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Detection of Parameters Enhancing the Performance of White-Rot Fungi for Degradation of Poly-Aromatic Hydrocarbons Through Design-of-Experiment Methodologies

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The aim of this work is to evaluate the tolerance and the growth capabilities of a white rot fungus, the *Pleurotus-Sajor Caju,* when exposed to Poly-Aromatic Hydrocarbons. The research was carried out by using *in vitro* systems developed on Petri dishes, where microbial strains are exposed to chemical pollutants, in particular pyrene and chrysene along with addition of surfactants, peptone, copper sulphate and lecithin that may promote fungal growth and tolerance. It was found that the fungal population growth is strongly inhibited by chrysene presence. On the other hand the pyrene has a mild negative impact on the micelyal growth, which seems to be positively influenced by the presence of Tween 80 and copper sulphate.

1. Introduction

The need for new regulations on environmental remediation of polluted sites has produced an increase of decontamination operations for soils, groundwater and sediments. The recovery processes are often directed to remove organic contaminants that are toxic and barely biodegradable. Among the several contaminants present in the environment, Poly-Aromatic Hydrocarbons (PAHs) are considered priority pollutants because they are one of the most ubiquitous class, and most of them are mutagenic and carcinogenic. Biological treatments applied to organic compounds removal showed to be attractive, mainly because they are not expensive and fulfil the most important properties required by the current regulations (Martins et al, 2012). The main obstacles for an efficient biodegradation of PAHs are their low bioavailability and recalcitrance (Leonardi et al., 2007). Among the different microbial consortia proposed in literature to be applied for bioremediation, white rot fungi have recently captured the interest of several researchers because of their ability to degrade an extremely diverse range of very persistent or toxic environmental pollutants. In particular, their degradation ability has been assessed under laboratory conditions for pesticides (Xiao et al., 2011), chlorophenols (Rubilar et al., 2011), synthetic dyes (Zhuo et al., 2011), drugs (Rodarte-Morales, 2012) and PAHs (Wen et al., 2011). The capacity for degrading such complex compounds depends on their ability to produce extracellular enzymes with low substrate specificity, such as lignin peroxidase, laccase, aryl-alchohol-oxidase and manganese peroxidase (Tortella et al., 2013).
In the present study, the ability of the white rot fungi *Pleurotus sajor-caju* to degrade PAHs has been investigated, focusing the research on its tolerance when exposed to pyrene and chrysene. The impact of different parameters affecting bioavailability (Tween 80) and promoting the fungal growth (lecithin, peptone and copper sulphate) have been also considered. The experimental campaign has been developed using a

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fractional factorial design which allowed a rigorous assessment of the effects of the different parameters here considered.

2. Materials and methods

2.1 Organism and culture conditions

Pleurotus sajor caju, a white-rot fungus growing spontaneously in eastern countries, was used in the described experimental campaign. The strain was maintained in malt-yeast-agar plates at 25 °C further mentioned as AMY; a voucher culture is permanently preserved at 4 °C in the collection of the Biochemistry Unit of the Department of 'Scienze Biomediche', Cittadella Universitaria di Monserrato' University of Cagliari. Disks 6 mm in diameter were transferred under sterile conditions in 9 cm Petri dishes containing the appropriate medium and PAHs (chrysene or pyrene); in this work, the impact of the following substances was investigated: copper sulphate (Bettin et al., 2008), peptone (Hanson, 2008) and soy lecithin (Pannu et al., 2004). Furthermore, in order to enhance the bioavailability of the contaminants, the addition of a surfactant (Tween 80) was considered (Roch and Alexander, 1995). The cultivations were maintained at 25 +/- 1 °C for more than 40 days. The temperature was controlled and monitored by using an electronically controlled incubator.

2.2 Data acquisition

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 are then post-processed by exploiting the Adobe Camera Raw® and Adobe Photoshop® software. The areas covered by the fungal population were calculated using an in-house written Matlab® code that binarizes the images and then computes the number of black pixels corresponding to the mycelium. Figure 1 reports an example of raw picture collected during the experiments and the related processed image.

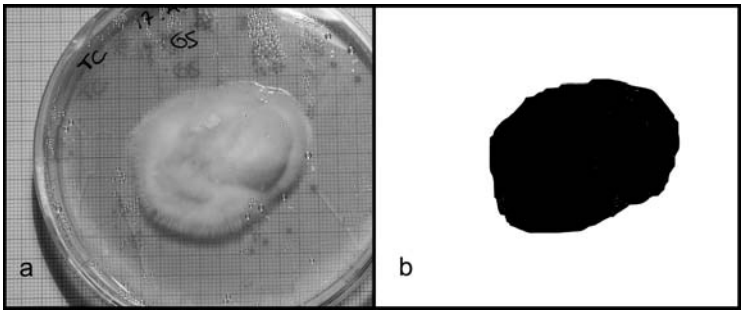

Figure 1: Pleurotus sajor-caju strains in a Petri dish; (a) original picture and (b) corresponding processed binary image containing only the mycelium area in black

2.3 Experimental design

The behaviour of the mycelia strains was thus monitored by jointly varying 6 factors, which are the concentrations of: (a) pyrene; (b) copper sulphate ($CuSO_4$); (c) peptone; (d) Tween 80; (e) soy lecithin; (f) chrysene.

Table 1: Levels at low (-1) and high (1) concentration.

Factor/Level	Low	High	Units
A=Pyrene	0	5	mg/kg
B=$CuSO_4$	0	0.05	mM
C=Peptone	0	1	%
D=Tween 80	0.25	1	%
E=Lecithin	0	3	%
F=Chrysene	0	5	mg/kg

A two level fractional factorial design of the experiments was adopted in order to obtain a good compromise between number of experiments and effective reliable information. In particular, a two level, six factors, one-quarter fraction factorial design, with resolution IV (2_{IV}^{6-2}) was used (for further details see e.g. Montgomery, 2013), leading to 16 different experimental conditions describing the influence of 6 parameters. 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 concentration levels used for each compound in the experiments are shown in Table 1.
Table 2 summarizes the generators of the fractional design, along with all the single effects and the second order combined effects that can be resolved and not confounded with the (2_{IV}^{6-2}) fractional design (for further details on the methodology see Montgomery, 2013).

Table 2: Generators, defining relations and alias structure, up to third degree interactions

	Terms		
Generators	E = BCD	F = ACD	
Defining relation	I = BCDE	I= ACDF	I= ABEF
Alias structure	A + BEF + CDF	B + AEF + CDE	C + ADF + BDE
	D + ACF + BCE	D + ACF + BCE	F + ABE + ACD
	AD + CF	AE + BF	AF + BE + CD
	BC + DE	BD + CE	

2.4 Growth model

Above all, two mathematical models were taken into account in order to describe the fungal population growth (Seber et al, 2003): the logistic and the Gompertz model. It was found that the Gompertz model (Cogoni et al, 2012), introduced in Equation 1, was the most suited to describe the growth dynamics.

$$\frac{dA}{dt} = \mu \cdot A(\log k - \log A). \quad (1)$$

In Equations (1), *A* is the mycelial population, μ is the specific growth rate and *k* is the carrying capacity (i.e. the asymptotic value as $t \to \infty$). The model was compared with the experimental data provided by the protocol introduced in Section 2.2, assuming that the measured area is a reasonable estimation of the *fungal population*. The model parameter estimation was performed for each experimental run by resorting to the nonlinear regression libraries provided with Matlab®. To evaluate the model performance the Mean Square Error Eq.(2) is considered.

$$MSE = \frac{1}{n}\sum_1^n \left(A_i - \hat{A}_i\right)^2 \quad (2)$$

where *n* is the number of experimental data collected at each experimental condition, A_i is the i-th experimental point and $\hat{A}_i$ is the model predicted value at the i-th point. The *MSE* scalar is the maximum likelihood estimation of the experimental variance, therefore the quality of fit increases as this scalar tends to zero. For the objective of the present work, the interest was mainly focused on the evaluation of the specific growth rate parameter μ.

3. Results

The Gompertz model provided a good description of the fungal population growth, with an average value for the MSE scalar equal to 0.863, with values ranging from MSE_{min}=0.032 to MSE_{max}=14.60. Figure 2 shows some representative comparisons between experimental data and model prediction for different experimental conditions: white circles refer to experimental points for the control condition (AMY, absence of the other compounds) compared with the model predictions (solid line); gray squares refer to experimental data collected at Tween 80 =1 %, pyrene=5 mg/kg, $CuSO_4$=0.05 mM, absence of peptone, lecithin and chrysene, compared with the model (dashed line); gray triangles refer to data collected at Tween 80 =1 %, lecithin=3 %, pyrene=5 mg/kg, absence of peptone, lecithin and $CuSO_4$, compared with the model (dashed line).
The point estimation of the μ scalar in Eq. (1) can be regarded as a representative index of the fungal population growth, therefore a statistical analysis on the μ values estimated for the different experimental runs

can give information on the effects of process conditions on the biological system. This task was accomplished by means of Minitab Statistical Software, used to calculate the main statistical parameters of the fitted models. An ANOVA test was performed and the related results are reported on Table 2. In more detail, for each source of variation (reported on the column 1), the table shows the corresponding degrees of freedom d.o.f. (column 2), the sum of squares of the source variation (column 3), the adjusted Mean Square Error (column 4), the F-ratio statistics (column 5) and the p-value associated to that source of variation (column 6). The effect contribution is considered significant when $p< 0.05$. The significant factors (i.e. with a p-value <0.05) are highlighted with the asterisk. The main outcomes of the analysis are listed below:

1. the compounds with statistically significant effects are: (i) peptone, (ii) $CuSO_4$, (iii) Tween 80 and (iv) chrysene concentrations.
2. interactions of pyrene with Tween 80 and lecithin are statistically significant.

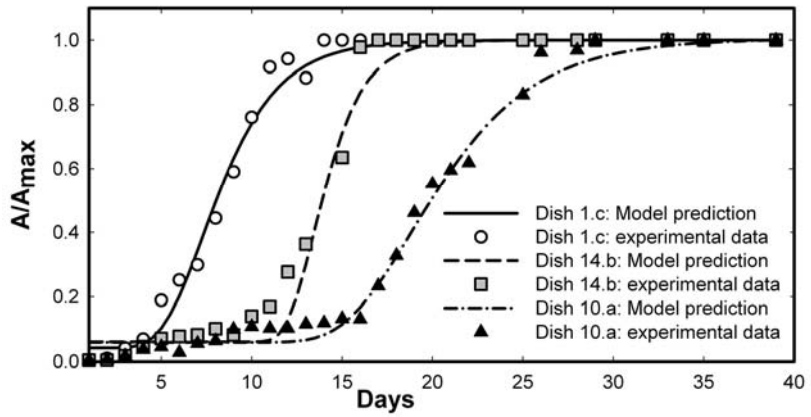

Figure 2: Normalized experimental growth curves compared with the model prediction.

Table 2: Analysis of Variance for the fungus 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	11.30	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**
Tween 80(*)	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	2.10	0.167
Pyrene·Tween 80(*)	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	4.00	0.063
$CuSO_4$·Tween 80	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·$CuSO_4$·Tween 80(*)	1	38.26	38.26	5.90	**0.027**
Residual Error	16	103.748	6.484		
Pure Error	16	103.748	6.484		
Total	31	754.557			

The dependence of the parameter μ on the process conditions can be described by the relationship, reported in Eq. (3), where only the significant effects selected with the ANOVA are taken into account. Eq. (3) can be used to optimize or estimate the mycelium growth rate, and it is written with the terms in order of significance.

$$\mu = 3.564 - 2.391 \cdot C - 1.992 \cdot F - 1.494 \cdot AE + 1.484 \cdot B + 1.277 \cdot D + 1.167 \cdot AD + 1.093 \cdot ABD \quad (3)$$

It can be easily seen that peptone (C) and chrysene (F) strongly reduce the mycelium growth rate, whereas copper (B) and Tween 80 (D) enhanced the response. Also two (AD and AE) and three factor (ABD) interactions significantly contribute to the mycelium growth as reported on Eq. (3), written in coded units. To measure how much variability is observed in the response values, the determination coefficient R^2 has been estimated, obtaining 86.25%.

The results are also confirmed by the Pareto chart calculated using the Yates algorithm (Morgan, 1991) and reported in Figure 3.a. For sake of clarity, also the significance threshold value (with a significance level α=0.05) is reported with the solid line. It was confirmed that the response of peptone, chrysene, copper, Tween 80 and the interaction between pyrene and Tween 80 are the main significant effects. On the other hand pyrene and lecithin do not affect significantly the system, meaning that the former seems well tolerated by *Pleur. Sajor-caju* strains, while the latter does not enhance fungal growth. On the other hand the presence of pyrene combined with Tween 80 has an impact of the fungus growth, at least for the concentration ranges here investigated. For sake of completeness, the main effects are shown in Figure 3.b, where significant effects are reported with solid lines, while the effects classified as not significant are reported with dashed lines. It was confirmed that: (i) copper and Tween 80 positively affect the system response; (ii) peptone and chrysene strongly inhibit the fungus growth and (iii), pyrene (slight negative impact) and lecithin (slight positive impact) are not statistically significant.

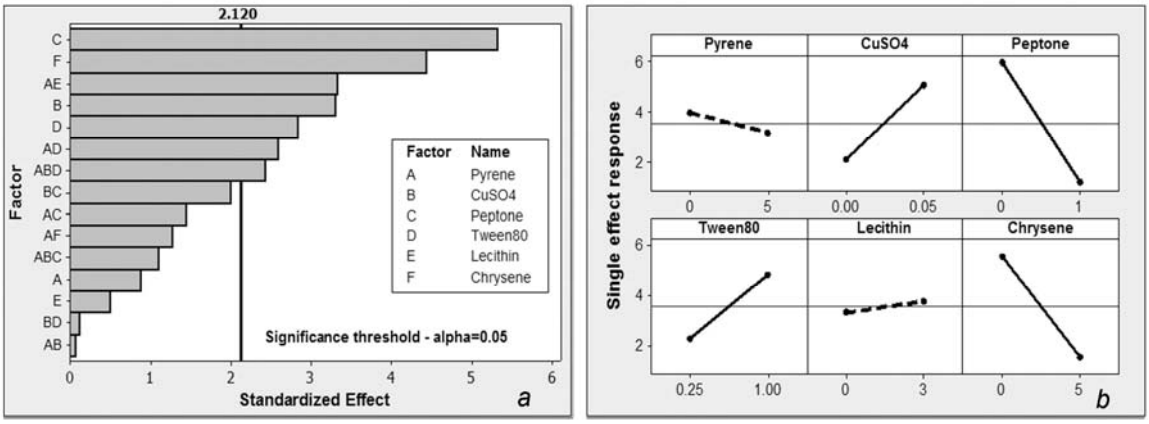

Figure 3: a) Pareto chart of standardized effects for the μ parameter; b) Main effect behaviour.

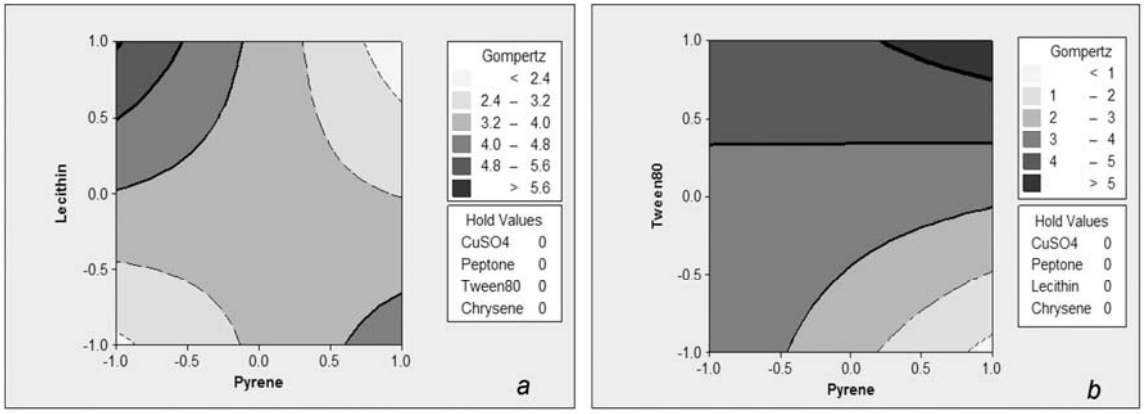

Figure 4: Contour plot for second order interaction: a) pyrene-lecithin, b) pyrene-Tween 80.

The significant interactions between the factors are shown in Figure 4, that indicates that lecithin does not favor mycelia growth when medium is contaminated by pyrene, whereas Tween 80 does. In particular, Figure 4a shows that high pyrene concentrations lead to higher growth rate when lecithin is absent in the medium, while its addition decreases growth rate. Figure 4b indicates that addition of Tween 80 favors fungal growth rate leading to the highest values of μ when both compounds are at the highest concentration levels.

4. Conclusions

An experimental campaign based on a (2_{IV}^{6-2}) fractional factorial design was carried out to analyze the impact of different medium composition on the *Pleurotus sajur-caju* growth when exposed to PAHs contaminants. As main result it was found that the mycelia can better tolerate pyrene than chrysene, furthermore Tween 80 and copper have 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. The statistical analysis also indicated the occurrence of interactions among the factors. In particular the influence of lecithin and Tween 80 on the process changes with the level of pyrene concentration in the medium. For the current investigation the impact of the additives to the medium does not seem to influence the ability of the mycelium to tolerate chrysene which had always a negative effect on the population growth rate.

References

Bettin F., Osorio da Rosa L., Montanari Q., Calloni R., Aparecida Gaio T., Malvessi E., Moura da Silveira M., Pinheiro Dillon A. J., 2011, Growth kinetics, production, and characterization of extracellular laccases from *Pleurotus sajor-caju* PS-2001, Process Biochemistry, 46,758–764.

Cogoni G., Grosso M., Baratti R., Romagnoli J.A., 2012, Time evolution of the PSD in crystallization operations: An analytical solution based on Ornstein-Uhlenbeck process. AIChE J., 58, 3731-3739.

Hanson, J.R., 2008, The Chemistry of Fungi, Royal Society of Chemistry, Cambridge, UK.

Leonardi V., Šašek V., Petruccioli M., D'Annibale A., Erbanová P., Cajthaml T., 2007, Bioavailability modification and fungal biodegradation of PAHs in aged industrial soils. International Biodeterioration & Biodegradation, 60, 165-170.

Martins F.F., Ferreira T.F., Azevedo D.A., Coelho M.A.Z., 2012, Evaluation of Crude Oil Degradation by Yarrowia lipolytica, Chemical Engineering Transactions, 27, 223-228.

Montgomery, D. C. 2013, Design and analysis of experiments, Hoboken, NJ: John Wiley & Sons, Inc.

Morgan E., 1991,Chemometrics, Open Learning in Experimental Design, Wiley, New York, USA.

Pannu J.K., Singh A., Ward O.P., 2004, Vegetable oil as a contaminated remediation amendment: application of peanut oil for extraction of polycyclic aromatic hydrocarbons. Process Biochemistry, 39, 1211-1216.

Roch F., Alexander M., 1995, Biodegradation of hydrophobic compounds in the presence of surfactants. Environmental Toxicology and Chemistry, 14, 1151-1158.

Rodarte-Morales A.I., Fejoo G. Moreira M.T., Lema, J.M., 2011, Degradation of selected pharmaceutical and personal care products (PPCPs) by white rot fungi. World J. Microbiol. Biotecnol., 27, 1839-1846.

Rubilar O., Tortella G., Cea M., Acevedo F., Bustamante M., Gianfreda L., Diez M. C., 2011, Bioremediation of a Chilean Andisol contaminated with pentachlorophenol (PCP) by solid substrate cultures of white-rot fungi. Biodegradation, 22, 31-41.

Seber G.A.F., Wild C.J., 2003, Nonlinear regression. Hoboken, NJ: John Wiley & Sons, Inc. Chapter 7.

Tortella G., Durán N., Rubilar O., Parada M., Diez M.C., 2013, Are white-rot fungi a real biotechnological option for the improvement of environmental health? Critical Reviews in Biotechnology. doi:10.3109/07388551.2013.823597.

Wen J., Zhang B., Liang H., 2011, Co-metabolic degradation of pyrene by indigenous white-rot fungus Pseudotrametes gibbosa from the northeast of China original. Itern. Biodeter. Biodegrad., 65, 600-604.

Xiao P., Moria T., Kameic I., Kiyotad H., Takagie K., Kondoa R., 2011, Novel metabolic pathways of organochlorine pesticides dieldrin and aldrin by the white rot fungi of the genus Phlebia. Chemosphere, 85, 218-224.

Zhuo R., Ma L., Fan F., Gong Y., Wan X., Jiang M., Zhang X., Yang Y., 2011, Decolorization of different dyes by a newly isolated white-rot fungi strain Ganoderma sp.En3 and cloning and functional analysis of its laccase gene. Journal of Hazardous Materials, 192, 855-873.