CHEMICAL ENGINEERING TRANSACTIONS
VOL. 49, 2016
A publication of AIDIC
The Italian Association of Chemical Engineering
Online at www.aidic.it/cet
Guest Editors: Enrico Bardone, Marco Bravi, Tajalli Keshavarz

ISBN 978-88-95608-40-2; **ISSN** 2283-9216
DOI: 10.3303/CET1649082

Biodegradation of Polycyclic Aromatic Hydrocarbons by *Pleurotus sajor-caju*

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Polycyclic aromatic hydrocarbons (PAHs) are considered priority pollutants because they have potentially dangerous effects on the environment and human health. Bioremediation has recently become attractive to restore polluted sites, because it is not expensive and fulfils the most important properties required by the current regulations. In this work the capability of a white-rot fungi, *Pleurotus sajor-caju*, was assessed for degradation of pyrene and chrysene. The main objective was to investigate the effects of pollutant concentration on the mycelium growth and find the conditions that can enhance the microorganism tolerance when exposed to pyrene and chrysene. The tests carried out in Petri dishes showed that chrysene inhibited mycelium growth, whereas pyrene was well tolerated. Experiments in liquid medium evidenced that the mycelium was able to degrade pyrene with a removal efficienty greater than 90%.

1. Introduction

Bioremediation has attracted much interest in the last decades, because it offers an economic and environmentally sustainable solution for restoring pollutant sites (Stenuit et al., 2008; Llado et al., 2013; Spigno and Tronci, 2015). The main obstacle to such approach could be the recalcitrant characteristics of pollutants, as it happens when dealing with high molecular weight polycyclic aromatic hydrocarbons (PAHs). In this case it is important to find a microorganism able to degrade complex molecular structure. With this regard, white-rot fungi show in nature this ability. These organisms, in fact, are able to degrade several organic pollutants with structure similar to lignin thanks to the high non-specificity of their extracellular lignin-modifying enzymes (Llado et al., 2013).
The present study was focused on the biodegradation of PAHs using the white rot fungi *Pleurotus sajor-caju.* It belongs to the class of *basidiomycetes*, and it is equipped with extracellular oxidative enzymes such as laccase (benzenediol: oxygen oxidoreductase, EC 1.10.3.2, LC), manganese – dependent peroxidase (Mn2+: hydrogen peroxidase oxidoreductase, EC 1.11.1.13; MnP) and the hydrogen peroxide-producing enzyme aryl alcohol oxidase (aryl alcohol: oxygen oxidoreductase, EC 1.1.3.7; AAO) (Curreli et al., 2004). Such enzymes are used by the mycelium for oxidation and depolymerisation of lignin and several lignin-derived compounds (Leonowicz et al., 1999). *Pleurotus sajor-caju* showed to be able to grow and degrade cotton stalks (Kerem et al., 1992), milling wastewater (Sanjust et al., 1996), anthraquinones (Sollai et al., 1996). Its capability to degrade PAHs has been recenlty described (Ipeaiyeda et al., 2015), on the other hand the use of white-rot fungi as *Pleurotus ostreatus*, *Trametes versicolor*, *Phanerochaete chrysosporium* is well documented (Tortella et al., 2015; Cohen et al., 2002).
The present study aimed to investigate the ability of *Pleurotus sajor-caju* to tolerate and degrade pyrene and chrysene in solid and liquid medium. The effects surfactants (Tween 80) and possible promoters of the

Please cite this article as: Saiu G., Tronci S., Grosso M., Cadoni E., Curreli N., 2016, Biodegradation of polycyclic aromatic hydrocarbons by pleurotus sajor-caju, Chemical Engineering Transactions, 49, 487-492 DOI: 10.3303/CET1649082

biodegradation (copper sulphate, lecithin, peptone) were also evaluated in order to find the optimal conditions for mycelial growth.

2. Materials and methods

Pleurotus sajor-caju was used in mycelium form, starting from a voucher culture permanently preserved at 4 °C in the collection of the Biochemistry Unit of the department of 'Scienze Biomediche', University of Cagliari.
Agar, malt and yeast extract (microbiological grade), chrysene (purity 99.9%) and Tween 80 were obtained from Fluka, whereas pyrene (purity>99%), acetonitrile (HPLC grade), dichloromethane (pesticide grade), acetone (pesticide grade) and hexane (analysis grade) were furnished by Sigma Aldrich.

2.1 Fungal growth in Petri dishes

For the experiments on solid media campaign the strain was maintained in agar-malt-yeast (AMY) into 9 cm Petry dishes at 25 °C with an electronic incubator. Appropriate medium and PAHs (chrysene and pyrene) for each experimental run were used, accordingly with the experimental design explained in the following section. Six millimeters disk plugs of fresh mycelium were added in each plate under sterile condition. The impact of copper sulphate (Bettin et al., 2008), peptone (Hanson, 2008) and soy lecithin (Bustamante et al., 2011) was evaluated and, in order to enhance bioavailability, Tween 80 was added (Roch and Alexander, 1995).

2.2 Experiments in liquid medium

Biodegradation experiments in liquid medium were carried out in 500-ml Erlenmayer flasks containing malt (2%) – yeast extract (0.5%) based medium. The medium contained also copper sulphate (0.05 mM), Tween 80 and pyrene at two different concentrations, 5 and 10 ppm. As in the solid plates, 6 mm plugs of fresh mycelium were added under sterile condition to the medium. Cultures were incubated for 50 days in an orbital shaker at 25 ºC and 300 rpm. The working volume was 250 ml of medium. Samples were withdrawn at different times to monitor pyrene biodegradation. All experiments were carried out in duplicate and two more control runs, containing only malt and agar, were performed.

2.3 Analytical methods

Aliquots of 4 ml of the suspension containing broth and mycelium were taken over time and filtered through a 0.22 µm filter and then analyzed by high performance liquid chromatography. The EPA 8310 method was used with the following equipment: Agilent 1200 infinity LC system, column Zorbax Eclipse plus C18 (4.6•100mm, 3,5 µm particle size). Elution was performed using a two solvent gradient (A: water – B: acetonitrile): the elution started with 40% of B followed by a linear gradient to 95% of B. The injection volume was 5 µl, and detection was performed through DAD at 230, 240, 254, 270, 350 nm with 400 nm reference wavelength and spectrum acquisition.
From another aliquot of the sample, 1 ml of volume, the suspended mycelium was separated by centrifugation and then treated as a solid. The mycelium was extracted using an accelerated solvent extractor, Dionex ASE 300, with the following operating condition: T 100 °C, P 1500 psi, heat 5 min, static 5 min, flush 60%, purge 60 sec, cycles 1, solvent acetone-dichloromethane 1:1.

3. Results

3.1 Fungal growth in solid medium

The first step of this investigation was devoted to assess *Pleurotus sajor-caju* tolerance towards the selected four-ring PAH compounds, namely chrysene and pyrene. These chemical species were selected because they showed low bioavailability and recalcitrance. The behavior of *Pleurotus* strain was thus monitored by jointly varying 6 factors, which are the concentrations of the following compounds: pyrene (pyr), chrysene (cry), copper sulphate ($CuSO_4$), peptone (pep), Tween 80 (T80), soy lecithin (lec). Tween 80 was added to enhance bioavailability while copper sulphate, peptone and soy lecithin should improve fungal tolerance to polyaromatic hydrocarbons (Bettin et al., 2008; Hanson, 2008).
The effects of the factors reported above were analyzed by exploiting a two level fractional factorial design. In particular, 16 experimental runs were carried out as result of a two level, six factors, one-quarter fraction factorial design, with resolution IV, $2_{IV}^{(6-2)}$ (for further details see e.g. Montgomery, 2013). Every treatment was repeated three times and three more control runs (with only AMY) have been also performed, leading to a total of 51 experiments. The experimental conditions are reported in Table 1. Fungus growth was monitored taking digital photos in raw format at regular interval of 24 h using a high resolution digital camera (Canon EOS 50d –

15.1 mp sensor). Every photo was suitably cropped and processed to jpeg format. The images were then post-processed by exploiting the Adobe Camera Raw® and Adobe Photoshop® software. The area covered by the fungal population was calculated using an in-house written Matlab® code that binarizes the images and then computes the number of black pixels corresponding to the mycelium.

Table 1: Lower and higher values of each compound for the fractional factorial design

Factor/Level	Low	High	Units
Pyrene (pyr)	0	$5.0 \cdot 10^{-3}$	g/kg
Chrysene (cry)	0	$5.0 \cdot 10^{-3}$	g/kg
Copper sulphate ($CuSO_4$)	0	$12.5 \cdot 10^{-3}$	g/kg
Peptone (pep)	0	10.0	g/kg
Tween80 (T80)	2.5	10.0	g/kg
Lecithin (lec)	0	30.0	g/kg

The Gompertz model (Seber and Wild, 2003) was used to describe the fungal population growth and it is reported in Eq(1).

$$\frac{dA}{dt} = \mu A(logk - logA) \quad (1)$$

In Eq(1) *A* is the mycelium population, μ is the specific growth rate and k is the carrying capacity (i.e. the asymptotic value as t →∞). The model was developed by considering that the fungal area is a proper estimation of the mycelium population. Exploiting the experimental data obtained with the conditions in Table 1, model parameter estimation was performed for each experimental run by resorting to the nonlinear regression libraries provided with Matlab®. For sake of brevity, only the comparison between model prediction and experimental data for eight of the sixteen conditions considered with the factorial design are shown in Figure 1, in the understanding that similar prediction capabilities were obtained in the other cases.

Table 2: Analysis of Variance for Pleurotus growth. All the scalars in table are dimensionless.

Source	d.o.f.	SS	MS	F-ratio	p-value
Main Effects	6	439.474	73.246	11.3(	0.000
Pyrene	1	5.102	5.102	0.79	0.388
$CuSO_4$	1	70.514	70.514	10.87	**0.005**
Peptone	1	183.003	183.003	28.22	**0.000**
Tween80	1	52.208	52.208	8.05	**0.012**
Lecithin	1	1.613	1.613	0.25	0.625
Chrysene	1	127.034	127.034	19.59	**0.000**
2-Way Interactions	7	165.221	23.603	3.64	0.015
Pyrene·$CuSO_4$	1	0.033	0.033	0.01	0.944
Pyrene·Peptone	1	13.614	13.614	2.1(	0.167
Pyrene·Tween80	1	43.596	43.596	6.72	**0.020**
Pyrene·Lecithin	1	71.441	71.441	11.02	**0.004**
Pyrene·Chrysene	1	10.513	10.513	1.62	0.221
$CuSO_4$·Peptone	1	25.927	25.927	4.0(	0.063
$CuSO_4$·Tween80	1	0.097	0.097	0.01	0.904
3-Way Interactions	2	46.115	23.057	3.56	0.053
Pyrene·$CuSO_4$·Peptone	1	7.855	7.855	1.21	0.287
Pyrene·$CuSO_4$·Tween80	1	38.26	38.26	5.9(	**0.027**
Residual Error	16	103.748	6.484		
Pure Error	16	103.748	6.484		
Total	31	754.557			

The ANOVA test was used to analyze the effects of process conditions on the specific growth rate and evidenced that (Table 2): i) compounds with statistically significant effects are: peptone, copper sulphate, Tween 80 and chrysene and ii) interactions of pyrene with Tween 80 and lecithin are statistically significant.

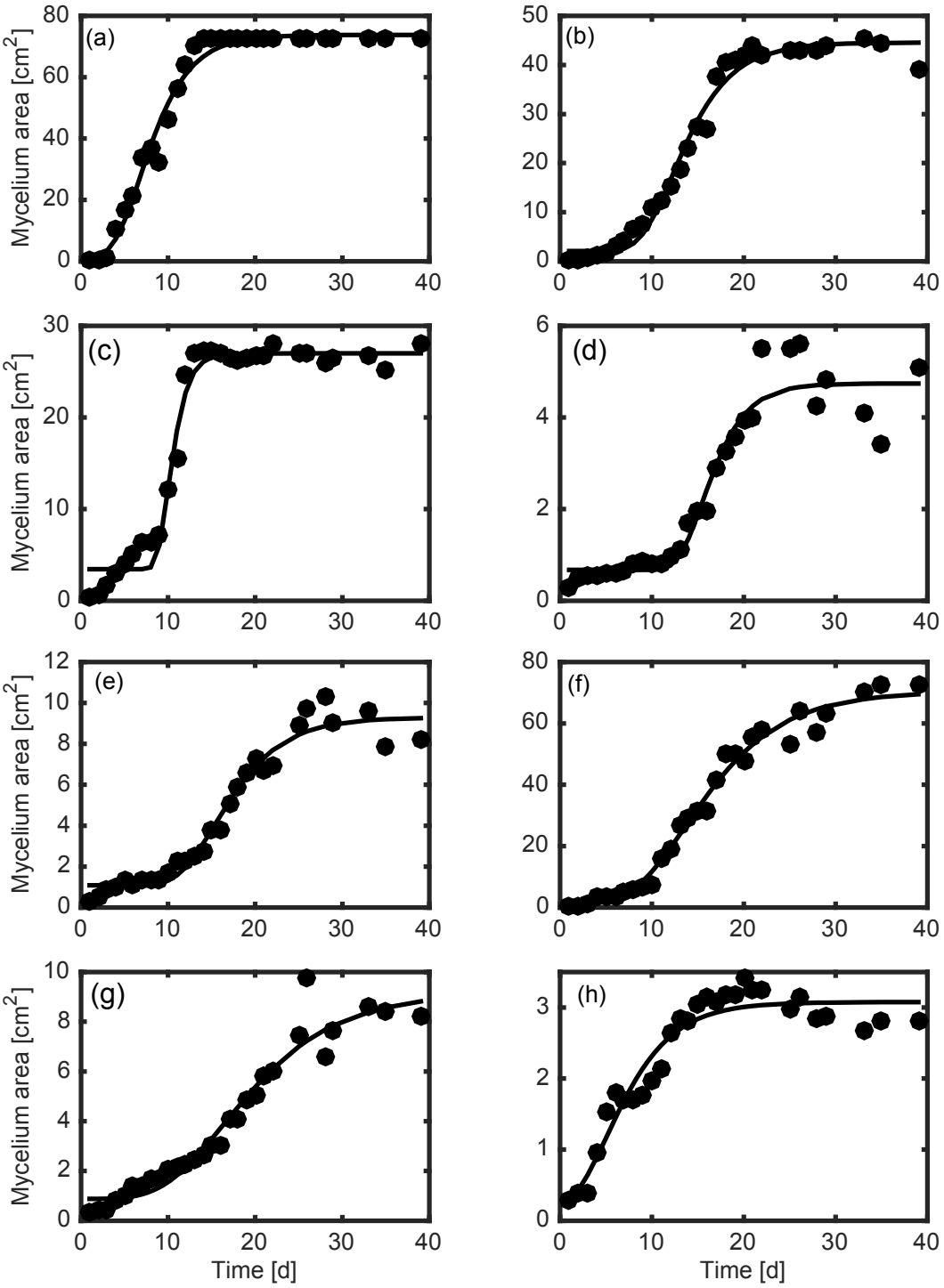

Figure 1: Comparison between experimental data (black circle) and model prediction (black line) for the control (a) and for the following conditions (concentration expressed in g/kg): (b) pyr =0, $CuSO_4$=0, pep=0, T80=10, lec=30, cry =5.0·10^{-3}; (c) pyr=0, $CuSO_4$ =12.5·10^{-3}, pep=10, T80=10, lec=30, cry=0; (d) pyr=0, $CuSO_4$=0, pep=10, T80=2.5, lec=30, cry=5.0·10^{-3}; (e) pyr=5.0·10^{-3}, $CuSO_4$=0, pep=0, lec=0, T80=2.5, cry=5.0·10^{-3}; (f) pyr=5.0·10^{-3}, $CuSO_4$=0, pep=0, T80=10, lec=30, cry=0; (g) pyr=5.0·10^{-3}, $CuSO_4$ =12.5·10^{-3}, pep=0, T80=2.5, lec=30, cry=5.0·10^{-3}; (h) pyr=5.0·10^{-3}, $CuSO_4$ =12.5·10^{-3}, pep=10, T80=10, lec=30, cry=5.0·10^{-3}.

Focusing on the specific growth rate to obtain information of the mycelium tolerance with respect to PAHs, the stepwise regression (Draper and Smith, 1998) was performed in order to evaluate which factors have a significant effect on the growth and quantify it. The obtained relationship is reported in Eq(2), where only the significant effects selected with ANOVA are taken into account.

$$\mu = 3.564 - 2.391x_{pep} - 1.992x_{cry} - 1.494x_{pyr}x_{lec} + 1.484x_{CuSO4} + 1.277x_{T80} + 1.167x_{pyr}x_{T80} + 1.093x_{pyr}x_{CuSO4}x_{T80} \quad (2)$$

In Eq. (2) x_i indicates the normalized concentration of the *i-th* compound. The determination coefficient R^2 was estimated, equal to 86.25 %, thus indicating a good agreement between data and model predictions. The stepwise regression evidenced that the presence of chrysene inhibited mycelium growth, therefore the degradation of such pollutant seemed quite difficult to occur, at least for the considered conditions. Conversely, fungal growth in presence of pyrene was not significantly reduced, meaning that it is well tolerated.

3.2 Pyrene biodegradation in liquid medium

The results obtained by exposing the mycelium to chrysene and pyrene in Petri dishes were used to assess the conditions for evaluating the behavior of *Pleurotus sajor-caju* in a liquid medium containing PAHs. Because the experimental data showed that pyrene was well tolerated by the mycelium, with greater population growth in presence of Tween 80 and copper sulphate, such compounds were added to the liquid medium. The following conditions were used: Tween 80 concentration equal to 7.5 g/l, copper sulphate concentration equal to 0.05 mM, pyrene concentration equal to 5 and 10 ppm.
Pyrene biodegradation profiles are reported in Figure 2 for the two pollutant loads investigated. Results shows that the four-ring compound is almost transformed within 20 days, and that, for the investigated range, the pyrene concentration does not significantly affect mycelium behavior.

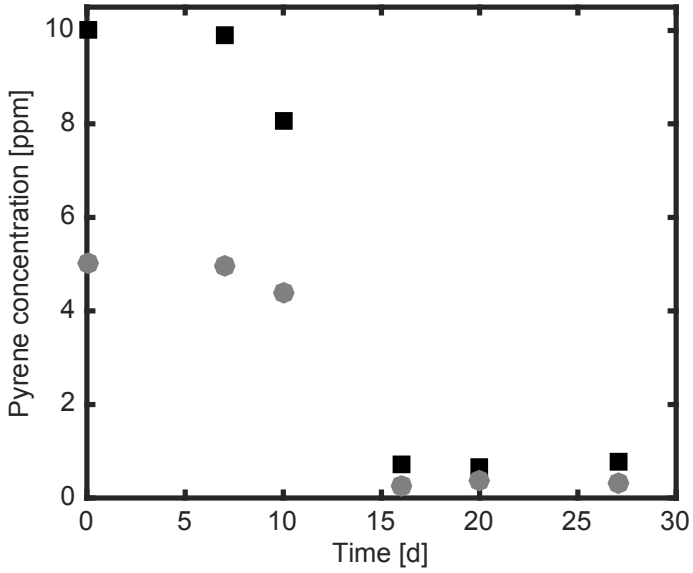

Figure 2: Pyrene biodegradation starting from a solution of 10 ppm (black square) and 5 ppm (grey circle).

4. Conclusions

This study investigated the use of *Pleurotus sajor-caju* to degrade two four-ring polycyclic aromatic hydrocarbons, pyrene and chrysene. First the mycelium tolerance was assessed by considering a solid medium and the experimental campaign was based on a $\mathbf{2_{IV}^{6-2}}$fractional design, aimed to evaluate the impact of different medium composition on *Pleurotus* growth when exposed to PAHs contaminants. As main result it was found that the mycelium could better tolerate pyrene than chrysene, furthermore Tween 80 and copper sulphate had a significant positive impact on the growth of fungal strains when exposed to pyrene. On the other hand, addition of lecithin showed a minor effect while peptone was detrimental for the process in the range here explored. According to the information gained with the first experimental runs, mycelium was used in a liquid medium containing pyrene, Tween 80 and copper sulphate. Pyrene biodegradation was obtained

starting from two different concentrations of the pollutant (5 and 10 ppm) and, in both cases, the compound was almost completely removed (removal efficiency >93%). It is important to underline that the absence of pyrene in the solid phase (mycelium) after 20 days was also assessed by GC-MS analysis, in order to be sure that the polycyclic hydrocarbon was definitely degraded by the organism.

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