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SORPTION-DESORPTION RESPONSE FOR PERYLENE UPTAKE BY WILD YEASTS ISOLATED FROM POLLUTED SEDIMENTS

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ABSTRACT

The industrial developments over the past few decades produced deliberately and/or accidentally released of xenobiotics to the environment, remaining these areas polluted for long periods. Bioremediation is a hard issue due to the complex mixture of the xenobiotics and to soil-sorbed contaminants that are not available for biotransformation. So microorganisms must be able to uptake the sorbed-molecules or facilitate the desorption by producing surfactants. The adsorption/desorption process was scarcely studied in fungi and sorbed-compounds fixed in diverse compartments with different desorption rates. Therefore, our aims were to isolate yeasts from polluted sediments, to evaluate the perylene bioavailability and to quantify their potential uptake. *Pseudozyma rugulosa* and *Centrolene petrophilum* grew on perylene cultures and their uptake were significant in relation to the other species. Different soils, type I, II and III, with diverse organic carbon, pH, cation exchange capacity, sand, silt and clay proportions were used. Soil type-III showed the higher perylene level in the aqueous phase and sorbed to particles, nevertheless showed the higher desorption rate. Perylene availability were in relation with the soil matrix and organic content, and the desorption coefficients were significantly related with the *P. rugulosa* and *C. petrophilum* kinetic parameters. These results were in relation to the biosurfactant production

by both fungal species. Desorption parameters significantly fitted perylene uptake, with R^2 = 0.97 for *P. rugulosa* and R^2 = 0.95 for *C. petrophilum*. Biosurfactants and extracellular enzymes production explained the perylene degradation by both yeasts, being the results confirmed by the surface tension measurements.

Keywords: Bioavailability, *Centrolene petrophilum*, Perylene, *Pseudozyma rugulosa*, Sorption/Desorption Mechanisms

INTRODUCTION

Most of the polycyclic aromatic hydrocarbons (PAHs) accumulate in nature because release rates from industrial effluents exceed dissipation rates, microbial and chemical degradation **[1, 2]**. Low biodegradation had been attributed to diverse factors, such as surface and subsurface effluent properties, chemical toxicity, high pollutants levels and limited bioavailability of the toxicant to microorganisms **[3, 4]**.

Perylene, dibenz [*de,kl*] anthracene, ($C_{20}H_{12}$, molar mass 252.31 g / mol) occurred as brown solid, with low water solubility (1.2 / 10^5 mmol / l) being its derivatives carcinogenic. Its molecule consists of two naphthalene molecules connected by a carbon-carbon bond at the 1 and 8 positions of both molecules. It is considered as a hazardous pollutant **[5]**.

Soil-sorbed PAHs are not available for biotransformation without prior desorption **[6, 7]**, so microorganisms must be able to uptake the sorbed molecules **[8, 9]** or facilitate the desorption by producing surfactants **[10, 11]**. While bacteria had been reported as biosurfactant producer to remove PAHs **[1, 12]**, fungal species had not been studied so far. Otherwise, yeasts and filamentous fungi had been reported as frequent organisms in heavily polluted habitats **[13, 14]**, but few researchers studied the fungal transformation of soil-sorbed PAHs **[15, 16]**.

The PAHs adsorption/desorption process was scarcely studied in fungal cultures **[17, 18]**, and sorption experiments alone did not predict desorption responses, due to hysteresis and irreversibility of the process, and that sorbed compounds fix in diverse compartments, each one with different desorption rates **[19, 20]**. Therefore, our aims were to isolate yeasts from polluted sediments, to quantify their potential to uptake perylene (Pryl) and to evaluate the bioavailability using a three-site desorption model.

MATERIALS AND METHODS

Sampled Sites and Chemical Analysis

Composite samples of surface sediments were taken from two different polluted streams from industrial areas, La Plata,

Argentina. The total organic carbon and total organic nitrogen levels were determined by CHN analyzer (Perkin-Elmer, Norwalk, CT) and Macro-Kjeldahl method, respectively. 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 **[21]**. Different soils, type I, II and III, with diverse organic carbon (OC) contents, sand, silt and clay proportions, pH and cation exchange capacity (CEC) **(Table 1)** were sterilized and suspended in sterile phosphate buffer (20 mM), at a ratio 1:40. To control the sterility. 0.1 g of each mix was placed on nutrient-agar plate, and incubated at 30ºC for 7 days.

Identification of Yeasts

Yeasts were isolated from dilution of sampled sediments, in a mineral medium supplemented with Pryl, added to test the yeast tolerance to the pollutant **[22]**. The isolates were identified by colony, cell morphologies, assimilation and physiological differences, and other tests, like D-glucuronate assimilation **[23]**, and coenzyme Q-system determination by HPLC were also done **[24]**.

Perylene Degradation Assays

Yeasts were cultivated on 40 ml liquid Sabouraud for 3 days at 180 rpm, 28ºC, till exponential growth to accumulate enough internal-C reserves. For degradation experiments, 1 ml was incubated in 500-ml flasks with 100 ml of a mineral medium (MM, **[24]**) with different Pryl aliquots (0, 20, 40, 60, 80, and 100 μg/ml). After 3 days incubation at 28 ºC and 180 rpm, cells were harvested by centrifugation, washed twice with sterile MM and the pellet was resuspended in MM to an optical density of 6 (600 nm).

Periodically, 1 ml of each flask was sampled to obtain the Pryl-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 (5mm) column (Merck, Darmstadt, Germany). The chemicals, Pryl and solvents were purchased by Aldrich-Chemie, and were of the highest purity available.

Surface Tension (ST)

Surface tension was evaluated to assess the uptake of soil-sorbed Pryl; ST allowed us to estimate biosurfactant production by a DuNoüy tensiometer. Yeasts responded to 5-50 mg/l Pryl levels by a capillary assay **[25]**. The cells harvested in lag phase were washed twice and resuspended in 20 mM phosphate buffer, then they were 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 cells that went into the capillaries with Pryl-solution after 1 h were enumerated by plate counts.

Perylene Bioavailability Assays

Soil-sorbed Pryl assays were performed with soil controls by triplicate. Two isolates able to grow on Pryl as C source were used in this study; inocula were prepared by culturing yeasts in liquid Pryl-medium at 180 rpm, 28 ºC, and cell growth was monitored at 600 nm absorbance with an spectrophotometer. Yeasts in lag phase were centrifugated, washed twice and resuspended in sterile phosphate buffer (20 nM, pH 7) to obtain a final cell density of 10^6 CFU/ml. Two mililiters of this cultures were 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 and 2 ml yeast culture; tubes were incubated at 20 rpm for 10 days.

At the 10^{th} day, the tubes were centrifuged to separate soil from the supernatant and analized to determine Pryl levels in sorbed or liquid phases by HPLC. Two control tubes were incubated in the same conditions, one without soil aliquots, another with 30 ml soil-suspension and then sterilized. Initial sorbed and fixed perylene 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 Pryl-levels and yeast densities.

Data Analysis

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

RESULTS AND DISCUSSION

Pseudozyma rugulosa and *Centrolene petrophilum* grew on perylene cultures and their uptake were significant in relation to other species, a maximum uptake rate of Vmax = 35 and 120 nM [(min^{-1}) .$(10^3 cell)^{-1}$] were observed in *P. rugulosa* and *C. petrophilum* assays, respectively. Moreover, both yeasts had not been already mentionated as conspicuous biodegrader in other researches. Soil type III showed the higher perylene concentration in aqueous phase and particles, and also showed the higher Pyrl desorption **(Figure 1, Figure 2)**. Desorption processes preceded the degradation of the hydrocarbon, being the experimental parameters and site fractions representative of the yeast uptake **(Figure 3)**. Cultures with different physical states like solid-liquid Pryl were composed of equilibrium (Seq), nonequilibrium (Sneq) and nondesorption (Snd) areas. Nondesorption sites were those with substrates that cannot be released to

solution, nonequilibrium sites showed a proportional release in relation to the gradient between these sites and the liquid phase, and the equilibrium areas release the Pryl to the liquid phase.

The Pryl uptake of both yeasts fitted to first-order kinetics with a Km = 0.0398 uM (R^2 = 0.97) and Km = 0.0240 uM (R^2 = 0.95) for *P. rugulosa* and C. *petrophilum*, respectively. Km was inversely related to the microbial affinity for the substrate, that is *C. petrophilum* could be more suitable to used in bioaumentation strategies. The ST differences between the controls and the *P. rugulosa* and *C. petrophilum* cultures indicated that biosurfactants were produced in both yeast experiments; 6.0 and 9.0 dynes/cm were the ST data in the *P. rugulosa* and *C. petrophilum* assays, respectively.

The results fixed to the equation: S = Seq + Sneq + Snd, where total substrate (S) were the sum of each fraction. The equilibrium phase was described by Seq = $f$eq K_F Ce^n; the nondesorption response followed the relation Snd = fnd K_F C^n, while the organic release from nonequilibrium followed the equation: d S neq / dt = α ($f$neq K_F C^n - Sneq). In the relations, K_F was the Freundlich sorption coefficient, n was the curvature constant, C was the Pryl liquid-phase concentration (mg/ l), Ce was the Pryl liquid-phase concentration in sorption equilibrium (mg/ l), t desorption time (min), α 1rst. order desorption 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 Pryl sorbed levels (mg/kg) in the solid equilibrium, nonequilibrium and nondesorption areas, respectively **(Table 2)**.

The perylene desorption parameters were obtained by the mentionated equations by nonlinear regression analysis of the experimental data **(Table 3)**.

The Pryl equilibrium fraction increased in relation with the OC content, ranging from 0.35, 0.63 and 0.73; the nonequilibrium Pryl fraction was similar among soil types and nondesorption sites decreased as OC content increased, ranging from 0.51, 0.23 and 0.13. So, the desorption efficient increased as the OC increased, being these observations consistents with the interactions between soil constituents and pollutant bioavailability.

The desorption profiles confirmed that in natural habitats the organic compounds fixed to the equilibrium (Seq), nonequilibrium (Sneq) and nondesorption (Snd) areas. That is to say that perylene was also observed in the nondesorbable fractions, therefore incomplete desorption was found. Similar results were obtained with other toxicants like pesticides **[26, 27]**

and organics bound to humic fractions **[28]**.

The perylene availability was in relation with the matrix and organic content of the sediments. The Pryl-desorption coefficients were significantly related with the *P. rugulosa* and *C. petrophilum* kinetic parameters, and this data depended on the yeast biosurfactant production **[29]**.

The presence of microorganisms able to mineralize higher-molecular-mass PAH with four and more annealed rings (hmw-PAH) are of particular interest since this PAH group are significantly higher genotoxic than the two-three ring PAHs **[30]**. However, the ubiquitous presence of potent and versatile mineralizing microflora in PAH-contaminated soils indicated that microorganisms are not the limiting factor for the hmw-PAH-detoxification **[31, 32]**. Perylene had not been extensively study as carbon source and in many studies no isolates were able to degrade perylene **[33]**; therefore our results are remarkable.

Different microbial mechanisms were proposed for organic soil-sorbed bioavailability, like: biosurfactants production **[11, 34]**, extracellular enzymes production **[35, 36]**, and fungal high affinity and cell adhesion to particles. The two first mechanisms explained the perylene degradation in this study by *C. petrophilum* and *P. rugulosa*, being the results confirmed by the surface tension measurements. The 3rd. situation, called direct-uptake, had been observed for bacterial pyrene, naphthalene and fluoranthene uptake but not for perylene **[37]**.

Table 1: Characteristics of the Soils Used in the Experiments

	OC (%)	Sand (%)	Silt (%)	Clay (%)	pH	CEC (cmol/kg)
Type I	**1.30**	**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**

Table 2: Pryl Sorption Parameters in the Soil Types Obtained by Each Equation

	Freundlich Equation			Linear Equation	
	K_F [(mg/kg)/(mg/l)]	n	R^2	Kd (l/kg)	R^2
Type I	**1.44**	**1.02**	**0.99**	**1.50**	**0.98**
Type II	**3.18**	**1.05**	**0.98**	**3.40**	**0.99**
Type III	**12.40**	**0.93**	**0.99**	**11.90**	**0.98**

Table 3: The Desorption Parameters Obtained by the Three-Site Model were (SD of the Data in Parentheses)

	*f*eq	*f*neq	*f*nd	α (min^{-1})	R^2

Type I	**0.35 (0.009)**	**0.14**	**0.51 (0.005)**	**0.0020 (0.003)**	**0.97**
Type II	**0.63 (0.008)**	**0.17**	**0.23 (0.003)**	**0.0025 (0.004)**	**0.96**
Type III	**0.73 (0.011)**	**0.18**	**0.13 (0.010)**	**0.0027 (0.009)**	**0.90**

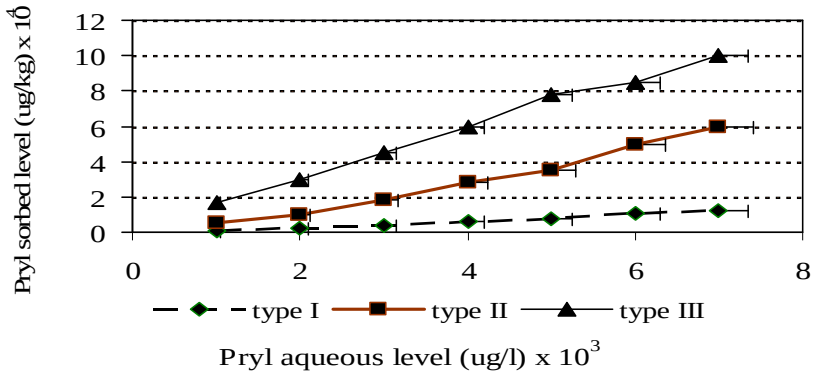

Figure 1: Perylene Levels in the Aqueous Phase and Sorbed to Particles

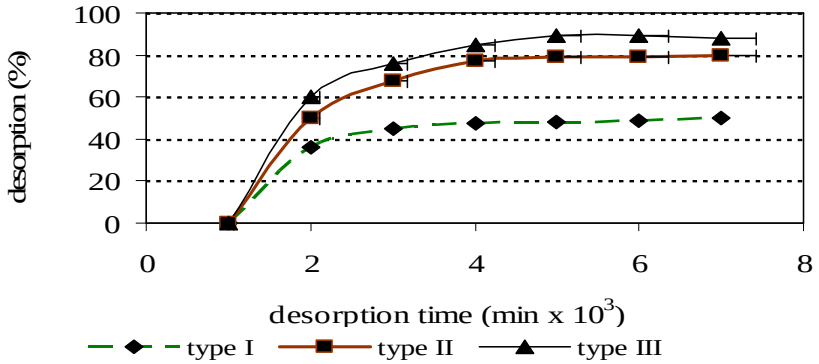

Figure 2: Perylene Desorption (%) for the Different Soil Types

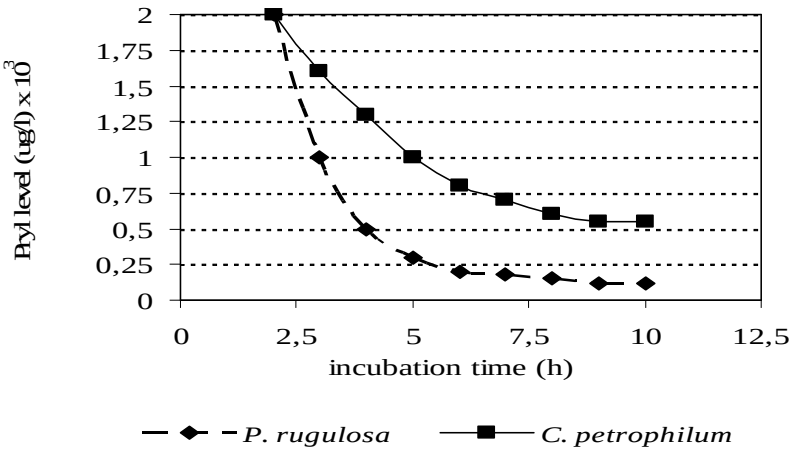

Figure 3: Pryl Biodegradation by *P. rugulosa* and *C. petrophilum* in Liquid Phase

CONCLUSION

In conclusion, the isolated yeasts contributed to perylene uptake and detoxification processes, in an environmental biotechnological context, yeasts may be useful in the treatment of PAHs polluted effluents. Future work in the area should be directed towards identifying other compounds and fungal species responsible for organics biotransformation.

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