladium viride* and the combination of both ability to transformed hydrocarbons. A conspicuous transformation was obtained for fluorantene by the three communities, pyrene was degraded with a minor rate and chrysene was the most recalcitrant substrate. Unless fluorene, *G. viride* showed less uptake capacity for pyrene and chrysene; this two aromatics were significantly transformed by the wild fungi and the mixed culture. The PAHs concentrations by the three assays decreased 30 up to 70 % of the original levels. The PAH transformation by wild microflora and *G. viride* was significant increased in the bioreactor, due to the higher ratio water-solid the agitation kept the heterogeneous solids and PAHs in suspension and increased the aeration rates.

Keywords: biodegradation - chrysene - fluoranthene - *Gliocladium viride* - pyrene - soil slurry reactor.

Abbreviations: higher-molecular-mass PAH (hmw-PAH) - fluoranthene (FLU) - pyrene (PYR) - chrysene (CHRY) -

Introduction

Higher-molecular-mass PAH (hmw-PAH) with four and more annealed rings are heterogeneous organic contaminants emitted during combustion of organic materials, natural fires, oil spills and diverse anthropogenic sources (Sing, 2006; Singh, 2012). PAHs have been thoroughly studied due to their toxicity and environmental persistence; the studies had been often limited to 16 PAHs, designated as priority pollutants by the United States Environmental Protection Agency (USEPA, 2009, 2011).

The hmw-PAH remediation depended on their low bioavailability, low aqueous solubility and high water-soil ratios, that promote their accumulation in the solid phases of terrestrial and aquatic environments (Couling, 2010; Das, 2012). Moreover, pollutant biodetoxification is usually limited by the existence of wild tolerant microorganisms; they produced extracellular or surface-active compounds which enhanced the emulsification of water insoluble compounds. These biosurfactants incremented the po llutant availability increasing by this way the bioremediation (Pattanathu *et al.,* 2008; Nikifiriva *et al.,* 2009)

Most of the studies evaluated soils detoxification by bacteria (MacLeod and Daugulis, 2005; Obayori et al., 2009) or ligninolytic fungi (Pointing, 2001; Novotny, 2004), althought imperfect filamentous fungi had been found as dominant species in polluted habitats (Romero *et al.,* 2005;2011). Therefore, the aims of this study were to assesse the degradation potential of fluoranthene (FLU), pyrene (PYR) and chrysene (CHRY) by filamentous fungi, to compare the detoxification ability of the indigenous fungi and isolated strains, and to evaluate the detoxification potential in a slurry reactor with polluted sediments.

Materials and methods

Fungi strain and inoculum. PAH degrading fungi were isolated from polluted sediments from an

industrial area, at La Plata, Argentina; soil samples were plated on a mineral medium with 300 mg/l n-hexadecane and 30 mg /l FLU as carbon (Romero *et al.,* 2010), pH 6 and 75 ml/l antibiotic solution (5.0 g streptomycin, 2.5 g chloramphenicol, 1.0 l distilled water) to avoid bacterial growth. The hydrocarbon-degrading fungi were selected on the basis of their prevailing growth on subsequent plating, and the dominant fungus was selected for further assays. Fungi were incubated at 28ºC, for 20 days, in darkness, and 5 plugs of active mycelium were used as inoculum in the biodegradation assays. The isolates were identified by colony, cells, conidia and ascospores morphologies in different culture media by scanning electron microscopy, and by Fourier transform infrared spectroscopy (Santos *et al.,* 2010).

Soil slurry assays. The sediments used in the bioslurry reactors were collected from heavily polluted streams near the industrial area. The soil texture were sand, clay and lime (69.0:18.5:12.5 %) with pH 6.8, composition: C-organic 7.80 %, N-organic 0.38 %, P-organic 0.09 % and 97.5 mg PAH kg soil^{-1}. The P_2O_5, K_2O content (calcium lactate extract), Fe and Mn contents were 20, 430, 580 and 40 ug.g soil^{-1}, respectively. Soil characteristics were determined according to the standard methods (APHA, 2009).

To evaluate the wild micoflora, fungus degradation activities and the combination of both, different treatments were assessed: (1) unsterile soil to evaluate the detoxification ability of the indigenous microflora present in the soil (IM), (2) unsterile soil inoculated with the selected fungal strain to assess the mixed transformation (MT), (3) sterile soil inoculated with the selected fungi (Gv) and (4) sterile soil without inoculation as an abiotic control (C), all the assays were done in triplicate

Soil was autoclaved (121ºC, 45 min) and amended with 500 mg/l n-hexadecane as cosubstrate and 70 mg/l of FLU, PYR and CHRY in dimelthylformamide as substrate; with a final individual-PAH level of 30 mg/kg soil. Then, it was homogenized by shaking and kept under a fume hood (24h) to evaporate the solvent; soil homogeneity was evaluated by PAH-levels measurements, by triplicate, obtaining concentrations of 30.97 ± 1.06, 29.97 ± 1.17 and 30.25 ± 0.98 for FLU, PYR and CHRY, respectively. A 5 l stirred reactor with a single turbine, pH and temperature sensors was filled with 3.5 l of the treated soil, at pH 6, 28 ºC and 150 rpm, air was blown into the reactor at 1 vvm, during 75 days.

Residual PAH-levels were periodically studied, obtaining average extraction efficiencies of 93.43 ± 4.92 %, 97.14 ± 4.47 % and 88.93 ± 3.55 % for FLU, PYR and CHRY, respectively. Efficiencies were determined by adding 2 g soil with 100 ml of a stock solution obtaining levels of 50 mg PAH/kg soil. Subsamples were extracted and put into vials for high performance liquid chromatography (HPLC) and PAH analysis (Romero *et al.,* 2001; 2002). Calibration curves were obtained by HPLC determinations of 1, 2, 4, 8 and 10 mg/l of each PAHs, curves were obtained by this standards and typical regression coefficients, ranging between 0.998-0.995.

To monitor the predominant fungi that grew in the bioreactor, 100 ml subsamples were analyzed each 5 days; they were culture in the same PAH-medium and culture conditions and compared with control plates.

Statistics: All the experiments and cultures were done by triplicate, three replicates were used for each PAH assays. The results are expressed as the arithmetic mean ± standard error of the mean; the Student´s two-tailed t-test was used to evaluate the differences between control (0 PAH) and experimental means, with $P < 0.5$ being considered significant.

Results and discussion

Gliocladium viride was selected for its hmw-PAH degradation potential, its prevailing growth on subsequent plating and due to the recently discover that a *Gliocladium* spp. isolated from the Patagonian Rain Forest, Argentina, had potential to produce bioalcohol and biodiesel (Strobel *et al.,* 2008; Strobel, 2011). *G.viride* showed a medium growth rate, *n*-hexadecane depleted after 6 days with a lag period of 2 days, and not modified parameters as pH, DO and redox potential, that were observed during this period.

To compare the degradation ability of the cultures, the percentage of each aromatic hydrocarbon respect to the initial PAH amount were calculated for each assays. While *G. viride* degraded the 6 %, 31 % and 63 % of PYR, CHRY and FLU, respectively; the indigenous microflora showed higher potential uptake for PYR (20 %), 30 % for CHRY and 50 % for FLU. The mixed cultures, that is *G. viride* plus wild fungi, transformed 21 % of CHRY, 31 % of PYR and 48 % of FLU. Therefore, the indigenous microflora showed higher degradation ability for PYR and the mixed culture for CHRY; being a remarkably results because both hydrocarbon were much more recalcitrant than FLU.

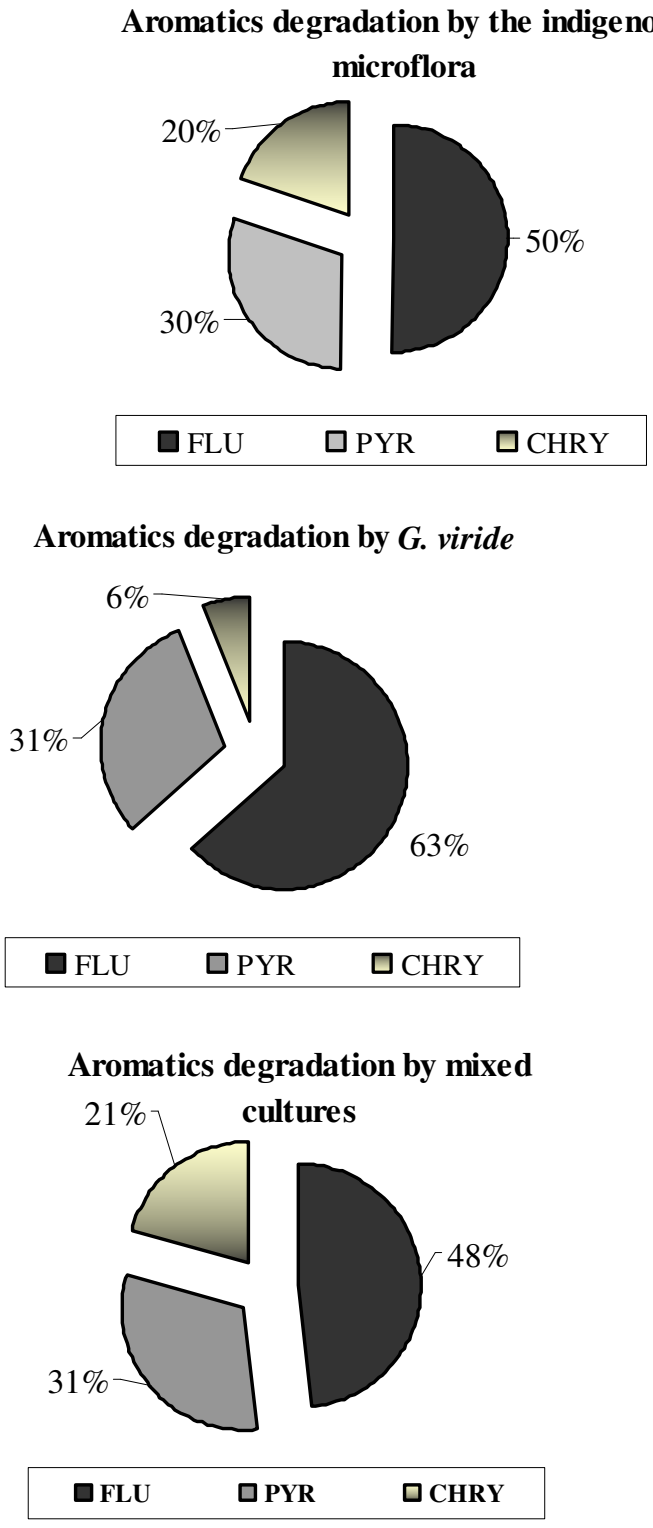

Figure 1: FLU, PYR and CHRY degradation (%) by the indigenous microflora, *G. viride* and the mixed cultures composed by indigenous microflora plus *G. viride* at the end of the experiment.

A conspicuous transformation was obtained for FLU by the three communities, showing the indigenous micoflora, *G. viride* and the mixed culture similar detoxification potential.

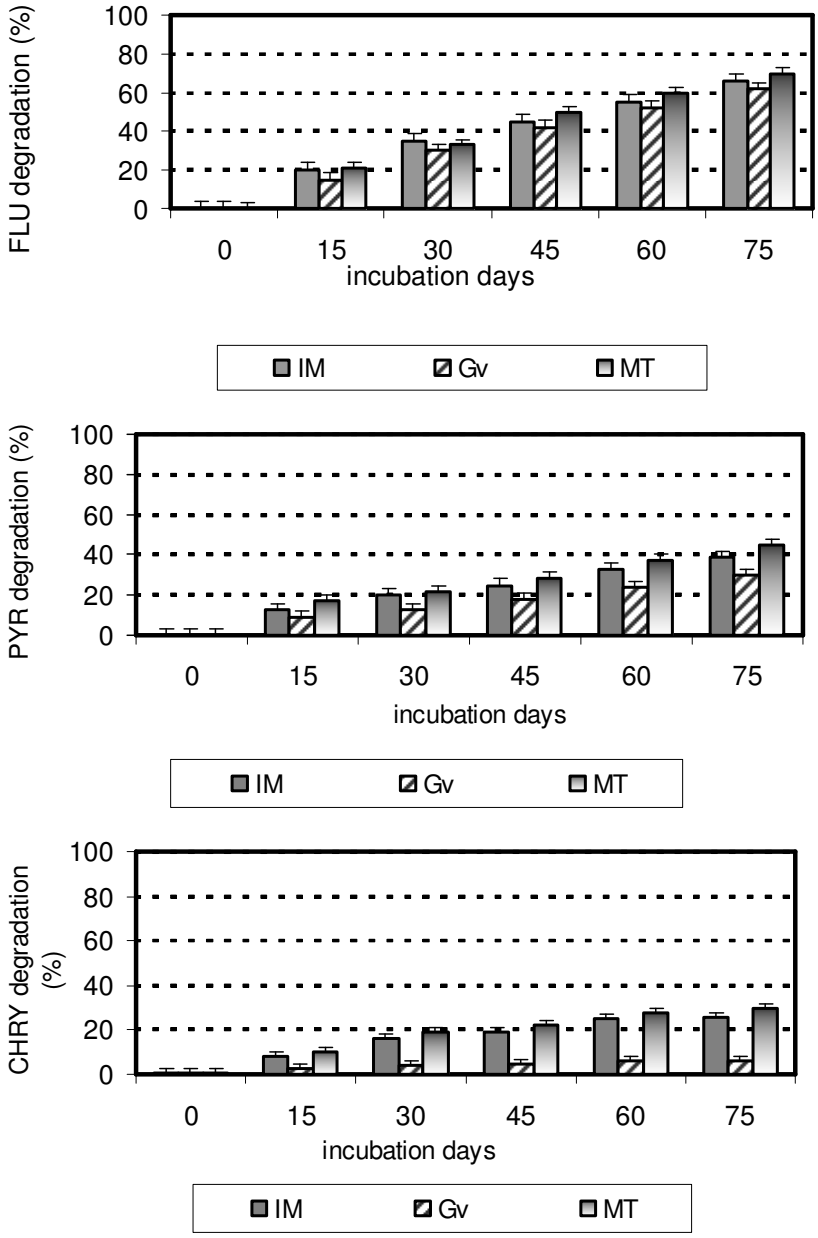

Figure 2: FLU, PYR and CHRY degradation (%) by the indigenous micoflora*, G. viride* and mixed culture.

PYR was degraded with a minor rate and CHRY was the most recalcitrant substrate. Unless FLU, *G. viride* showed less uptake capacity for PYR and CHRY.PYR and CHRY were significantly transformed by the wild fungi and the mixed culture. The PAHs reduction percentage was calculated from the initial levels of each pollutant at day 0 (Fig. 2).

G. viride was tolerant to the shear stress, aeration requirements and competition with indigenous microbes with conspicuous growth in unsterile sampled soils. Taking into account the filamentous morphology in an aerobic environment, the bioreactor allowed better mass transfer by improving oxygen and nutrient supply due to the stirring system, viscosity, as well as tolerable shear; also, it maintained the slurry homogeneity and prevented the solid separation. The significant biodegradation obtained in the bioreactor by the three biological communities was due to the air sparging through the bottom, the mixing degree provided by the impellers that favoured the PAH-dissolution from soil matrix into the aqueous phase.

PAH-degradation in the mixed culture, endogenous micoflora as well as the *G. viride* assays showed that the PAHs concentrations decreased 30 up to 70 % of the original levels, depending on the PAH. Aromatic uptake by the three cultures showed

significant degradation rate of the PAHs during all the incubation time.

The assayed hydrocarbons, FLU, PYR and CHRY, differred in their physico-chemical properties, like aqueous solubility, vapour pressure and molecular weight, these features determined the environmental behaviour and they PAH-low aqueous solubility caused the low degradation rates. These limitations could be improve in bioreactors cultures. Filamentous fungi had been assayed as PAHs degrader in Erlenmeyer-flasks, however, the application in slurry system for hmw-PAH transformation had only been studied in white-rot fungi (Baborova *et al*., 2006).

PAHs transformation by white-rot fungi had previously been demonstrated by many researchers (Zheng and Obbard, 2002; Valentín *et al*., 2006); similar PYR uptake had been shown by *Pleurotus ostreatus* and *Trametes versicolor* (Novotny *et al*., 2004). Also the ligninolytic fungus *Phanerochaete chrysosporium* transformed PAHs with significant coefficients up to 40 % for fluoranthene, pyrene and chrysene (Zheng and Obbard, 2001); *Bjerkandera* spp. was able to transformed dibenzothiophene, FLU, PYR and CHRY in soil slurry reartor (Valentin *et al.,* 2007).

Among filamentous fungi, *Aspergillus* spp (Da Silva et al., 2003)*., Penicillium* spp. (Leitão, 2009), *Cladosporium* spp., *Paecilomyces spp.* and *Talaromyces* spp. (Romero *et al.,* 2005; Romero *et al.,* 2010) showed significant transformation potential to breackdown aromatic hydrocarbons.

Other researchers had studied the PAH degradation in cocultures of indigenous soil bacteria and white-rot fungi (Canet *et al.,* 2001; Andersson *et al*., 2003) and the combinations of ligninolytic fungi with native microflora (Cerniglia and Sutherland, 2001, Giubile *et al.,* 2009). The mentionated researches presented data of the micoremediation impact on the wild microbiota but not in bioreactors. In this study we evaluate the improvement on the PAH transformation by wild microflora in the present of filamentous fungi in bioreactors

Bioreactors had been implemented usually with mixed microbial populations and ligninolytic fungi (Khiyami *et al*., 2006; Valentin *et al.,* 2007; Haritash *et al.,* 2009) this studies confirmed that the active mixing and aeration in the slurry reactors increasing the performance. Therefore, our results contribute and enlarged the PAHs treatments confirming that soil filamentous fungi enhanced the PAH transformation in soil reactors.

In conclusion, the PAH transformation by wild microflora and *G. viride* was significant increased in soil bioreactor, due to the higher ratio water-solid, the agitation kept the heterogeneous solids and PAHs in suspension and increased the aeration rates. Moreover, PAHs transformation is limited by the dissolution into the aqueous phase and the contact of the pollutants with the microorganisms, both aspects were improved in the bioreactor.

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