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Modelling and Optimization of Poly-Aromatic-Hydrocarbons Biodegradation by Bulab 5738

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In this work, the bacterial consortium Bulab 5738 was used to simultaneously remove pyrene, phenanthrene and catechol from aqueous solutions. The bacterial population growth was estimated by means of optical density measurements while HPLC was used to quantify the pollutant concentration in the solution. The obtained data were used to model the systems, in term of biomass population and substrate concentration. The effects of pollutant concentration values were analysed using the outputs of a full factorial experimental design.

1. Introduction

Polycyclic aromatic hydrocarbons (PAH) are highly toxic pollutants, with potential harmful effects on human health and environment, and difficult to remove by traditional treatments (Carta and Desogus, 2013). Bioremediation represents an environmentally sustainable method for treating polluted sites, although its application is still constrained by factors such as the unpredictable endpoint PAH concentrations and the lack of adequate monitoring tools to ensure active biodegradation processes (Vila et al., 2015). Bacteria and fungi showed to be able to efficiently remove PAHs, as reported in different studies on bioremediation (e.g., Palanisamy et al., 2014; Khan et al., 2013; Lu et al., 2011; Tronci and Spigno, 2015; Lee et al., 2015; Saiu et al., 2015, 2016), but their ability is negatively affected as the number of aromatic rings increases or when different organic compounds are present in the polluted soil. Recent studies showed that bacteria consortia may improve PAH degradation, because the presence of different microorganisms may result in enhanced PAH utilization, since metabolic intermediates produced by some organisms may work as substrates for the others (Mrozik, 2003; Zhong et al., 2011; Janbandhu and Fulekar, 2011).
This study is focused on the evaluation of the bioremediation capability of the bacterial consortium provided by Buckman Laboratories International and named Bulab 5738. In more detail, the goal is to understand the consortium behaviour when the only source of carbon is represented by pyrene, phenanthrene and catechol. The presence of a surfactant (Tween 80) has been also considered for determining the process conditions leading to the best results in term of degradation rate and pollutant removal. The experimental conditions were selected using a full factorial experimental design, considering two levels for the compounds concentration values. The obtained experimental results were processed by statistical tools to rigorously reveal the impact of the pollutants on the bacterial population growth and on their removal efficiency.

2. Methods

2.1 Chemicals and culture medium

Phenanthrene, pyrene and catechol (all >99% purity) used in degradation experiments were purchased from Sigma Aldrich. Tween 80, a non ionic surfactant, was supplied by Fluka. The mineral medium contains the

following salts: NaCl, $MgSO_4$, $(NH_4)_2SO_4$, $FeCl_3$, $CaSO_4$, Na_2HPO_4, KH_2PO_4. All the salts were added in large excess to supply the needs of nutrients for the microorganisms growth and to be a not limiting factor for it.

2.2 Microorganisms

The microorganisms used in this study were the mixed bacterial culture Bulab 5738 containing many different species of bacterial strains including *Rhodococcus*, *Aeromonas*, *Streptomyces*, *Bacillus*, *Pseudomonas*. The mixed bacterial culture appears as a light brown granular powder containing lyophilized microorganisms on a substrate of cereal bran and freeze dried enzymes.

2.3 Analytical methods

The bacterial growth was determined by UV–visible spectrophotometer (JENWAY 7300) at 600 nm by measuring the absorbance of the cell suspensions, as already done by Carta and Desogus (2010) and Carta et al. (2006).
Samples of the solution containing the pollutant were taken at the beginning and at the end of the bioremediation experiments in order to assess whether the chemicals had actually been degraded or not. Aqueous pollutants were quantified using high-performance liquid cromatography. The samples were only filtered using 0.20 µm filter before the analysis. 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 solvents 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.

3. Experimental design

The capability of the bacterial consortium to degrade the pollutants was monitored by jointly varying the concentrations of pyrene (PYR), phenanthrene (PHE) and catechol (CAT), which range from 5 to 15 mg/l. Each experimental run was replicated twice, leading to 16 experiments carried on in 250 *ml* Erlenmeyer flasks. The factorial design 2^3 is reported in Table 1.

Table 1: Factorial design

Run	PYR (mg/l)	PHE (mg/l)	CAT (mg/l)
1	5	5	5
2	15	5	5
3	5	15	5
4	15	15	5
5	5	5	15
6	15	5	15
7	5	15	15
8	15	15	15

Two different models were considered to describe the growth of the bacterial population in terms of absorbance (A) measured at different sampling time: i) a Gompertz growth model reported in Eq. 1 and ii) a zero-order kinetic model reported in Eq. 2

$$\frac{dA}{dt} = \mu_1 A(logA_\infty - logA)$$
$$A(t_0) = A_0 \quad t_0 \leq \lambda \tag{1}$$

$$\frac{dA}{dt} = \mu_2 \tag{2}$$

In Eq. 1, the parameter λ is the lag time, which is the interval of time where the growth rate is almost null. The statistical analysis was then performed by using the estimated parameters μ_1 and μ_2 as outputs of the experiments.

4. Results

The absorbance values registered at the different sampling time are reported in the left panel of Figure 1, in the understanding that they represent an indirect measurement of the microbial population. For sake of clarity, only one replicate is shown.

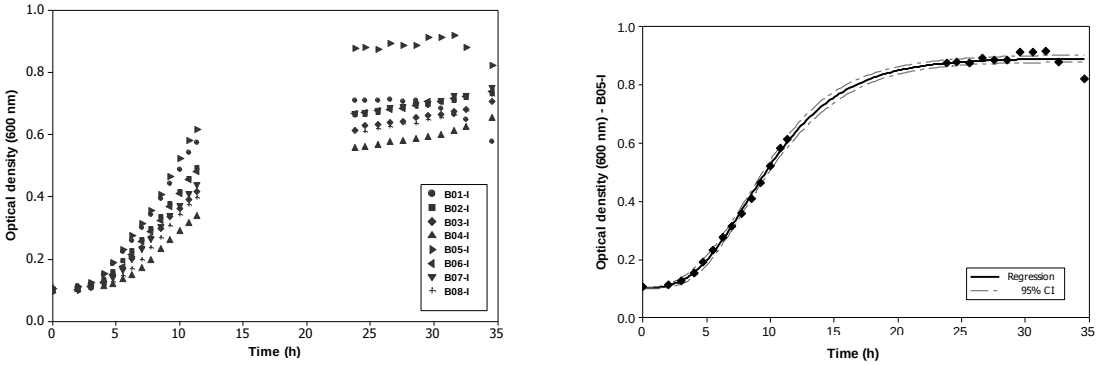

Figure 1: Absorbance values of the samples collected during the experimental runs in Table 1 (left panel) and comparison between model estimation (continuous line) and experimental data B05-I (blach diamond) along with the confidence interval (right panel).

It is r evident a diauxic growth behaviour that can be explained by considering the kind of molecules present in the solution. First, bacteria are reasonably able to degrade the simplest compound, which is catechol. Then, they can metabolize phenanthrene and pyrene, which are the most refractory compounds in the solution.
The Gompertz model (Eq. 1) showed to quantitatively describe the first growth step, with an average value for the Mean Square Error (MSE) scalar equal to $1.71 \cdot 10^{-5}$, with values ranging from $MSE_{min}=2.64 \cdot 10^{-6}$ to $MSE_{max}=3.91 \cdot 10^{-5}$, whereas the zero-order kinetics (Eq. 2) demonstrated to be more adequate for fitting the final experimental data (average value for the MSE scalar equal to $7.87 \cdot 10^{-3}$, with values ranging from $MSE_{min}=1.62 \cdot 10^{-4}$ to $MSE_{max}=1.61 \cdot 10^{-2}$). The parameter estimation was carried out by means of the Least Squares Method (Levenberg-Marquardt algorithm) and the obtained values are reported in Table 2. As illustrative example, the comparison between the model estimation and the experimental data for the run B05-I is shown in the right panel of Figure 1.

Table 2: Parameters estimated for the Gompertz model (λ and μ_2) and for the zero-order kinetics (μ_2)

Run	λ [h]	μ_1 [h^{-1}]	μ_2 [h^{-1}]
B01-I	3.93775	0.07294	0.00000
B02-I	4.01294	0.06252	0.00639
B03-I	4.16928	0.05638	0.00758
B04-I	5.34434	0.05069	0.00839
B05-I	4.11486	0.07925	0.00000
B06-I	4.37582	0.06498	0.00675
B07-I	4.39583	0.05902	0.00754
B08-I	5.28789	0.06260	0.00927
B01-II	3.88736	0.07385	0.00000
B02-II	3.97566	0.06245	0.00652
B03-II	4.30350	0.05752	0.00692
B04-II	5.30830	0.05245	0.00795
B05-II	4.03412	0.07863	0.00345
B06-II	4.49460	0.08017	0.01167
B07-II	4.40283	0.06106	0.00815
B08-II	5.22947	0.06108	0.00825

A statistical analysis on μ_1 and μ_2 estimated for the different experimental runs can give information on the effects of the process conditions on the biological system. An ANOVA test was performed (Montgomery, 2013) and the related results are reported on Table 3 and Table 4 for the parameters μ_1 and μ_2, respectively.

In more detail, for each source of variation (reported on the column 1), the table shows the corresponding degrees of freedom (DoF), the sum of squares (SS) of the source of variation, the adjusted Mean Square (MS) error, the F-ratio statistics and the p-value associated to that source of variation. The effect contribution is considered significant when p< 0.05. The significant factors (i.e. with a p-value <0.05) are put in bold type. The main outcomes of the analysis are listed below:

i. all the compounds are statistically significant for μ_1 whereas the higher order interactions are not;
ii. pyrene and phenanthrene have an impact on μ_2 along with their interaction. The impact of catechol is negligible, thus confirming that it has mainly consumed during the first phase of the experiments.

Table 3. Analysis of variance for the parameter μ_1

Source	DoF	SS	MS	F-ratio	p-value
PYR	1	0.0001087	0.0001087	7.16	**0.028**
PHE	1	0.0008121	0.0008121	53.51	**0.000**
CAT	1	0.0002101	0.0002101	13.85	**0.006**
PYR-PHE	1	0.0000468	0.0000468	3.09	0.117
PYR-CAT	1	0.0000343	0.0000343	2.26	0.171
PHE-CAT	1	0.0000013	0.0000013	0.09	0.778
PYR-PHE-CAT	1	0.0000017	0.0000017	0.11	0.744
Residual error	8	0.0001214	0.0000152		
Pure error	8	0.0001214	0.0000152		
Total	15	0.0013367			

Table 4. Analysis of variance for the parameter μ_2

Source	DoF	SS	MS	F-ratio	p-value
PYR	1	0.0000622	0.0000622	26.09	**0.001**
PHE	1	0.0000535	0.0000535	22.46	**0.001**
CAT	1	0.0000080	0.0000080	3.36	0.104
PYR-PHE	1	0.0000367	0.0000367	15.38	**0.004**
PYR-CAT	1	0.0000003	0.0000003	0.11	0.749
PHE-CAT	1	0.0000027	0.0000027	1.14	0.318
PYR-PHE-CAT	1	0.0000003	0.0000003	0.11	0.748
Residual error	8	0.0000191	0.0000024		
Pure error	8	0.0000191	0.0000024		
Total	15	0.0001826			

The dependence of the parameters μ_1 and μ_2 on the process conditions can be described by the relationships reported in Eq. (3) and (4), respectively, where only the significant effects selected by the ANOVA are taken into account.

$$\mu_1 = 0.06472 - 0.002607 \cdot c^{PYR} - 0.007124 \cdot c^{PHE} + 0.003624 \cdot c^{CAT} \quad (3)$$

$$\mu_2 = 0.0062 + 0.0020 \cdot c^{PYR} + 0.0018 \cdot c^{PHE} - 0.0015 \cdot c^{PHE} \cdot c^{PYR} \quad (4)$$

In Eqs. (3) and (4), c^i represents the initial concentration of the compound. It can be easily seen that pyrene and phenanthrene strongly reduce the population growth rate μ_1, whereas catechol has a positive effect. On the other hand, during the second phase represented by the parameter μ_2, the two PAHs increases the population growth rate, with a synergic effect shown by the positive sign of the coefficient related to the interaction term. This behavior indicates that bacteria are able to adapt to pyrene and phenanthrene, which initially had an inhibitory effect on the population growth, and they become able to use PAH as carbon source.
The results are also confirmed by the Pareto chart calculated using the Yates algorithm (Morgan, 1991) and reported in Figure 2.a and 3.a for μ_1 and μ_2, respectively. 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 all the pollutants affect the first phase of bacteria population growth, whereas the second phase depends on the concentration of PAHs and on their interaction. The main effects are also shown in Figure 2.b and 3.b.

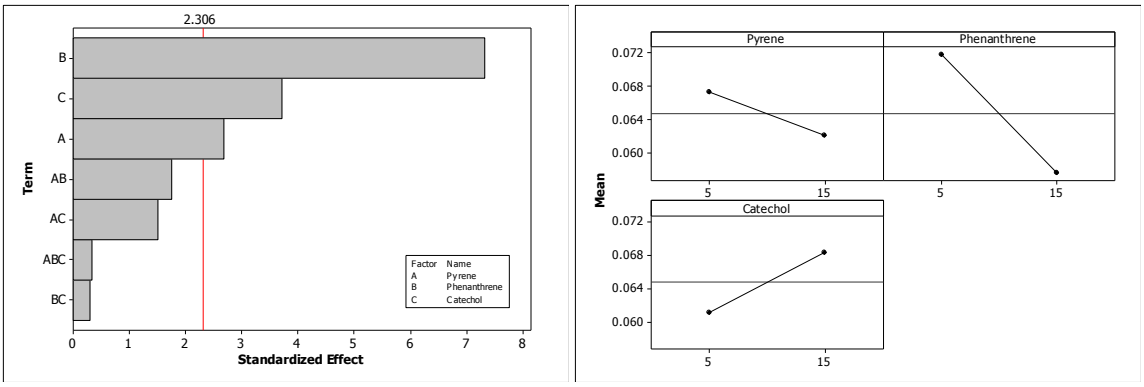

Figure 2: a) Pareto chart of standardized effects for the μ_1 parameter; b) Main effect behaviour.

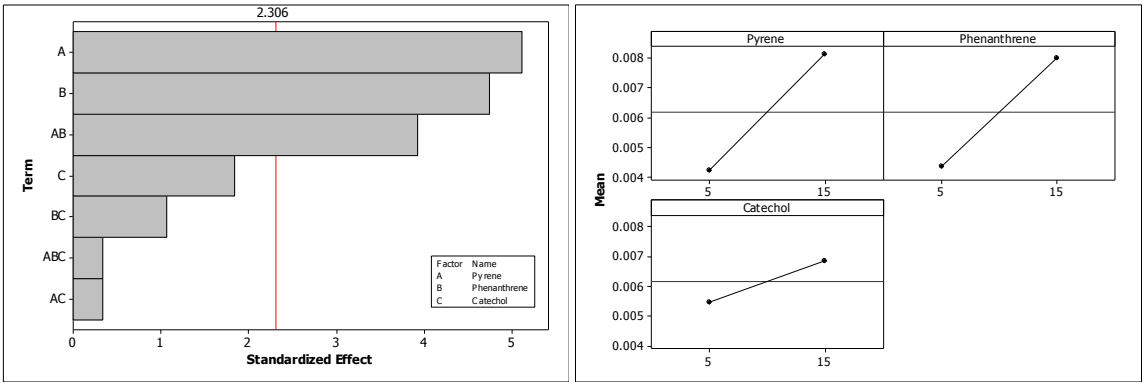

Figure 3: a) Pareto chart of standardized effects for the μ_2 parameter; b) Main effect behaviour.

The solutions were also analysed by HPLC to measure the pollutant removal efficiency (RE_p) in Eq. (5)

$$RE_p = \frac{C_p^i - C_p^f}{C_p^i} \tag{5}$$

where the superscripts *i* and *f* indicate respectively initial and final concentration for the pollutant *p*.
The results are reported in Table 5, where the data regarding catechol confirm that it is easily degraded by the consortium for each investigated condition. The higher removal efficiency of phenanthrene (83.1%) is observed for the second run (02-II), which corresponds to the lowest PAHs initial concentration (5 g/l) and the highest catechol initial concetration (15 g/l). On the other hand, the minimum value for RE_{PHE} (38.8%) is obtained when the initial concentration of all the compounds is equal to 5 g/l (Runs 01-I,II). Also pyrene showed a similar behaviour, as it presented the higher removal efficiency (75.7%) when the initial concentration of the two PAHs was at the lowest level (5 mg/l) and that of catechol was maximum (15 mg/l) (Run 02-I), whereas the lowest removal (efficiency of 18.3%), like for phenanthrene, occurred during Run 01-I, when all the components started from the lowest concentration (5 mg/l).
In all the experiments the average value of the removal efficiency for both pyrene and phenanthrene was about 55%.

Table 5. Removal efficiency (percentage) for the components in all the experimental runs.

Run	RE_{CAT}	RE_{PHE}	RE_{PYR}	**Run**	RE_{CAT}	RE_{PHE}	RE_{PYR}
01-I	100.0	38.6	27.6	**01-II**	100.0	40.1	18.3
02-I	100.0	76.6	75.7	**02-II**	100.0	83.1	73.8
03-I	100.0	49.2	53.2	**03-II**	100.0	44.6	55.3
04-I	100.0	52.5	55.3	**04-II**	100.0	44.8	62.6
05-I	100.0	70.9	47.5	**05-II**	100.0	69.5	39.9
06-I	100.0	40.4	74.8	**06-II**	100.0	48.3	75.4
07-I	100.0	60.0	55.6	**07-II**	100.0	52.8	56.7
08-I	100.0	55.1	61.4	**08-II**	100.0	49.0	61.2

5. Conclusions

In this paper it is presented and discussed the degradation ability of the bacterial consortium Bulab 5378 towards phenanthrene and pyrene, aided by the presence of catechol, in terms of the biomass growth rate and of the removal efficiency for each compound. The diauxic behaviour of the growing bacterial populations probably indicates two distinct phases: at first, they degradate the less stable molecular structure (catechol); after this, the microorganisms have become adapted to the more recalcitrant species phenanthrene and pyrene, which are finally (but not completely) degraded.

The statistical analysis of the experimental data shows that all the components influence the bacterial growth rate: to be precise, catechol has a positive effect, whereas phenanthrene and pyrene exercise a negative one, decreasing the growth rate, when catechol is still present in the suspension; after this, also the two PAHs (singularly and together in a synergistic way) give a positive contribute to the development of the bacterial populations.

The chemical analyses on the solutions always showed an about complete degradation of catechol, whereas phenanthrene and pyrene were consumed with an average removal efficiency of 55%, ranging from 38.6 to 83.1% for phenanthrene and from 18.3 to 75.7% for pyrene, depending on their initial concentration and on the relative abundance of catechol, which increases the degradation rate for the two PAHs in the second growth phase.

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