

Fractal parameters of *Talaromyces wortmannii* as indexes of aromatic hydrocarbon pollution

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Fungal morphologies by fractal geometry were studied mainly for industrial applications, but the relation with environmental factors had been scarcely described. Fungi are conspicuous degraders, so, our aims were to isolate filamentous fungi from contaminated effluents, to apply fractal geometry to fungal growth in fluid medium and to evaluate fractal parameters as pollution indexes in aromatic hydrocarbons presence. *Talaromyces wortmannii* was selected due to its remarkable growth with hydrocarbons, byphenil and dibenzofuran. The clump diameters with naphthalene, phenanthrene and anthracene were 1.45-1.75 mm, while with pyrene, fluranthene and benzo[a]pyrene the main morphology was larger pellets. Fractals increased with the concentrations and aromatic-rings of the pollutants, with more pronounced effects on DMF than in DSF; both values were higher for condensed pellets and lower for free filamentous forms, as the mycelium become hydrophobic. In our case, hydrophobicity depended from the hydrocarbons, yielding larger aggregates. Fractals were an effective tool to test the pollutant effects on fungal growth. In mycoremediation technologies, the mycelial growth is a significant factor that affected most of the methodologies; therefore our results could be important to decide the strategies towards the contaminated effluent remediation.

Keywords: fractal geometry, *Talaromyces wortmannii*, aromatic hydrocarbons, pollution indexes, benzene ring numbers, pellet formation, mycoremediation strategies.

INTRODUCTION

Fractal geometry had contributed to understand a plethora of natural events. In biology, it was used to describe food structure (Barrett and Peleg, 1995), plant morphogenesis (Fleury, 1999), spinal cord cultures (Neale et al., 2003), pulmonary physiology (Suki, 2002) and neurosciences phenomena (Kiselev et al., 2003). The mycelial morphologies by fractal dimension (FD) were studied in fungi mainly for industrial applications, as citric acid production (Papagianni, 1999; Papagianni and Matthey, 2006), antibiotics (Vecht-Lifshitz et al., 1990), proteins (Wang and Ridgway, 2004), enzyme secretion (Papagianni et al., 2002; Papagianni and Moo, 2002) and metabolites formation (Papagianni, 2004).

Moreover, the relation between fractals and pellet

formation with pathogenicity and metabolites production had been documented in actinomycetes (Vecht-Lifshitz et al., 1990), imperfect filamentous fungi (Amanullah et al., 2000) and yeasts (Wenland, 2001). However, the fungal morphology in relation with environmental factors had been scarcely described (Romero et al., 2005; Romero et al., 2010).

In mycoly, fractals were applied to evaluate growth on soil substrates (Boddy et al., 1999; Pachepsky et al., 2000), but in liquid systems were only studied in relation to fermentations (Gibbs et al., 2000; Papagianni, 2004) although there are numerous fluid natural and man-made habitats where fungi had remarkable activity as biodegraders. Therefore, the aims of this study were to isolate filamentous fungi from contaminated effluents, to apply fractal geometry to fungal growth in fluid medium and to evaluate fungal fractal parameters as pollution indexes in the presence of polycyclic aromatic hydrocarbons (PAHs).

MATERIAL AND METHOD

Fungi isolation and assays.

Filamentous fungi isolates were cultured on mineral medium with aromatic hydrocarbons as sole C source, pH 6.8 and 18 g/l agar and incubated at 28°C for 7 days (Romero et al., 2009). Spores were collected from mature culture plates, and spore suspensions diluted with sterile medium were used to inoculate the bioreactors with 10⁵ spores/ml media. The filamentous fungi were identified by scanning electron microscopy (Romero et al., 2005). The assays were implemented with different concentrations, 0.010, 0.050, 0.075 and 0.100 mg/l of low-aromatic ring hydrocarbons, like naphtalene (naph), phenanthrene (phe), anthracene (an), and high-aromatic ring hydrocarbons, as pyrene (pyr), fluoranthene (fluo) and benzo[a]pyrene (bap). Aromatic-agar plates were used to evaluate fungal growth on solid surface, and the development in fluid media was assessed in bioreactors.

A stirred 5 l tank equipped with two 6-bladed rushton-type impellers (52 mm), operating at a stirrer speed of 400 rpm was used for the assays. Temperature was maintained at 28°C, air-flow rate was 1 vol/vol/min air/medium (vvm), pH was controlled by the addition of 2 M NaOH and 20% H₂SO₄ solutions, and polyethylene glycol (MW 2000, Sigma) was used as antifoam. Cultures were done by triplicate during 150 h incubation time, with 3 controls: 1st. inoculated but without aromatic substrates, 2nd. without inoculum but with aromatic substrates, 3rd. neither inoculum nor substrates. Dry weights were determined by filtering 20 ml culture through pre-weighed glass fiber filters (grade GF/C, 4.25 cm, Whatman International, UK), washing and drying in a microwave oven, 15 min at low power, and left in a dessicator for 24 hours before reweighing.

Image analysis and fractal dimension

Fungal morphology was studied by semi-automatic image analysis system, with an Olympus microscope (Olympus, NY, USA) operated as phase contrast with a CCD camera (Sony, Cambridge, UK), a PC with a Pentium processor, a frame-grabber and a monochrome image with 640-460 pixels and 255 gray level to obtain the digital forms. I-photo, Creative Art (USA) program was used for image capturing. Subsamples were diluted with mineral medium to acquire unentangled mycelium, measurements were obtained for 50-200 randomly selected images of individual hyphae and pellets. Pellets were placed on a colony counter to capture its images by CCD camera, in grey shades, and the average sizes were obtained by aimage analysis program (Advancce American Biotechnology, USA).

Fractals were evaluated by the box-counting method (Obert et al., 1990), and binary images were employed to

remove foreign particles and to correct optical errors (Paul and Thomas, 1998). Fungal filamentous growth was estimated by the relationship: $N(L) = a L^F$ (eq. 1), where: L was the side lenght grid, N the box numbers overlapped by the mycelium, a and F were proportionality constant and fractal value, respectively. The logarithmic form of eq. (1) was: $\log N(L) = F \log L + \log a$ (eq. 2), and for a fractal object like mycelial growth, the logarithmic relation of the overlapped boxes (N) was linear to $\log L$ of box with a F slope (Lejune and Baron, 1996). Fractal values (F) and slope were acquired by linear regression of the experimental results. Surface fractal dimension (DSF) and mass fractal dimension (DMF) were obtained by the relation and criteria proposed by Papagianni (2006). Thirty (30) subsamples were processed and the fractal average values were obtained with different aromatic pollutants as C-substrates. Only relations with determination coefficients (R^2) > 0.95, representing good linear correlations were used, the data with minor coefficients were rejected.

RESULTS

Filamentous fungi were isolated from chronical polluted sediments of an industrial area, La Plata, Argentina. *Talaromyces wortmannii* SBUG-M410 (Strain Collection of the Institute for Microbiology of Greiswald University, Germany) was selected for this assays due to its remarkable growth and higher rates in PAHs-plates, and confirmed as byphenil and dibenzofuran degrader in a previous research (Romero et al., 2005). The captured images showed fungal forms ranging from simple mycelial hyphae to clumps, and finally to condensed pellets; and the equation obtained with the experimental results were significant. The log-log relation of N versus L was linear within upper and lower limits (Figure 1). Six μ m (mean diameter) and 30 μ m was applied to the upper and lower limit, respectively. The mycelial forms was quantified by total mycelium lenght (Lt), average tip numbers (Nt) and average growth unit (Gu), being Gu estimated by: $Gu = Lt / Nt$.

The growth unit showed remarkable changes with each aromatic and the physical state of the substrato; even more, as the benzene-ring numbers increased, complex branched mycelia developed. Higher Gu values were observed when the aromatic concentrations decreased, being F inversely related to Gu and directly to the benzene ring numbers of the pollutants. Simple but longer filamentous were obtained with naph, phe and an, and incremented Gu c.a. 60 %. Branched morphologies of pellet-forming conditions developed with pyr, fluo and bap, with a Gu increase of 40 %, fluctuating between 0.18-0.30 mm. An inverse relation was obtained between benzene ring numbers and Lt, being the free hyphae slightly shorter in bap experiments, Lt fluctuated around

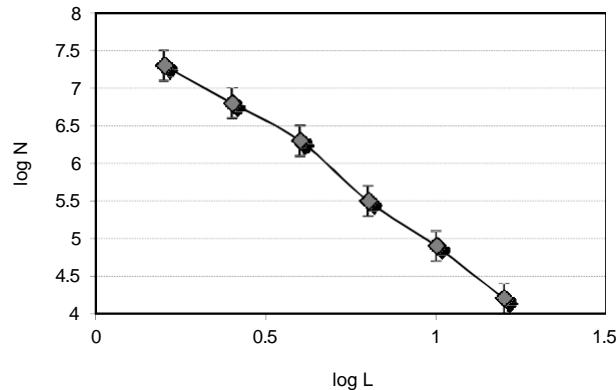


Figure 1. Relation between grid length (L) and grid numbers (N) overlapped by *T. wortmannii*.

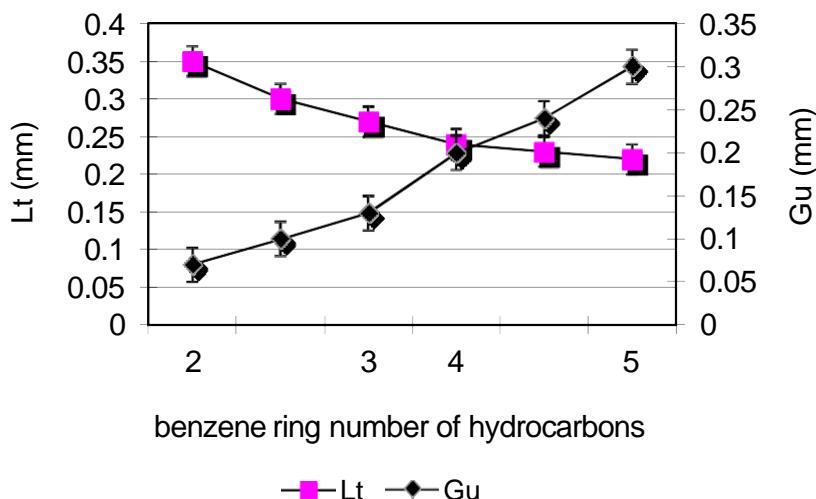


Figure 2. Effects of the hydrocarbons on the total mycelium lenght (Lt) and average growth unit (Gu).

0.22-0.25 mm in pyr, fluo and bap cultures (Figure 2).

The typical *T. wortmannii* morphologies under contaminated conditions were obtained in phenanthrene cultures at 65 h incubation time, and was clumps with significant tips numbers. Lt were less affected by the pollutants, however, a significantly decrease was observed with the increase of the benzene rings of the hydrocarbons.

Fractal nature of *T. wortmannii* mycelia was estimated by surface fractal dimension (DSF) and mass fractal dimension (DMF), for covered and enclosed area. DSF discriminated between samples which were fractal at their boundaries with entirely plane-filled interiors, DMF described cases where the interior filamentous had gaps. Both FD were appropriate descriptors of the fungal growth.

The compactness of the aggregates in relation with the number of benzene rings of the xenobiotics showed

significant results; in naph, phe and an assays the clump diameters were 1.45-1.75 mm, while in pyr, fluo and bap presence the main morphological forms were larger and condensed ones, 1.90-2.50 mm (Figure 3).

FD gave the distribution of the hyphal mass in the space, and in pellets the hyphae filled the center of the particles. The FD of the aggregates increased with the concentrations and aromatic rings of the pollutants.

The effects were more pronounced on DMF, that ranged from 1.15 to 2.30, while DSF values fluctuated around 1.10 to 1.40 (Figure 4). DMF and DSF showed a significant relation with the aggregate density, being higher for condensed pellets, lower for diffuse clumps and slightest for free filamentous forms, showing a significant reduce for dispersed morphologies.

This self-similarity held over a range of PAHs concentrations in the bioreactors, but broke down when the substratum changed, from fluid to solid ones. The FD

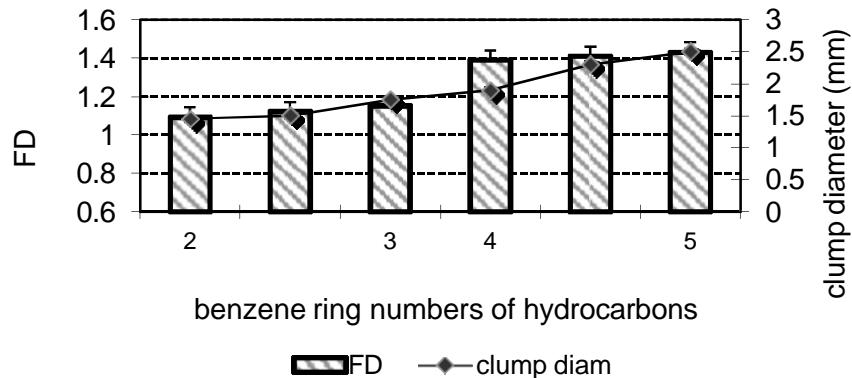


Figure 3. Fractal values and clump diameters in relation to the aromatic rings of the tested hydrocarbons.

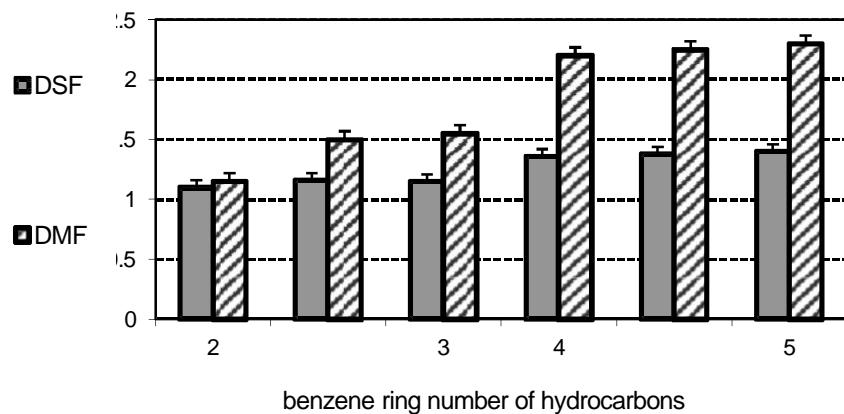


Figure 4. D_{MF} and D_{SF} data for *T. wortmannii* morphologies in relation to the benzene ring number of the hydrocarbons.

data obtained in the reactors and for solid conditions, on agar-plates, showed significantly different values; however, in cultures with a single hydrocarbon and with similar levels of others, FD did not change so much. It was a worthy features, as in natural and man-made habitats, the physical states of the wastes and effluents showed conspicuous changes along the times and space.

DISCUSSION

In the reactors, fluid cultures, the fungal forms varied between pellets and free filaments, and the compactness fluctuated among diffuse, fluffy, smooth and tight types depending on the hydrocarbon presence and levels. The mycelium less condensed grew mainly in 2 dimensions, whereas clumps developed in three-dimensions. Hyphae brought together by adhesive forces determined by surface properties, or repulsed each other producing free mycelia. Hydrophobic cells excluded water, and formed more branched filamentous, larger aggregates with

higher fractal values. Hydrophobicity was biologically regulated by different cultural and environmental factors, like oxygen, $^{\circ}T$, pH, carbon substrates and other components like xenobiotics influence.

The addition of high benzene ring hydrocarbons increased FD and pellet diameters; on the other hand, the cultures with low-aromatic ring hydrocarbons exhibited lower fungal fractal parameters, less numbers and pellet dimensions, as the mycelium become more hydrophobic. In our case, hydrophobicity depended from the aromatic hydrocarbons presence, and yielded larger and condensed aggregates.

The liquid media of the bioreactors was similar to sludges, fluid wastes and polluted waters, thus the FD could be used as indicators of sewage contamination levels. By the FD values obtained with *T. wortmannii*, we evaluated that fractal geometry was valuable to describe different growth in pollutant presence; moreover, FD values could be compared with other descriptors like classic morphological parameters. Since fractals gave a measure of the complexity degree and mass filling properties of an object, we confirmed that higher PAHs

concentrations contributed to the development of condensed clumps and could be used as pollution indexes.

In mycology, FD was used to describe colonies shapes in *Sordaria macrospora* and *Ashbya gossypii* (Obert et al., 1991), *Trichoderma viride* (Ritz and Crawford, 1990), *Pycnoporus cinnabarinus* (McLaren et al., 1996), *Aspergillus niger* (Papagianni, 2006) and *Cryphonectria parasitica* (Willyerd et al., 2009). Fractals had been mainly performed on solid surfaces to predict and enhance the yield of different by products (Huang et al., 2002).

From environmental point of view, fungal morphology had been controlled by physical, chemical and biological factors of the habitats (Klein and Paschke, 2004), and especially by pollutant presence (Pérez-Armendáriz et al., 2010; Matejczyk et al., 2011). The complexity and heterogeneity of industrial and urban effluents due to the contaminant levels, xenobiotic bioavailability and other environmental variables had not been easily measurable. This paper studied an innovative applications of FD to measure fungal parameters in relation to organic contaminants as indicators of polluted areas. The relation of heavy metals with the fractal geometry of *Ashlya bisexualis* was already assessed (Lundy et al., 2001), but the influence of organics by in FD of fungi had not been researched.

The adhesive properties of the cell wall induced pellet formation in *Streptomyces tendae* (Veich-Lifshitz et al., 1990), also different carbon sources affected *Aspergillus niger* morphology (Doohyun, 1999). The cell to cell interactions and mycelial growth were related to bioflocs release, so both features could be affected by aromatic compounds.

Filamentous fungi morphology in pollutants presence is difficult to assess by experimental data alone, due to the complexity of natural habitats, microscopic growth scale and complex mycelial developments (Singh, 2006; Graeme et al., 2007). Therefore fractal geometry provided us a powerful and efficient method to describe the morphological patterns under PAHs pollution.

In conclusions, fractal dimensions were an effective tool to test the pollutant effects on fungal growth. In mycoremediation technologies, the mycelial growth is a factor that significantly affected most of the proposed methodologies; so, our results could be important to decide the strategies towards the remediation of contaminated effluents.

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