Sorbed Anthracene Degradation by Sophorolipid Producing Yeasts

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Abstract: The organic pollutan adsorption/desorption process by microbial degradation had been less studied than metal ones. The sorption assays alone did not predict desorption, due to hysteresis, irreversibility, fixed compounds in different sites, with diverse desorption rates. Most of the studies dealt with bacteria rather than filamentous fungi and yeasts. So, our aims were to isolate yeasts from polluted sediments, to quantify its potential to uptake anthracene (An) and to evaluate the bioavailability by a desorption model. Yeasts were isolated from hydrocarbon-polluted samples, 40-isolates grew in anthracene-plates. Molecular characterization was achieved by sequence analysis of the ITS1-5.8S rRNA-ITS4 and 26S rRNA regions; morphological and physiological determination were also done. *Candida parasilopsis, Pichia anomala* and *Rhodothorula mucilaginosa* were the prevalent yeasts. An-degradation was assessed in soil-systems with 0, 50, 100, 150, 200 and 250 µg An/l, 3 differentes sorbens types, organic carbon, organic nitrogen, PAHs, sand:silt:clay, pH and cation exchange capacity. Sophorolipids excretion were confirmed by HPLC, UV-detector with active fraction at 9.669 min (RT 9.646 min = sophorolipid-standard). A desorption model with equilibrium, nonequilibrium and nondesorption areas, was applied to explain the experimental data, An-transformation was greater in the organic liquid-phase than in the soil-sorbed ones; the desorption-coefficients and soil components were negatively correlated with the kinetic parameters. The An-release depended on the sophorolipid excretion, soil matrix and particles sizes. Desorption parameters significantly fitted the yeast uptake, with R^2 = 0.97, R^2 = 0.90 and R^2 = 0.97 for *C. parasilopsis, P. anomala* and *R. mucilaginosa, respectively.*

Keywords: Anthracene, bioavailability, biodegradation, *Candida parasilopsis,* desorption model *Pichia anomala, Rhodothorula mucilaginosa,* sophorolipid.

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) accumulate in nature because release rates from industrial effluents exceeded dissipation rates, chemical and microbial transformation [1,2]. Low biodegradation had been attributed to diverse factors, such as surface soil and effluent properties, chemical toxicity, pollutant concentrations and low availability of the toxicants [3-5]. Soil-sorbed PAHs had been considered unavailable for biotransformation without prior desorption, so microorganisms must uptake sorbed molecules or facilitate desorption by surfactant production [6,7].

Yeasts secret different biosurfactants, like sophorolipids (SLP), liposan, carbohydrat-protein-lipid complex, mannoprotein, mannosylerythritol, erythritol and mannose-lipids [8,9]. SLP are the most promising ones, due to its low toxicity, high biodegradability, excreted in great amounts, low foam formation, high detergency, selective and specific activity at wide range of temperature, pH and salinity, produced from renewable sources or industrial residues, easy to recover [10,11].

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The organic pollutant adsorption/desorption had been less studied than to metal process [12,13]. Sorption tests did not predict desorption responses, due to hysteresis, irreversibility of the process, and fixed compounds in compartments with different desorption rates [14-16]. Most of the studies dealt with bacteria rather than filamentous fungi and yeasts, therefore, our aims were to isolate yeasts from polluted sites, to quantify the anthracene (An) uptake by cometabolism, to evaluate the An-bioavailability and to obtain the desorption parameters.

MATERIALS AND METHODS

Sampled Sites and Yeasts Isolation and Identification

Yeasts were isolated from sediment samples taken from the industrial area, La Plata, Argentina. Total organic carbon (TOC), total organic nitrogen (TON; CHN analyzer, Perkin-Elmer, Norwalk, CT) and polycyclic aromatic hydrocarbons (PAHs, FTIR-Perkin-Elmer), sand:silt:clay proportions, pH and cation exchange capacity (CEC (cmol/kg) of the sediments were determined (Table **1**) [17].

Yeasts were isolated under selective conditions in agar-mineral medium (MM, [18]) with 75 µg An/l as C-source. They were characterized by colony, cell morphologies, assimilation, physiological differences,

ISSN: 1927-3037/16

Table 1: Characteristics of the Soils Used in the An-Degradation Experiments

	TOC (mg/kg soil)	TON (mg/kg soil)	PAHs (mg/kg soil)	sand:lime:clay	pH	CEC (cmol/kg)
soil I	9.8	4.3	102.1	47:21:32	5.5	7.10
soil II	5.6	2.0	84.6	58:15:25	5.9	24.40
soil III	4.5	1.2	40.7	48:28:24	6.0	33.00

D-glucuronate, reproduction and coenzyme Q-system by HPLC [19].

Yeasts were identified by sequencing two different rRNA genes and DNA isolation [20]. The amplifications were performed with Corbett Cool Gradient Palm Cycler CGI-96: DNA strands were sequenced by ABI 3130XL automated sequencer (Applied Biosystems, USA) of ITS1-5.8S rRNA-ITS4 and 26S rRNA regions. PCR amplicons were synthesized using primers, Fermentas, Germany). Amplification was performed for 40 PCR cycles, denaturation at 95°C for 2 min, annealing at 58°C for 2 min, and extension at 72°C for 2 min, final extension for 10 min. For 26S rRNA, the primers NL1 (5'-GCA TAT CAA TAA GCG GAG GAA AAG–3') and NL4 (5'-GGT CCG TGT TTC AAG ACG G-3') were used for amplifications, for 35 PCR cycles with denaturation at 95°C for 1 min, annealing at 58°C for 1 min, and extension at 72°C for 2 min, final extension for 10 min. Sequences were compared by BLASTN and aligned with related species sequences [21,22].

Anthracene Degradation Assays

Three yeast species were selected for this assays, due to its conspicuous development in An-plates. They were precultivated in 50 ml Sabouraud for 48 h, at 28ºC, 2 days, till exponential growth to accumulate enough internal-C reserves. Then, 1 ml yeast suspension were inoculated in 6 flasks with 100 ml MM plus 0, 50, 100, 150, 200, 250 µg An/l MM and 2% C16, pH 5.4, by triplicate. After 15 days incubation time at 28ºC and 180 rpm, cells were centrifugated (5000 g, 5 min), washed twice with sterile MM, and resuspended in MM to an optical density (OD) 600 nm, by triplicate.

Yeast cell concentration (cells/ml), dry cellular weight (DCW, g/l) and OD600 were obtained subsampling at 0, 1^{st}., 2^{nd}., 3^{rd}., 4^{th}., 5^{th}., 7^{th}., 10^{th}., 13^{th}. and 15^{th} days. A correlation curve was plotted with OD600 and DCW against cell concentration. A correction factor 1.0-1.3 was used for NucleoCounter® YC-100. Correlation between OD, DCW and yeast cell count was 1 OD600, within linear range ca. 0.3-0.6 g DCW /l = ca. 1×10^6 to 5×10^6 cells/ml.

Simultaneously, 1 ml of each flask were subsampled to obtain An-levels by HPLC (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 analizsed by a diode array detector, in triplicate [12,23]. The chemicals, An and solvents were purchased by Aldrich-Chemie, and were of the highest purity available.

An-Desorption Assays

Different sterilized soils were used to obtain the desorption factors (Table **2**). The assays were carried out in 100 ml tubes with 30 ml sterile soils plus 2 ml sterile phosphate buffer plus 1 ml inoculumn yeasts, 100 µg An/l MM and 2% C16, pH 5.4. Tubes were incubated at 180 rpm, 15 days, in darkness, at 28ºC. At the 15^{th}. day, the tubes were centrifugated, to separate sediments from supernatants, and An-final levels were determinated in sorbed and liquid phases by HPLC. Three controls were incubated in the same conditions, one inoculated but without soil, other inoculated with 30 ml soil suspension and then sterilized, the 3^{rd}. one uninoculated with 30 ml soil suspension.

Once a day, 1 ml subsamples were withdrawn from each tube to quantify unsorbed and fixed An-levels, yeast cell concentration, DCW and OD600. Initials An-concentrations were determined in soil samples and controls. All the subsamples were analized by triplicate.

Biosurfactant presence were obtained from the same An-degradation cultures by surface tension (ST) measurements. Cells harvested in late log phase were washed twice with 20 mM phosphate buffer,

resuspended in 20 mM phosphate buffer plus 10 µM EDTA, placed in a U-shaped tube to be observed by microscopy. Uninoculated tubes were used as controls, all the measurements were made by triplicate.

Subsamples were taken and extracted with ethyl-acetate to determine their oil-displacement activities, and kept for the HPLC linear gradient with UV detector system. The most active fraction eluted was obtained at RT 9.669 min; standard sophorolipids showed a peak at RT 9.646 min, m=z of 648.760 and 650.816 were found. M/z of 648.760 corresponded to a sodium adduct of C18 sophorolipid which is added 23 Da of the sodium molecule, whereas the m=z of 650.816 corresponded to C20:1 sophorolipid [24,25].

Data Analysis

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

RESULTS

The isolates were identified as *Candida batistae, Candida bombicola, Candida glabrata, Candida guillermondi, Candida maltosa, Candida sphaerica, Candida parasilopsis, Cryptococcus albidus, Cryptococcus diffluens, Pichia anomala, Pichia kudriavzevii, Rhodotorula bogoriensis, Rhodotorula glutinis, Rhodotorula mucilaginosa and Saccharomyces cerevisiae. C. parasilopsis, P. anomala* and *R. mucilaginosa* were frequent in the An-plates, so they were selected for this study. They were identified using amplification and sequencing of the Internal Transcribed Spacer ITS1 and ITS4 region of ribosomal DNA (rDNA) (Table **3**). ITS-region is short, readily amplified by PCR using universal single primer pairs complementary to conserved regions. Morphological and physiological characterization of the isolations accorded (ca. 78 %) with the sequence analysis.

Biphasic cultures, with soluble and particulate An, were similar to environmental situation than one-phase ones; moreover, pollutants settled in equilibrium, nonequilibrium and nondesorption areas. In nondesorption sites, toxicants did not discharge to solution, nonequilibrium area showed a proportional release rate between solid and liquid phases, and in the equilibrium ones An-particles dissolved in the liquid.

The three-site desorption model fitted the experimental data, being the parameters and site fractions representative of yeast uptake (Figure **1**). A first-order kinetics was observed in the *C. parasilopsis* and *R. mucilaginosa* assays, with a Km value = 0.0375 min^{-1} (R^2 = 0.97) and Km = 0.0450 min^{-1} (R^2 = 0.98), respectively, while *P. anomala* cultures followed Michaelis-Menten kinetics with a Km = 0.0740 min^{-1} (R^2 = 0.95). Km value assessed the yeasts affinity for the substrate, so in *C. parasilopsis* and *R. mucilaginosa* experiments higher An-degradation would be expected, in relation to *P. anomala.*

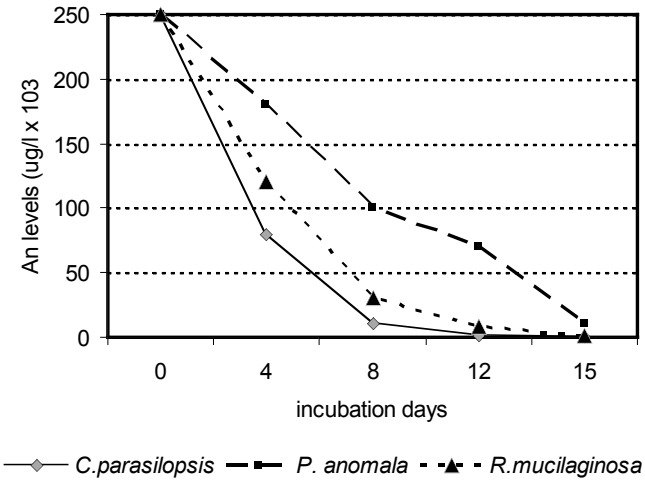

Figure 1: An-biodegradation curves in *C. parapsilopsis, P anomala* and *R. mucilaginosa* in liquid phase.

Equilibrium (Seq) partitioning was described by Seq = $f$eq K_F Ce^n; nondesorption (Snd) responded to Snd = fnd K_F C^n; and nonequilibrium (Sneq) sites followed the relation: d Sneq / dt = α ($f$neq K_F C^n - Sneq). In the equation K_F was the Freundlich sorption coefficient, n: isotherm curvature constant, C: An liquid-phase concentration (mg/ l), Ce: An liquid-phase concentration (mg/ l) in sorption equilibrium, t: desorption time (min), α: 1st. order desorption rate coefficient (min^{-1}) for nonequilibrium areas, $f$eq, $f$neq and fnd: were equilibrium, nonequilibrium and nondesorption fractions, Seq, Sneq and Snd were An-sorbed levels (mg/kg) in the equilibrium, nonequilibrium and nondesorption areas, respectively (Table **4**). K_F and n were obtained from the sorption isotherm, fnd was the plateau of the desorption profile, while $f$neq, $f$eq and α were calculated by nonlinear regression analysis of the desorption experimental data, for each yeasts cultures.

The profiles confirmed that the three processes occurred; equilibrium was confirmed by the An-levels in the liquid phase; An did not increase although its molecules were liberated from soil particles due to fungal degradation. More An was sorbed to soils with

Table 2: An-Desorption Parameters Obtained by the Three-Site Model. (Data are the Arithmetic Mean of the Three Yeasts Cultures for Each Soil Type; ± SD)

	*f*eq	*f*neq	*f*nd	α (min^{-1})	R^2
soil I	0.35 (± 0.009)	0.33 (±0.010)	0.35 (±0.005)	0.0020 (±0.003)	0.97
soil II	0.43 (± 0.008)	0.38 (±0.008)	0.20 (±0.008)	0.0025 (±0.004)	0.96
soil III	0.75 (± 0.011)	0.41 (±0.009)	0.10 (±0.010)	0.0027 (±0.009)	0.90

higher TOC, TON and PAHs contents (Table **1**); so more An desorption could be expected (Figure **2**). The An-*f*eq ranged from 0.35, 0.43 and 0.75; *f*neq were similar among soil types (0.33; 0.38, 0.41) (Table **3**), and *f*nd decreased as TOC, TON and PAHs increased, ranging from 0.35, 0.20 and 0.10.

Moreover, An-sorbed to particles by photodimerization under shear stress, formed a complex solid with 2 An-molecules, a reversible process. Desorption coefficients α (min^{-1}), were consistent with the interactions between soil constituents, yeasts presence, pollutant levels and availability, and were negatively correlated with the kinetic parameters, Km and maximum degradation rate (Vmax).

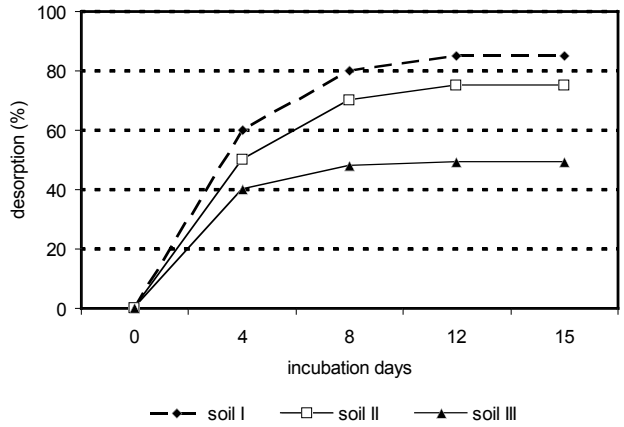

Figure 2: An-desorption (%) during the desorption assays for the different soil types.

An-liberation depended more on the yeast surfactant excreted, than to soil particles to which they were bound. The 1st. condition explained the An-availability of this study, confirmed by the surface tension measurements, by other hand, the 2nd. process, called direct-uptake, was not significant.

The ST differences between controls and yeasts cultures indicated that biosurfactants presence. From thermodynamics view, ST was the excess of free energy/unit area in the interface liquid-vapour interface. The van der Waals interactions were 0.027 ± 0.004; 0.031 ± 0.005 and 0.058 ± 0.004 N/cm^2, for *C. parasilopsis, R. mucilaginosa* and *P. anomala,* respectively. ST was the integrating anisotropic tangential stress along the perpendicular direction to the interface An-media and air-flask, depended on temperature, composite media, An-levels and yeasts densities. SLP was liberated to the medium as extracellular carbon storage, as secondary metabolites and did not have function for cellular growth, development and reproduction [5,26].

Different fungal mechanisms determined the bioavailability of organic soil-sorbed pollutants, like: biosurfactants production, extracellular enzymes excretion, cometabolism, high substrate affinity (low Km), high Vmax and cell adhesion to particles [27-29]. SLP had been especially reported in *Candida* spp. and *Rhodothorula* spp. cultures [30-32], in accordance with our results, and PAHs-degradation was in relation to its metabolic abilities [33].

Yeasts are preferred than others microorganisms for biosurfactants due to their GRAS (generally regarded as safe) status, that is, they do not induce

Table 3: Sequence Analysis to Identify the Yeast Species by *ITS* Regions

yeast species	primer sequence	product size	annealing temperature	% agreement
P. anomala	5'-ACGTCATAGAGGGTGAGAAT-3' 5'-AAACACCAAGTCTGATCTAATG-3'	197 bp	57 º	97.5
C. parapsilopsis	5'-GAGGGTGTCAGTTCTTTGT-3' 5'-GTGAGCTGCGAGAGTC-3'	224bp	56º	96.5
R. mucilaginosa	5'-GCGCTTTGTGATACATTTTC-3' 5'-CCATTATCCATCCCGGAAAA-3'	169 bp	54º	95.0

Table 4: The An-Sorption Parameters by each Yeast Assays ((Data are the Arithmetic Mean of the Thre Yeasts Cultures for Each Soil Type; ± SD)

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

toxicity or pathogenic reactions. Yeasts produce biosurfactants in higher concentrations, faster growth rate than filamentous fungi, resistant to unfavorable environments, useful in biological treatment of effluents and PAHs degradation [34,35]. *Candida albicans* [31], *Candida bombicola* [30,36], *Candida batistae* [37], *Candida glabrata* [38], *Candida kuoi* [39], *Candida tropicalis* [26,27], *Pichia anomala* [2,40], *Hansenula angusta* [17], *Rhodotorula bogoriensis* [32,41], *Rhodotorula minuta* [17], *Rhodotorula glutinis* [33,42] and *Wickerhamiella domercqiae* [43,44] had been confirmed as SLP producers.

Biosurfactant physiological roles are not completely elucidated, a generalization is difficult because they presented different chemical structures and superficial properties [45,46]. Therefore, each biosurfactant could perform diverse functions, providing a wide range of advantages for microorganisms in its ecological niches [47-49]. However, the use of biosurfactants in bioremediation and the optimization of large-scale production is needed, as well as studies of alternative C-sources derived from agroindustrial wastes [50-52].

In conclusions, it is important to evaluate the possible *in-situ* production in polluted sites to speed up soil restoration, to isolate wild microorganisms that excreted biosurfactant, to study the factors and conditions to optimize biosurfactant production and use in field studies. Like any important subject in science, there are more questions than answers; research in biosurfactants for use in *in-situ* bioremediation of polluted sites is still an innovative subject that needs more reliable scientific data.

ACKNOWLEDGEMENTS

This work was supported by grants from the National Council of Scientific and Technological Research – CONICET, and from the National University of La Plata (UNLP), Fac. Ciencias Veterinarias, Cátedra de Micología Médica e Industrial, Argentina.

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Received on 09-12-2015 Accepted on 06-01-2016 Published on 02-03-2016

DOI: http://dx.doi.org/10.6000/1927-3037.2016.05.01.4