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Full Length Research Paper

Effects of the sorption/desorption process on the fluoranthene degradation by wild strains of *Hansenula angusta* and *Rhodotorula minuta*

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The fluoranthene degradation was predicted by the sorption/desorption process as its fungal transformation was in relationship with the bioavailabit. Toxicants availability is significant to assess as their bioremediation and persistence in the contaminated environment depended on the physical, chemical and textures of the polluted sediments that fixed the organic xenobiotics. In most of natural and man-made habitats, the aromatic hydrocarbons had been found sorbed to soil particles that inmobilized the compounds and diminished the microbial attack. Therefore, wild yeasts from hydrocarbon polluted areas were isolated, and their potential as fluoranthene degraders were evaluated in different texture soils and organic matter contents. *Hansenula an*gusta and *Rhodotorula minuta* were isolated from industrial effluents and used in desorption experiments; the obtained Flu uptake parameters explained the efficiency of both yeasts to biotransform Flu sorbed to soil particles. *H. angusta* and *R. minuta* degraded Flu by bioemulsifiers production; evenmore, they were highly efficient to uptake fluoranthene in the biphasic cultures and were dominant in the sampled polluted sediments. The potential application of biosurfactants produce by indigenous yeasts in PAHs recovery from the polluted environments was demonstrated by the percentage of fluoranthene removal and by the stability of the surface tension.

Keywords: Bioavailability, fluoranthene, *Hansenula angusta, Rhodotorula minuta* - desorption process, biosurfactants.

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) accumulate in nature because release rates from industrial effluents exceed the rates of dissipation, microbial and chemical degradation (Jacques et al., 2008; Coccia et al., 2009).

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Abbreviations

PAHs, polycyclic aromatic hydrocarbons; Flu, fluoranthene; OC, organic carbon; CEC, cation exchange capacity; MM, mineral medium; ST, surface tension.

Low biodegradation had been attributed to diverse factors, such as surface and subsurface soil/effluents properties, chemical toxicity, high concentrations of the pollutants, limited bioavailability of the toxicant to the degrading species (O'Donnel et al., 2007; Wang et al., 2008), and deleterious conditions of the areas for microbial survival or proliferation (Yong and Mulligan, 2006; Nasr et al., 2009).

Reliance on indigenous strains may be inappropriate; and an alternative approach would be inoculation of the polluted soils with filamentous fungi and yeasts possessing the appropriate metabolic, physiological and kinetic potential in conjunction with manipulation of the soil parameters, to enhance the survival, activity and proliferation of the degrading species (Tang et al., 1998;

Fen et al., 2000). Once more, the isolation, culture and potential activity determination of wild fungi able to degrade xenobiotics become conspicuos (Al-Tahlan et al., 2000). The advantages of fungi as degrading species releace on the production of large biomass, special metabolic activities, exoenzymes production, wide adaptation to diverse environmental factors, resistance and longevity (Noordman et al., 1998; Romero, et al., 2010). However, successful bioremediation was still dependent on achieving an acceptable rate and extent of the degradation, and they depended on the kinetic parameters of the inoculant strains in conjunction with the bioavailabilty of the toxicants, being this factor in relation with the physical state of the pollutant (Romero et al., 2002; Mulligan, 2005).

Microbial naturally produced biosurfactants or surface active compounds have similar properties but are less toxic, biodegradable, and can be produced in-situ at the contaminated site (Mulligan, 2009). For these reasons, biosurfactants have gained increased attention; moreover, they could be produced from cheap materials and were effective under extreme conditions (Nitschke and Costa 2007; Sànchez et al. 2009). Many micro organisms, including bacteria, yeasts and filamentous fungi, could produce extracellular or membrane-associated surface-active compounds which were used to assist the enhancement of emulsification and dispersion of waterin-soluble toxicants. The bioemulsifiers could reduce surface tension at the air-water interface and they could assemble into a wide variety of morphologically different structures.

Otherwise, the interactions between microorganisms, contaminants and biosurfactants had been interpreted from a functional perspective, considering that the main natural role attributed to biosurfactants is their involvement in hydrocarbon uptake (Paria, 2008; Perfumo et al., 2010).

Soil-sorbed PAHs had been considered unavailable for biotransformation without prior desorption (Park et al., 2001; Park et al., 2003), therefore fungi must be able to uptake the sorbed molecules by direct uptake (Calvillo and Alexander, 1996; Tang et al., 1998), or to facilitate the desorption in some manner. While different bacterial species had been extensively reported as producing effective biosurfactants to remove PAHs (Rodrigues et al., 2006; Obayori et al., 2009; Thavasi et al., 2009; Nasr et al., 2009), fungi had not been studied so far. By other hand, yeasts and filamentous fungi had been reported to be frequent organisms in heavily polluted habitats (Romero et al., 2005; Romero et al., 2009), so, they ought to develop mechanisms to survive in an advantageous ecosistems with the presence of organic pollutants.

Few studies dealt with fungal transformation of soil-sorbed PAHs, and the diverse factors that mediated the hydrocarbon availability. Therefore, our aims were to isolate yeasts from oil polluted sediments, to quantify their potential to degrade fluoranthene (Flu) and to examine the PAHs bioavailability in relation with the partitioning between soil solution and sorbed to soil-particles.

MATERIALS AND METHODS

Sampled sites and chemical analysis. Composite samples of the surface sediments were taken from two different natural areas, Regatas Station, Zanjon Station and two artificial channels that received industrial effluents, Channel Este, Channel Oeste and drain to Rio de La Plata, La Plata, Argentina. The total organic carbon concentrations of the sediment were determined by CHN analyzer (Perkin-Elmer, Norwalk, CT.) and the Macro-Kjeldahl method was employed to measure the amount of total organic nitrogen. The total PAHs concentrations were analized by a FTIR-Perkin-Elmer, by triplicate; the ultrasonic extraction was realized with Cl4C. A cell with BrK window, 0.35 mm thick, was employed for these determinations (APHA, 1992).

Different types of sorbents were used, soil type I, II and III, with diverse organic carbon (OC) contents, sand, silt and clay proportions, pH and cation exchange capacity (CEC, (Pageet et al., 1982). The sorbens were sterilized and suspended in sterile phosphate buffer (20 mM) at a ratio 1:40 to prepare the extracts; before each experiment, 0.1 g of each mix was placed on nutrient-agar plate, incubated at 30ºC for 7 days to verife sterility.

Isolation and identification of yeasts. Yeast species were isolated under selective conditions from dilution samples of the sediments, in a mineral medium supplemented with An and benzene, the first substrate was added to test the yeast tolerance to the pollutant and the second one as source of carbon and energy. The isolates were identified by colony, cell morphologies, assimilation and physiological differences, with additional tests, like D-glucuronate assimilation in liquid medium, and coenzyme Q-system determination by HPLC were also done (Kurtzman and Fell, 1998). In all the cases, the Yeast identification PC program were used to confirm the results.

Fluoranthene degradation. Yeasts were precultivated on 40 ml of liquid Sabouroud media, for 48h at 5g and 30 ºC, for 2 days, till exponential growth to be sure to accumulate enough internal-C reserves. Then, 1 ml of this culture was incubated in 500-ml shake flasks with 100 ml of a mineral medium (MM) containing, per liter, 200 mg KH_2PO_4, 800 mg K_2HPO_4, 200 mg $MgSO_4$ 7 H2O, 100 mg $CaSO_4$, 5000 mg $(NH_4)_2SO_4$, 1 mg $(NH_4)_2$ MoO_4 4 H_2O, supplemented with 2 % glucose and 1 ml vitamin solution, and pH 5.4. After incubation for 3 days at 30 ºC and 180 rpm on a rotary shaker, cells were harvested by centrifugation (5000g, 5min), washed twice with sterile MM and the pellet was resuspended in MM to an optical density of 6 (600 nm). Different aliquots of Flu

Table 1: Sampled soil features, OC, CEC, pH and particle size distributions used in the Flu-degrading assays.

	OC (%)	sand (%)	silt (%)	clay (%)	pH	CEC (cmol/kg)
type I	1.30	59.1	32.1	8.8	5.3	7.10
type II	3.28	54.6	24.0	21.4	6.8	24.40
type III	7.80	64.2	20.7	15.1	6.0	43.00

stock solution, 20, 40, 60, 80 and 100 µg, was added to 100 ml MM with the yeast suspensions, and 2 ml added to the Flu-assays.

Surface tension (ST) measurements were also evaluated as this characteristic affected uptake of soil-sorbed Flu. ST allowed us to estimate whether biosurfactants were producing during the yeast cultures, and ST were determined by a DuNoüy tensiometer. By a simple capillary assay, yeast response to Flu levels of 5 and 50 mg/l were determined (Al-Tahlan et al., 2000). Yeast cells harvested in late log phase were washed twice with 20 mM phosphate buffer, resuspended in 20 mM phosphate buffer plus 10 µM EDTA and placed in a U-shaped tube to be observed by microscopy. Uninoculated tubes were used as controls, and all the measurements were made by triplicate. The yeast cells that went into the capillaries with Flu-solution after 1 h were enumerated by plate counts.

Inoculum of yeasts without Flu in MM was used as controls, and the assays were made in triplicate. Periodically, 1 ml of each flask was sampled to estimate the Flu-levels by HPLC analysis (Hewlett-Packard, Bad Homburg, Germany), apparatus 1050 M equipped with a quaternary pump system, a diode array detector 1040 M series I, and an HP Chemstation. The separation was achieved with a LiChroCart 125-4 RP-18 end-capped (5 mm) column (Merck, Darmstadt, Germany). The initial solvent composition was 30% CH_3OH - 70% H_3PO_4 (0.1%), reaching 100% methanol after 14 min at a flow rate of 1 ml/min. The UV-visible absoption spectra of degradation products were determinated in a diode array detector (Romero et al., 2005). The chemicals, fluoranthene and solvents were purchased by Aldrich-Chemie, and were of the highest purity available.

Fluoranthene bioavailability assays. To assess the availability of soil-sorbed Flu for the degrading yeasts, the assays were performed with soil extract controls and soil slurries. Two yeasts isolates able to grow on Flu as sole C source were used in this study. Inocula were prepared by culturing yeasts in liquid Flu-MM with 150 rpm shaking, at 27 ºC, and cell growth was monitored by measuring absorbance at 600 nm with an spectrophotometer. Yeasts in lag phase were centrifugated, washed twice with sterile phosphate buffer (20 nM, pH 7), and resuspended in the buffer, to obtain a final cell density of 10^8 CFU/ml. Two mililiters of each yeast culture was used in the desorption experiments.

The desorption assays were carried out in 50 ml tubes with 28 ml sterile soil type I, II or III, plus 5 ml sterile phosphate buffer plus 2 ml yeast culture; tubes were incubated at 20 rpm for 10 days in darkness. At the 10^{th} day, each tube was centrifuged to separate soil from the supernatant; both were analized to determine Flu-final levels in sorbed and liquid phases by HPLC. Two control tubes were incubated in the same conditions, one without soil aliquots, and another with 30 ml soil suspension and then sterilized. Initial fixed Flu-concentrations were determined in the soil samples and in the control ones. Once a day, 1 ml subsamples were withdrawn from each tube to quantify Flu-levels and yeast densities.

Data analysis: The experimental data were analyzed by ANOVA, and fitted to the model by Quasi-Newton Technique (Robinson, 1985) and SAS guide (SAS, 1985), and the regression analysis (R^2) expressed the goodness of the results.

RESULTS

The yeasts *Hansenula angusta* and *Rhodothorula minuta* grew with Flu as sole C source and their uptake were significant in relation to the other species present in the Flu-agar plates. Besides, both yeasts had not been mentionated as PAHs degraders yet.

The soil features, like OC, CEC and particle size distributions were evaluated in the Flu-degrading assays (Table 1). Soil type III had more OC content and clay proportion than type I and II, therefore higher amount of organic pollutant could be sorbed to the soil particles. The Flu-sorption isotherms for the soil types showed that more Flu were found in the sorbed fraction in soil type III, than in the others (Figure 1). This fact confirmed the relationship between the OC content and soil texture with the availability of the organic compounds, and the posible bioremediation of the polluted sediments.

Similar Flu desorption amounts was obtained in the assays with the isolated yeasts, being both species effective to remove Flu from the soil particles. All the same, small quantities of residual Flu were observed at the end of the experiments in relation with the soil type. The desorption Flu of soil type I were less than the Flu released from type II and III in both yeast cultures; and this fact was due to the Flu-amount sorbed to the particles, being significantly minor in the soil texture with more sand than clay material (Figure 2).

Biphasic cultures, with two different physical states with

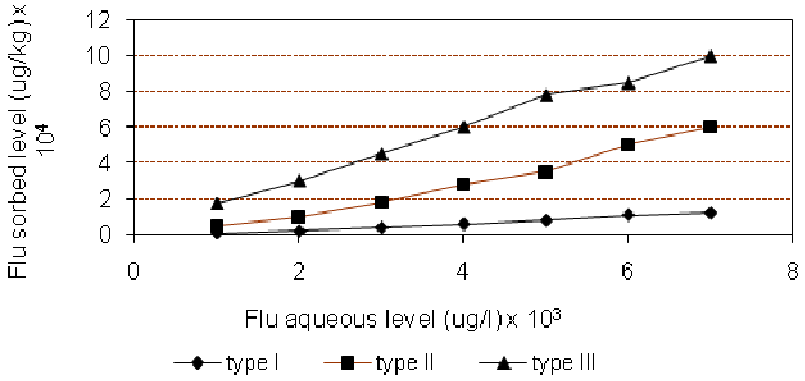

Figure 1: Flu-sorption isotherms for the different soil types

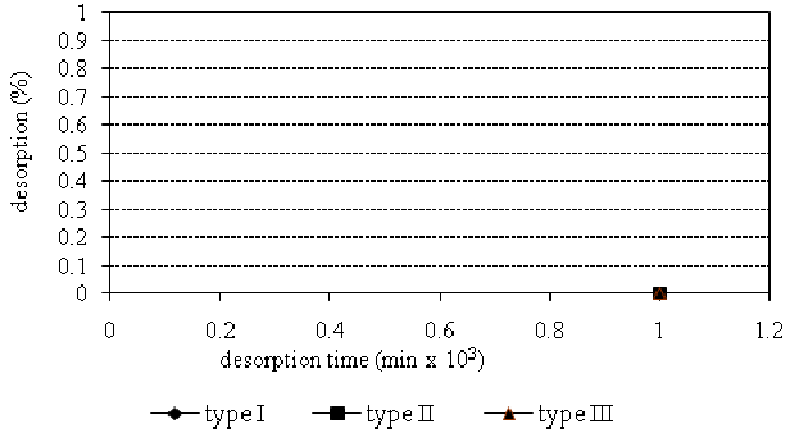

Figure 2: Flu-desorption (%) during the desorption assays with *H. angusta*

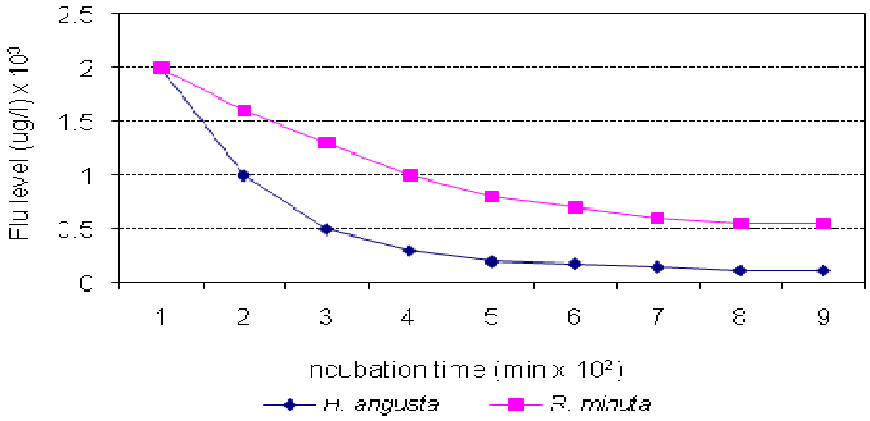

Figure 3: Flu biodegradation data by *H. angusta Y. lipolytica* and *R. minuta* in liquid phase.

Flu fixed to particles and in liquid phase were implemented, as it was much more similar to natural habitat than the monophasic ones.

The biphasic cultures were composed of equilibrium, nonequilibrium and nondesorption areas. Nondesorption sites were defined as those containing substrates that cannot be released to solution, nonequilibrium sites showed a proportional release rate in relation to concentration gradient between these sites and the liquid phase, and the equilibrium areas release the Flu to the liquid phase. The three-site desorption model fitted the experimental data, being the parameters and site fractions representative of the yeast uptakes (Figure 3).

The Km values for *H. angusta* and *R. minuta* with each

soil types fluctuated between 10.45 to 12.40 (R^2 = 0.90-0-95) and 9.59 to 11.90 (R^2 = 0.95-0.98) for each yeasts, respectively. The differences in ST between the controls and the *H. angusta* and *R. minuta* cultures indicated that biosurfactants were produced in both yeast experiments; 6.0 and 9.0 dynes/cm were the ST data in the *H. angusta* and *R. minuta* assays, respectively.

The total Flu concentration of the cultures was the sum of the equilibrium, nonequilibrium and nondesorption areas, represented by S = Seq + Sneq + Snd. Equilibrium partitioning was described by Seq = $f$eq K_F Ce^n; nondesorption responded to Snd = fnd K_F C^n, and the release from nonequilibrium sites followed the 1rst. order: d S neq / dt = α ($f$neq K_F C^n - Sneq), where K_F was the Freundlich sorption coefficient, n was the isotherm curvature constant, C was the Flu liquid-phase concentration (mg/ l), Ce was the Flu liquid-phase concentration (mg/ l) in sorption equilibrium, t desorption time (min), α 1rst. order desorption rate coefficient (min^{-1}) for nonequilibrium areas, $f$eq the equilibrium site fraction, $f$neq the nonequilibrium fraction, fnd the nondesorption fraction, and Seq, Sneq and Snd were the Flu-sorbed levels (mg/kg) in the solid equilibrium, nonequilibrium and nondesorption areas, respectively. The fnd were obtained from the sorption isotherm and represented the plateau of the desorption profile, while $f$neq, $f$eq and α were calculated by nonlinear regression analysis of the desorption experimental data.

The Flu-equilibrium fraction, $f$eq, increased in relation with the OC content, ranging from 0.30, 0.62 and 0.70; the nonequilibrium Flu-fraction, $f$neq, was similar among soil types and nondesorption sites, fnd, decreased as OC content increased, ranging from 0.45, 0.27 and 0.15. So, desorption coefficients increased as the OC increased, being these observations consistents with the interactions between soil constituents and pollutant bioavailability.

The degradation was limited to the dissolved Flu present initially, and to the desorbing Flu amount during the experiments. No direct uptake from Flu-sorbed particles was observed with *H. angusta* and *R. minuta*. The diverse degreess of availability were in relation to the association of Flu with soil matrix and OC content. The desorption rates of fluoranthene and soil constituents were negatively correlated with the kinetic parameters of *H. angusta* and *R. minuta*, this data seemed to dependent on the differences between the yeast biosurfactant production and in second order to soil texture and OC content of the habitats.

Sorption experiments alone did not predict desorption responses, due to hysteresis and irreversibility of the process, the sorbed compounds fixed in to diverse compartments, each one with different dissociate and/or dissolution rates. So, desorption assays and comparison with controls cultures were implemented. Three types of desorption were confirmed; equilibrium was evidenced by high solution of Flu-levels at the first incubation time for the soil types. However, some Flu-nondesorbable fractions were observed, because a complete reversible desorption was not obtained.

DISCUSSION

The reported efficiency of PAHs biodegradation ranged from 6.0 to 82.0 % for soil fungi, and 0.2 to 50.0 % for soil bacteria (Das and Chandran, 2011). Different mechanisms were proposed to explain the PAHs bioavailability, like biosurfactants, extracellular enzymes production, fungal high substrate affinity and cell adhesion to particles (Cha et al., 2008; Ge et al., 2008). Direct uptake of pyrene, naphthalene and Flu at the aqueous/non-aqueous interface had been demonstrated by bacteria, besides enhanced bioavailability was obtained for hydrophobic compounds, i.e. Flu (Ghosh et al., 2001; Kumar et al., 2008); although this ability had not been extensively studied in fungi.

Bioemulsifiers had great potential for stabilising emulsions between hydrocarbons with liquid and/or solid media, thus increasing the surface area available for biodegradation. Filamentous fungi and yeast had produced specific kind of surfactants (Kiran et al., 2009); besides biosurfactans had been reported in different *Candida* spp., namely, *C. bombicola* (Shah and Prabhune, 2007), *C. spherical* (Sobrinho et al., 2008), *C. lipolytica, R. mucilaginosa, Geotrichu*m spp., *Trichosporon mucoides*, *Rhodothorula* spp. (Luna et al., 2009) and *Lipomyces starkeyi* (Sanino et al., 2010). Among bioemulsifiers, phospholipids, mannosylerythritol lipids, lipopolysaccharide and polyol-lipids were produced by yeasts (Pattanathu et al., 2008); sophorolipids were obtained in *C. bombicola, C. apicola, Centrolene petrophilum* and *R. bogoriensis* cultures (Kim et al. 2002; Van Bogaert et al., 2007); and mannosylerythritol lipids were produced by *Pseudozyma* yeasts, *P. aphidis, P. antarctica* and *P. rugulosa* (Imura et al., 2007; Konishi et al. 2007a, b). The low-foaming sophorolipids from *C. bombicola* was suitable due to their high detergency ability, low cytotoxicity and high biodegradability and general environmentally acceptable properties (Hirata et al., 2009).

In accordance with our results, and their significant capacities to degrade aromatics were in relation to this enzimatic abilities (Sarubbo et al., 2007)

Therefore, we concluded that *H. angusta* and *R. minuta* degraded Flu by bioemulsifiers production; evenmore, they were highly efficient to uptake Flu in the biphasic cultures and were dominant in the sampled polluted sediments.

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