Biodegradation of lindane by *Pleurotus ostreatus* via central composite design

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Abstract

The degradation of lindane was studied in liquid-agitated cultures using a commercial strain of the fungus *Pleurotus ostreatus* as the biodegrading organism. The biodegradation was accomplished with the action of extracellular oxidative enzymes, produced by the fungus to decompose woody substrates. Enzyme activities of manganese peroxidase and laccase were measured in a liquid mineral medium. An orthogonal Central Composite Design of experiments was used to construct second-order response surfaces with the fungus growth, final pH and the lindane biodegradation as optimization parameters. The initial lindane concentration, the nitrogen content, the incubation time and the temperature were used as design factors. Optimal conditions found for all these parameters will be used for the continuation of this project aiming at the bioremediation of contaminated sites with persistent organic pollutants such as lindane.

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1. Introduction

Lindane is an organochlorine pesticide that has been extensively used for the control of agricultural and medical pests. It poses serious environmental hazards due to its toxicity and bioaccumulation arising from its persistence in water, soil and sediments. White rot fungi appear to have a great potential in the degradation of a wide range of organopersistent pollutants. This capability is based on the production of extracellular peroxidases (manganese and lignin peroxidase and laccases) that catalyze the breakdown of organic compounds (Gadd, 2001; Hattaka, 1994). Most studies concerning organopollutant degradation have been done using *Phanerochaete chrysosporium*. It has achieved extensive degradation of chlorophenols, PAHs, PCBs in the laboratory. However, there has been an interest in screening new species, which can produce higher levels of ligninolytic enzymes and a greater ability in degrading pollutants.

*Pleurotus ostreatus* has been tested for its ability to degrade lindane under different conditions. This mushroom has proved to be an efficient PAH degrader in liquid cultures and in soil–lignocellulose systems (Sheeja and Murugesan, 2002). It is the first time that *Pl. ostreatus* has been used in lindane degradation, although other strains of *Pleurotus* such as *florida*, *sajor-caju*, *eryngii* and different white rot fungi have been found to degrade this pesticide (Arisoy, 1998; Singh and Kuhad, 1999; Tekere et al., 2001). It was also examined for its ligninolytic activities and in comparison with *Ph. chrysosporium*, it did not express lignin peroxidase, which means that MnP and laccase are involved in lindane degradation. The secretion of ligninolytic enzymes was demonstrated in liquid cultures using defined media and temperature.

Before fungi can be used for the bioremediation of contaminated sites, the fundamental environmental and nutritional factors that affect biodegradation must be first
studied in the laboratory. Although statistical design of experiments can save a lot of time and money, decreasing considerably the number of trials needed to investigate a multi-variable phenomenon like this one, only limited application of this methodology is found in the literature (Bezazel et al., 1996). This is very useful when screening probable factors, but mainly it has been proved valuable when second-order models have to be studied such as the one of this study.

In the present study, the biodegradation of lindane was studied in agitated liquid cultures of a commercial strain of the fungus _Pl. ostreatus_ using the central composite design as a second-order methodology. The factors (variables) investigated were the initial lindane concentration, the nitrogen content (in the form of asparagine), the incubation time and the temperature. The optimization parameters selected were the fungus growth (biomass), the final pH and the lindane biodegradation. Expressions of the latter parameter investigated were the biodegraded mass of lindane, the extent of biodegradation relative to initial lindane mass and the extent of biodegradation relative to initial lindane mass and to final biomass.

### 2. Materials and methods

#### 2.1. Culture conditions

_Pl. ostreatus_ is commonly cultivated in Greece and used in mushroom production. Fresh tissue was aseptically transferred on Potato Dextrose Agar (PDA) and maintained at 4°C. Cultures were aseptically maintained at 25°C on PDA, composed of (g/l): peeled potatoes 200, dextrose 20 and agar 15. The inoculum was prepared by transferring four agar plugs (1 cm diameter) grown on PDA into 250-ml flasks containing 50 ml Malt Extract Broth (MEB) and incubated at 25°C under stationary conditions. MEB contained (g/l) malt extract 17, mycological peptone 3, KH2PO4 0.46, K2HPO4 1, MgSO4·7H2O 0.05, ammonium tartate 0.22, 2,2-dimethylsuccinic acid 2.90, glucose 5, thiamine 0.1, Tween 80 0.1%, veratryl alcohol 1.5 mM, trace elements (10 ml). The trace elements composed of (mg/l): MnSO4 33, Fe2(SO4)3 50, ZnSO4·7H2O 43, CuSO4·7H2O 80, H2MoO4 50. Ten-milliliter aliquots were added to 90 ml Kirk’s media (in 250-ml Erlenmeyer flasks). The cultures were further incubated at 25°C.

#### 2.2. Enzyme assays

The liquid medium used for enzyme activities, was the Kirk’s medium composed in g/l of KH2PO4 0.20, CaCl2 0.01, MgSO4·7H2O 0.05, ammonium tartate 0.22, 2,2-dimethylsuccinic acid 2.90, glucose 5, thiamine 0.1, Tween 80 0.1%, veratryl alcohol 1.5 mM, trace elements (10 ml). The trace elements composed of (mg/l): MnSO4 33, Fe2(SO4)3 50, ZnSO4·7H2O 43, CuSO4·7H2O 80, H2MoO4 50. Ten-milliliter aliquots were added to 90 ml Kirk’s media (in 250-ml Erlenmeyer flasks). The cultures were further incubated at 25°C. Samples (1 ml) were aseptically removed from the liquid cultures at various times and tested for enzyme activities. Three replicates were performed. The culture filtrates were used for the estimation of manganese peroxidase, lignin peroxidase and laccase.

Laccase activity was determined spectrophotometrically as described by Szklarz et al. (1989). The activity was measured over 4 min at 30°C and one unit (U) of activity was defined as 1 μmol of syrigaldazine oxidized in 1 min (at 525 nm, 30°C). Lignin peroxidase activity was measured according to the method of Tien and Kirk (1988), with one unit representing 1 μmol of veratryl alcohol oxidized in 1 min (at 310 nm, 26°C). Manganese peroxidase activity was measured using the method of Paszczynski et al. (1988) based on the formation of quinone of syrigaldazine at 525 nm. One unit (U) of activity was defined as 1 μmol of syrigaldazine oxidized in 1 min. The activities were expressed in U/L.

#### 2.3. Samples treatment in the statistical experiment

Each 125-ml Erlenmeyer flask contained 45 ml of Basal Medium under different concentrations of asparagine and 5 ml homogenised mycelium. The flasks were agitated (90 rpm) and aseptically incubated in the respective temperatures according to the statistical design. Two replicates were performed for each experiment and one control consisted of uninoculated flask. At the end of each experiment, the fungal biomass was separated from the medium by filtration. Filtrate, biomass and flask were extracted separately for lindane. Biomass was first dried in air, then 2 ml of hexane was added and the suspension mixed vigorously for 10 min. In the flask, 1 ml of hexane was added and was shaken for 2 min. For the filtrate extraction, 0.5 ml sample was diluted in 1.5 ml deionized water and mixed with 10 ml hexane for 10 min. The upper phase was collected and stored with the extracts of flask and biomass in darkness at 4°C until further analysis. For all the procedures, the chemicals and solvents used were of the highest purity available.

#### 2.4. Gas chromatography analysis

For the analysis of the samples, a gas chromatograph (Shimadzu GC-17A) equipped with an Electron Capture Detector and 30 m×0.32 mm Optima-5 column. The injector, detector and column temperatures were 270, 280 and 60°C, respectively. The N2 flow rate was programmed at 50 ml per min, 1 μl samples were injected each time for the analysis.

#### 2.5. Statistical design of experiments

A central composite design was applied with four design factors, namely the initial lindane concentration (_X_1), the nitrogen content supplied from asparagine (_X_2), the incubation time (_X_3) and the incubation temperature (_X_4). To simplify the recording of the conditions of an experiment and processing of the experimental data, the
factor levels are so selected that the upper level corresponds to +1, the lower level to −1 and the basic level to zero. For factors with a continuous determination region, this can always be done with the aid of the transformation:

\[ x_j = \frac{x_{\tilde{j}} - x_{j0}}{j} \]

where \( x_j \) = coded value of the factor, \( x_{\tilde{j}} \) = natural value of the factor, \( x_{j0} \) = natural value of the basic level, \( J_j \) = variation interval and \( j \) = number of the factor.

Simply speaking the coded values ±1.483 give the corresponding values of each factor in proportionality with the unit variation intervals selected for each factor.

Obviously, this is not the only fuzzy point in the experimental design used in our work. Since it was not possible to fully describe the method in this paper due to space limitations, we consider that elementary knowledge of this statistical method is a prerequisite. The coded levels and the natural values of these factors set in the statistical experiment are shown in Table 1.

The optimization parameters selected were the fungus growth (biomass) and various expressions of the lindane biodegradation, as follows:

- \( Y_1 = M_t \) Fungus growth (mg)
- \( Y_2 = M_{de} - M_t \) Biodegraded mass of lindane (μg)
- \( Y_3 = (M_{de} - M_t)/M_{de} \) Extent of biodegradation relative to initial lindane mass (μg/g)
- \( Y_4 = (M_{de} - M_t)/M_{de}M_t \) Extent of biodegradation relative to initial lindane mass and to final biomass (μg/g/g)
- \( Y_5 = \text{pH} \) Final pH

Two replicates were run at the center point and the whole statistical experiment was performed twice in two blocks. The number of trials (\( N \)) for the central composite design selected was based on the number of the design factors \( k=4 \) as follows:

\[ N = 2^k + 2k + 2 = 26 \text{ trials} \]

For one repetition of the design matrix, this gives 52 trials. For a full second-order factorial design, if chosen, the number of trials needed would be instead:

\[ N = 3^k + 1 = 82 \text{ trials} \]

A repetition of this design in two blocks would give then 164 trials. Obviously, the decrease in the number of trials using a central composite design to obtain a second-order model instead of a full factorial design is significant and this is why the former approach was selected.

After running these 52 trials, the corresponding quadratic models for the above optimization parameters were first computed, from which the outliers (statistically unacceptable measurements) were determined and excluded from the subsequent calculations. The insignificant effects (factors and interactions) having \( p \)-values less than 0.05 were excluded, since these are significantly different from zero at the 95% confidence level.

The statistics used to determine whether the so constructed models were adequate to describe the experimental data were the adequacy of the model, the lack-of-fit test and the adequacy of precision. The computations were performed with the aid of the Design-Expert software (version 6.0.10).

### 3. Results and discussion

#### 3.1. Enzymes production in the liquid medium

Laccase and manganese peroxidase were measured, but there was no expression of lignin peroxidase. At the initial growth stages, a lag phase with almost no activity was observed until a maximum activity 28.1 U/L on the thirteenth day, decreasing later by the end of growth. Laccase activity showed a higher value in comparison with MnP, namely 53.7 U/L.

#### 3.2. Fungus growth and biodegradation

The reduced models obtained after running the 52 trials of the central composite design and excluding the insignificant effects are the following:

\[ Y_1 = 83.67 + 29.07X_3 - 36.38X_4 \]  
(2)

\[ Y_2 = 0.0799 + 0.0320X_1 + 0.0014X_2 - 0.0121X_4 
- 0.0278X_1X_4 + 0.0260X_2X_4 \]  
(3)

\[ Y_3 = 0.3294 - 0.0004X_2 + 0.1028X_3 - 0.0161X_4 
+ 0.1139X_2X_4 \]  
(4)

<table>
<thead>
<tr>
<th>Response</th>
<th>Adequate model ((p&lt;0.05))</th>
<th>Lack of fit ((p&gt;0.05))</th>
<th>Adequate precision ((\text{ratio}&gt;4))</th>
</tr>
</thead>
<tbody>
<tr>
<td>( Y_1 )</td>
<td>( p=0.0013 )</td>
<td>( p=0.2065 )</td>
<td>Ratio=7.770</td>
</tr>
<tr>
<td>( Y_2 )</td>
<td>( p=0.0014 )</td>
<td>( p=0.0924 )</td>
<td>Ratio=7.961</td>
</tr>
<tr>
<td>( Y_3 )</td>
<td>( p=0.0232 )</td>
<td>( p=0.0938 )</td>
<td>Ratio=6.114</td>
</tr>
<tr>
<td>( Y_4 )</td>
<td>( p=0.0015 )</td>
<td>( p=0.2746 )</td>
<td>Ratio=8.934</td>
</tr>
<tr>
<td>( Y_5 )</td>
<td>( p=0.0001 )</td>
<td>( p=0.8006 )</td>
<td>Ratio=10.097</td>
</tr>
</tbody>
</table>

Table 2

Statistics used to test the adequacy of the reduced models
All these models were tested with the statistics shown in Table 2 and were found adequate at the 95% confidence level. A model is considered adequate (significant) if its $p$-value is lower than 0.05, i.e., the chance that a model F-value this large could occur due to noise is less than 5%. When the estimated $p$-value of the lack-of-fit is less than 0.05, there is statistically significant lack-of-fit at the 95% confidence level, i.e., the model does not adequately represent the data. The “adequate precision” statistic measures the signal to noise ratio. A ratio greater than 4 is desirable. In addition, no “leverage points” (remote observations) were found potentially having disproportionate leverage on the parameter estimates, predicted values, and the usual summary statistics. No points were also identified that were potentially influential due to their location in the factor space (Cook’s distance). Consequently, all the computed models pass the above major statistics and can be used to navigate the design space.

From these models, only the first one (the growth model) was found to be a simple first-order model that can be easily investigated. This model suggests that within the factor space chosen, the fungus growth is independent of the pesticide and the additional nitrogen concentrations. The growth increases proportionally with the time, but decreases proportionally with the temperature. Thus, we expect an optimum (maximum for this response) at the maximum time and the minimum temperature of the design. Maximum time looks quite logical and an optimum temperature of around 15.6 °C for Pl. ostreatus is in agreement with the observations of many mycologists saying that this species prefers cold weather in the range 5–22 °C depending on the strain (Laessoe, 1998; Courtecuisse, 1999; Villaescusa and Gil, 2003; Fragoeiro and Magan, 2002). We should take into account here that the prediction variance is not the same everywhere in the region of experimentation. Thus, the standard error of design is reasonably uniform over a relatively large portion of the design space, but it increases rapidly near the boundaries of this space as it is shown in Fig. 1 for the case of the fungus growth model. Consequently, predictions of the optimization parameters at the extreme values of the factors are not expected to be very accurate.
The rest of the models were second-order (quadratic) models for which the effect of every factor is not obvious. To visualize these effects only two factors at a time can be plotted against one response. From the three biodegradation responses ($Y_2$, $Y_3$ and $Y_4$), the more complete is the extent of biodegradation relative to initial lindane mass and to final biomass ($Y_4$) whose plots against the factors $X_1$ (lindane content) and $X_4$ (temperature) are shown in Fig. 2 for both contour and three-dimensional depictions. The dependence of the response $Y_5$ (pH) on the factors $X_3$ and $X_4$ is depicted in Fig. 3.

The optimal natural values of the optimization parameters obtained from all the models inside the experimentation region with the corresponding levels of factors were calculated and are shown in Table 3. It is concluded from this table that the fungus growth is favored at the lowest level of the toxic compound, at intermediate nitrogen content, at the highest level of the time and at the lowest level of the temperature.

The optimum conditions (maxima) for the various expressions of biodegradation of lindane vary a lot, but with regard to the more complete response $Y_4$ (the extent of biodegradation relative to initial lindane mass and to final biomass), there are significant differences with the maximal growth response $Y_1$ for the factors $X_2$ (nitrogen content) and $X_4$ (temperature). Thus, a higher nitrogen concentration and a higher temperature than the ones for the maximum growth are needed for the maximum relative biodegradation $Y_4$ which is 25.8 mg of biodegraded lindane per initial gram of lindane per final gram of the fungal mass.

In this work, no significant new peaks were observed in the chromatograms obtained, which is evidence for fast intermediate reaction rates in the pathway of biotransformation and possible mineralization of the pollutant.

The decrease of pH is a biological characteristic of fungi as an effort to enhance low pH favored catalytic oxidations of substrates and as a reaction to environmental stress caused by the presence of a toxic compounds like lindane. It is not a great surprise then that the optimum conditions (minimum) for pH are not found at the minimum value of the toxic compound content. In fact, this compound seems to trigger the reduction mechanism of pH.

In field applications, the fungus is expected to behave differently, due to environmental stress and phenomena such as antagonism and competition via interaction with native organisms (other fungi, bacteria and protozoa), therefore, a field pilot study is imperative in this as well as in every bioremediation project.

4. Conclusions

The central composite design selected as a response surface methodology proved to be suitable for performing

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**Table 3**

<table>
<thead>
<tr>
<th>Optima →</th>
<th>$Y_1$ (growth)</th>
<th>$Y_2$ (degraded lindane)</th>
<th>$Y_3$ (degraded lindane)/ (initial lindane)</th>
<th>$Y_4$ (degraded lindane)/ (initial lindane)/ (biomass)</th>
<th>$Y_5$ (pH)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$X_1$ (mg/L)</td>
<td>2.03</td>
<td>7.94</td>
<td>2.28</td>
<td>2.03</td>
<td>4.46</td>
</tr>
<tr>
<td>$X_2$ (g/L)</td>
<td>0.56</td>
<td>0.14</td>
<td>0.11</td>
<td>1.89</td>
<td>1.87</td>
</tr>
<tr>
<td>$X_3$ (days)</td>
<td>12.45</td>
<td>12.45</td>
<td>12.45</td>
<td>12.45</td>
<td>12.45</td>
</tr>
<tr>
<td>$X_4$ (°C)</td>
<td>15.6</td>
<td>15.8</td>
<td>15.6</td>
<td>24.5</td>
<td>16.8</td>
</tr>
</tbody>
</table>

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Fig. 3. Response surfaces for $Y_5$=pH against $X_3$=lindane content and $X_4$=time depicted as contour (A) and three-dimensional (B) plots. The factors $X_1$ and $X_2$ were kept at their basic (zero) levels.
bioremediation studies in complex systems where the growth of microorganisms in a hostile medium, the production of degradative enzymes by the microorganism, the toxicity of the pollutant to the microorganism, the heterogeneity of the system’s components and other factors do not permit a straightforward study and as a consequence the black-box of Cybernetics appears to be indispensable. The selected fungus (Pl. ostreatus) had a maximum growth of 181 mg at the factors combination of the lowest level of lindane content (2.03 mg/L), intermediate nitrogen content (0.56 g/L), the highest level of time (12.45 days) and the lowest level of temperature (15.6 °C).

The maximum biodegradation of lindane, expressed as the extent of biodegradation relative to initial lindane mass and to final biomass (Y₄) was found equal to 25.8 mg/g/g (degraded lindane/initial lindane/biomass), at the region of 2.03 mg/L lindane, 1.89 g/L nitrogen content, 12.45 days and 24.5 °C.

The optimum (minimum) for pH was found at the conditions of 4.46 mg/L lindane, 1.87 g/L nitrogen content, 12.45 days and 16.8 °C.

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References