Fluoranthene degradation induced morphological and functional changes in *Dipodascus ingens* biotypes

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ORIGINAL

Diferenciación morfológica y funcional de *Dipodascus ingens* durante la degradación de fluoranteno

Resumen. En los últimos años, los estudios sobre biorremediación han demostrado que las levaduras suelen ser dominantes en efluentes industriales, mostrando desarrollo dimórfico y variaciones fisiológicas conspicuas relacionadas con el grado de contaminación. Los objetivos del presente trabajo fueron caracterizar las levaduras capaces de degradar hidrocarburos y evaluar el dimorfismo, agregación y patrones enzimáticos durante la transformación de fluoranteno (FA) en cultivos de *Dipodascus ingens*. Una variabilidad significativa en las formas celulares y desarrollo de pseudomicelio se observó en las cuatro cepas; el contenido de quitina y la actividad quitin-sintasa confirmaron dichas variaciones intraespecíficas, obteniéndose mayor variabilidad, pseudohifas y agregados celular en los cultivos con FA. *D. ingens* desarrolló formas miceliales y celulares, y esta plasticidad morfológica propició la colonización del sustrato. La fase levaduriforme tuvo mejor dispersión en medios fluidos mientras que las pseudohifas colonizaron las superficies sólidas, rasgo particularmente ventajoso en los ensayos de degradación de contaminantes, ya que éstos, debido a su baja solubilidad, permanecen en forma particulada en los medios de cultivo. Los rasgos permitieron agrupar las cepas, interpretándose la aparición de los biotipos como una respuesta adaptativa al habitat adverso.

Palabras clave: Actividad quitin-sintasa, *Dipodascus ingens*, flouranteno, sedimentos contaminados, variabilidad fenotípica.

Abstract. In recent years, bioremediation researches pointed out the increasing yeast populations associated with industrial discharge, and the occurence of physiological and morphological variations correlated with the pollution levels. So, the aims of this study were to characterize the yeasts with aromatic hydrocarbon degradation ability and to report the dimorphic transition, aggregation profiles and enzyme patterns during fluoranthene (FA) transformation in *Dipodascus ingens* cultures. A significant variability in cell forms and pseudomycelium development were observed in the isolated strains; the chitin synthase activity and chitin content confirmed the differences between the biotypes; moreover, pseudohyphae formation and aggregation profiles were dominant features in the aromatic hydrocarbon cultures. This study joined the strains in groups, particular traits were found for D1-D3 and D2-D4 biotypes, and the variability was considered an adaptative response to adverse habitats. *D. ingens* existed in cell and mycelial phases, and this plasticity ensured a better competitiveness in substrate degradation. Yeast phase dominated in liquid substrates whereas mycelial forms colonized solid ones, being this trait an advantage to transform toxicants, as most of them remained as particles in the degradation experiments.

Key words: chitin synthase activity, *Dipodascus ingens* strains, different phenotypes, flouranthene, polluted sediments.

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Introduction

Fluoranthene (FA), a tetracyclic aromatic polycyclic hydrocarbon (PAH), is commonly found as a component in PAH-polluted sites originated from petrogenic or pyrogenic sources. FA occurs in high levels in urban areas due to incomplete combustion of fossil fuels and has been shown to be cytotoxic, mutagenic and potentially carcinogenic [7, 14]. Many microorganisms were point out as biodegraders of PAHs up to three rings, however, less had confirmed as capable to metabolize larger and more recalcitrant molecules [9, 21].

Bacteria, pure strains and strain associations oxidized or co-oxidized FA [3,4, 8]; on the contrary, incidental PAHs metabolism by fungi had been mentioned and just ligninolytic species [12, 17]. Indeed, yeasts were reported as widespread organisms in polluted habitats due to their low susceptibility to environmental changes, advantage to survive stress conditions and able to transform diverse organic and inorganic pollutants [2, 20].

Different colony forms, dimorphic growth, hyphal development, including traits for taxonomic assignment, and physiological features were correlated with environmental and nutritional adverse situations [6, 26]. Therefore, the aims of this study were to characterize the hydrocarbon transforming yeasts isolated from polluted sediments, and to report the significance of dimorphism, aggregation profiles, chitin synthase activity and chitin content during flouranthene transformation in *Dipodascus ingens* cultures**.**

Materials and methods

Isolation and identification of fluoranthene degrading yeasts. Sediments from natural and artificial channels that drain to Rio de La Plata (Argentina) were diluted and plated to isolate FA-degrading yeasts by spread-plate methods, by triplicate. They were purified by streaking the colonies on agar-mineral medium (MM)[19] with 50 ppm fluoranthene (MM-FA) as carbon source. The samples and two control-sets (MM-sterile and MM-without fluoranthene plates) were also incubated at 27°C, 120 rpm for 20 days. The same MM-FA medium was employed to enumerate all the degrader yeasts.

Yeasts were identified using traditional morphological and biochemical tests, indeed additional assays like hexadecane, ethanol, glycerol, 2-keto-D-gluconate and arbutin assimilation and growth patterns were also done [10, 23]. Guanine-cytosine (GC) values were quantified by bouyant density in CsCl gradients generated by ultracentrifugation [11]. DNA was obtained from cell cultures on potato dextrose agar; 0.25 g biomass was added to 2 ml polypropylene tube with 0.5 g glass beads (0.5 mm) and 0.5 ml 120 mM K_2HPO_4 buffer (pH 8.0). Tubes were shaken 30 s at 5000 rpm in a minibeadbeater, after centrifugation at 15 min, 14000 rpm at 4ºC, the pellet was washed and resuspended in 1001 sterile deionized water.

The organic carbon and nitrogen concentrations of the sediments were determined by CHN analysis (Perkin-Elmer, Norwalk, Conn.). Total hydrocarbon and fluoranthene levels were analysed by FTIR-Perkin Elmer, in triplicate; the ultrasonic extraction was realized with Cl_4C. A cell with BrK window, 0.35 mm thick, was employed for this determination [1].

Chitin measurements. Yeast phase developed in YPD (1% yeast extract; 2% peptone; 2% dextrose) at 30ºC; and hyphae were induced by resuspending 2.0 x 10^7 cells/ml in pre-warmed 5% newborn calf serum at 35ºC, for 6 h. Chitin content in both forms were assayed by treating chitosan with nitrous acid, depolymerized and deaminated [18] and by acid-hydrolysis technique [27].

Chitin synthase activity (CSA). CSA were determined in yeasts and hyphaes. Mixed membrane fractions (MMFs, [16]) were employed to resuspend the pellets in 50

mM Tris/HCl (pH 7.5) with 30% glycerol. The Coomassie Protein-Assay was used to evaluate the MMFs-protein levels, and the enzyme assays were performed with and without trypsin pre-treatment. Standard trypsin preparation (100 ng trypsin / l MMF) incubated for 5 min at 30ºC; and trypsin activation was stopped by 150 ng inhibitor / l MMF. The CSA was carried out in 50 l containing: 50 g MMF protein, 25 mM *N*-acetylglucosamine, 50 mM Tris/ HCl (pH 7.5), 10 mM $MgCl_2$ and 1mM UDP-*N*-acetylglucosamine, which included 25 nCi UDP-[U-^{14}C]*N* -acetyl glucosamine. After 30 min, the reactions were stopped with 1 ml 66% (v/v) ethanol. Mixtures were filtered through GF/C filter, presoaked in 10% (v/v) TCA, and the tubes were rinsed with 1ml 1% (v/v) Triton X-100. Each filter was then washed with 2 ml 66% (v/v) ethanol, dried at 80ºC and placed in a vial for liquid scintillation counting.

Dimorphism. The yeast-to-hyphae transition was performed in control and in FA-media inoculated with 2 x 10^7 cells/ml incubated at 120 rpm, in triplicate, for 21 days. Cell density was measured at OD_{660} nm (LKB-Ultrospec II model 4050). Periodically, 5 ml control and FA-flasks were fixed with 6% (w/v) folmaldehyde in 0.2 M $K_3(PO_4)$ buffer (pH 6.5). Cell shapes, hyphaes and aggregation profiles were monitored by phase-contrast microscope, patterns and morphologies were assessed by couting at least 300 cells during the whole life cycle, by triplicate.

Statistical analyses. Data from the degradation assays, yeast densities, morphological features, enzyme activity and sampled sites characteristics were evaluated by ANOVA, the significance levels were set at P = 0.01.

Results and discussion

Thirty-eight different yeast species were isolated from PAHs-contaminated sites, being all of them able to grow in fluoranthene as only carbon source (Table 1). *Dipodascus ingens* (Rodrigues de Miranda : de Hoog, M.T. Smith & Guého) was chosen to assess fluoranthene effect on its phenotyphic features as the species showed morphological and functional variations, and was not mention as PAHs degrader, althought it was first isolated from sulphite, asphalt and phenolic industrial wastes [10, 23].

The pollution level of the studied sites led us to range them, indeed, Channel Este and Oeste received industrial discharges, Regatas St. was open to the Rio de La Plata and Zanjón St. was a polluted stream with organic wastes (Fig. 1). In accordance to the hydrocarbon, organic carbon, nitrogen and organic matter concentrations, the sites were different from each other ($P < 0.01$). Differences in the *D. ingens* strains from each sampled sited were observed, and four biotypes D1,D2, D3 and D4 where identified, being D1-D3 frequency higher in the heavily contaminated Este and Oeste channels (Fig. 2).

In the control experiments, *D. ingens* cell sizes were spheroidal, appeared in pairs or in chains, with a primitive pseudomycelium; cylindrical arthroconodia and ellipsoildal asci, containing 2-4 hyaline ascospores were obtserved. In prolonged cultures, 90 days incubation time, mostly

Table 1.Yeasts species isolated from the polluted sediments.

Aureobasidium pullulans	*Hansenula angusta*
Candida albicans	*H. polymorfa*
C. famata	*Pichia anomala*
C. guillermondi	*P. cactophila*
C. intermedia	*P. membranaefaciens*
C. krusei	*P. opuntiae*
C. tenuis	*P. pinus*
C. terreus	*Rhodotorula aurantiaca*
C. tropicalis	*R. glutinis*
Cryptococcus albidus	*R. graminis*
C. laurentii	*R. mimuta*
Debaryomyces carsonii	*R. mucilaginosa*
D. castellii	*R. rubra*
D. hansenii	*Squizosaccharomyces pombe*
D. maramus	*Yarrowia lipolytica*
Dipodascus aggregatus	*Williopsis californica*
D. ingens	*W. mucosa*
Exophiala spp.	*W. pratensi*
Geotrichum spp.	
G. candidum	

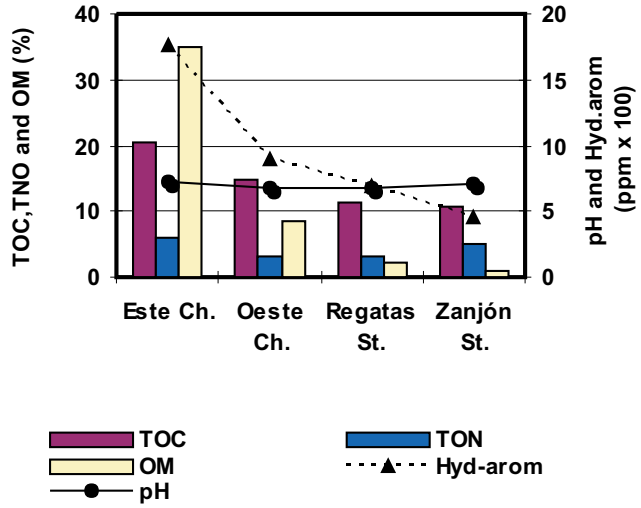

Figure 1. Chemical characteristics of the sampled sites: TOC= total organic carbon, TON= total organic nitrogen, OM= organic matter (% of 10 g dry weight), pH and aromatic hydrocarbon levels.

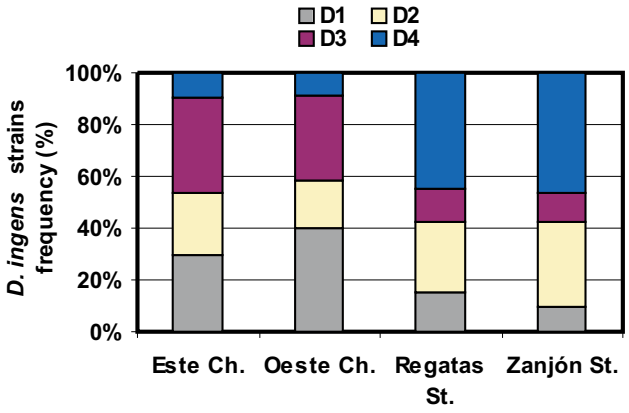

Figure 2. Frequency of the *D. ingens* strains in each sampled sites.

chlamydospores appeared in all the assays. The GC values ranged between 39.2-38.7 mol % (S.D. 0.20 mol %), confirming that the 4 strains belong to *D. ingens***.** However, in the FA-degradation assays, significant fluctuations of the physiological and morphological traits were observed.

Chitin synthase activity and chitin content were different in each biotypes. The CSA values were higher in hyphal membranes and trypsinized cells in relation to untreated ones. Hyphal and yeast D1 and D3-membranes showed major activities, intermediatte and low values characterized the D2 and D4 forms, respectively (Fig. 3a). The chitin content obtained for yeast cells were lower than the

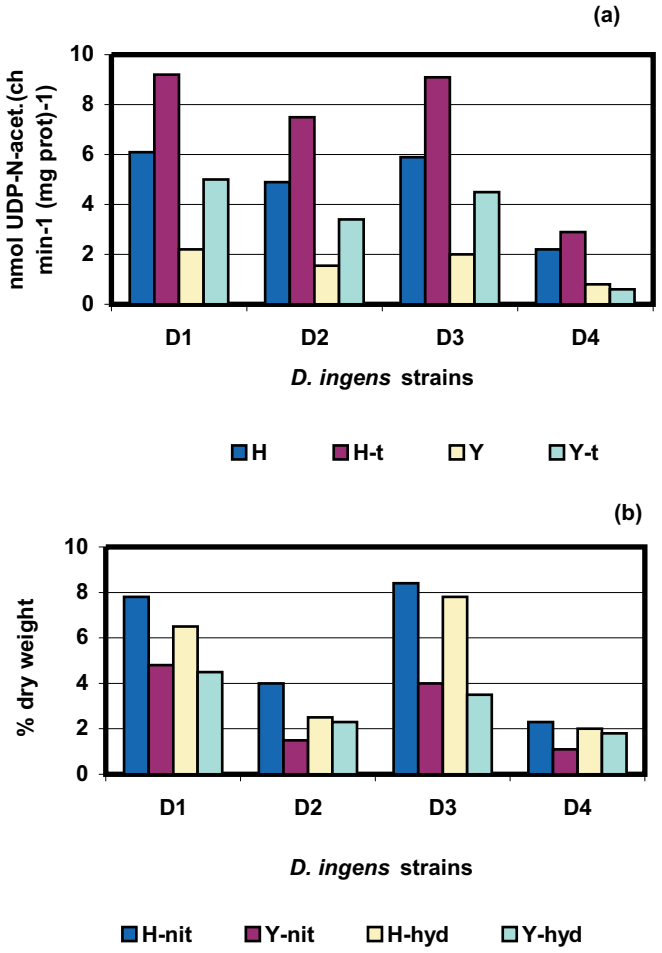

Figure 3. (a) Chitin synthase activity and (b) chitin content in the *D. ingens* strains.

hyphal forms, with both methods (Fig. 3b).

Pseudohyphae formation during the *D. ingens* life cycle, cell density and morphology were periodically examined in FA and control flasks. Whereas controls showed dimorphic growth without a lag phase for all the biotypes, the hydrocarbon cultures showed different patterns (Fig. 4a). With FA, the lag phases lasted 12 and 24 h and the exponential ones delayed 12 h and 36 h in D1-D3 and D2-D4 cultures, respectively. The stationary phase, was reached in the D1-D3 assays at 48 h incubation time when the OD_{660} reach 0.20 - 0.30; in the D2-D4 cultures it was a short one. D1-D3 developed a double growth in respect to D2-D4, in concordance with the FA-residual levels, that were less in the D1-D3 cultures at the end of the experiments (Fig. 4b).

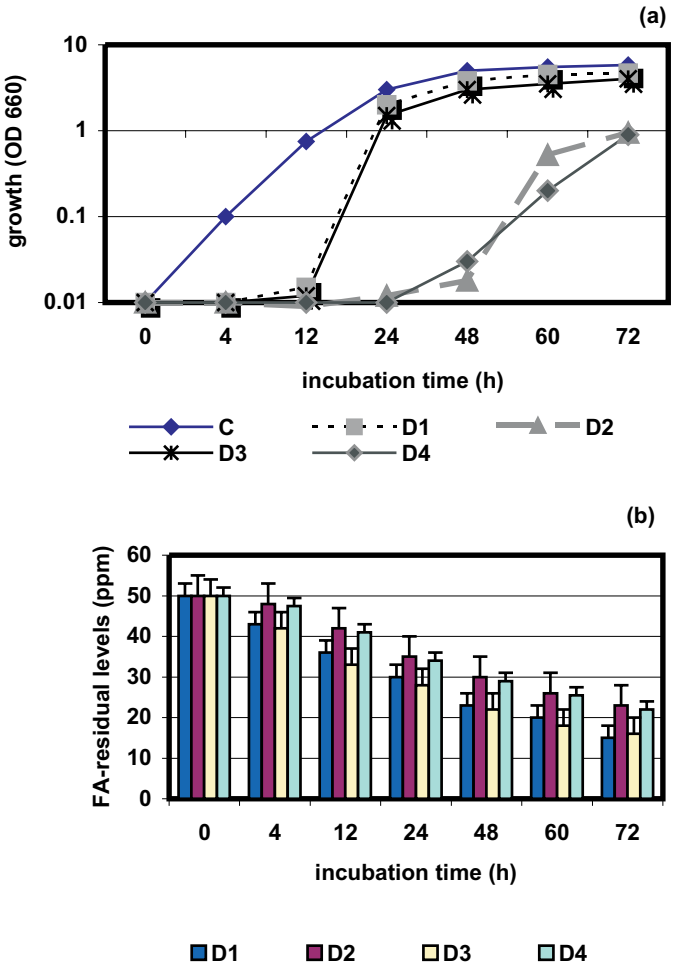

Figure 4. (a) Growth curves and (b) FA-residual levels for each *D. ingens* strains culture.

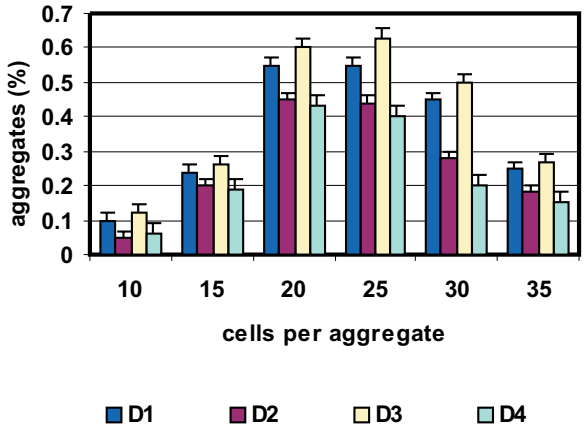

Figure 5. Aggregation profiles in the exponential phases of *D. ingens* strains in FA presence.

While a primitive pseudomycelium was confirmed in the controls, well-developed pseudohyphae appeared in FA-presence. D1 and D3 showed more elongated cells, whereas cylindrical and ellipsoidal ones dominated D2-D4 flasks.

Low cells per clumps were obtained in the control cultures, and a significant increase was observed in the FA-assays; greater aggregates developed in D1-D3 in respect to D2-D4 (Fig. 5). Thus, in accordance to dimorphism, aggregation patterns and life cycles, the biotypes reacted in different ways in the FA degradation assays. This study joined the strains in groups, particular traits were found for D1-D3 and D2-D4 biotypes and the variability was an adaptative response to the FA-presence and the pollution levels of the sampled sites, showing the former group higher frequency in the more contaminated channels.

Yeasts characterization isolated from contaminated sites is difficult since their morphological and physiological variability, and life phases were environmental controlled [24]. Reproducing forms, cell wall synthesis, assimilating functions and enzyme systems depended upon environmental and nutritional situations [13]. *Yarrowia lipolityca* switched from yeast-to-mycelium in hydrocarbon presence [28], ethanol effects were studied in *Candida tropicalis* [25], hyphal transition under adverse circumstances was reported in *Saccharomyces cerevisiae* [5] and *Candida albicans* [15, 22], and five *C. krusei* strains were isolated from petroleum polluted sediments [19].

In conclusion, the results pointed out that yeast cell and mycelial phases, and the morphological plasticity ensure a better competitiveness to substrate degradation. Yeast phase were dominated in fluid substrates whereas the mycelial forms colonized solid ones, being this trait an advantage to biotransform toxicants, as most of them remained as particles in the degradation experiments, due to their low water solubility.

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