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# Bioremediation of a soil contaminated by lindane utilizing the fungus *Ganoderma australe* via response surface methodology

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**Abstract**

Mixtures of a sandy soil and wheat straw were doped with the organochlorine insecticide lindane in glass tubes and were inoculated with the polypore fungus, *Ganoderma australe*. An evaluation of bioremediation process effectiveness was searched and five parameters identified for the solid-state system. Fungi growth is a function of temperature and requires moisture for a proper colonization. These microorganisms need inorganic nutrients such nitrogen and phosphorus to support cell growth and it is also appropriate to know the range of concentration and toxicity of the used insecticide. Thus, an orthogonal central composite design (CCD) of experiments was used to construct second order response surfaces. Five design factors, namely temperature, moisture, straw, lindane content and nitrogen content and seven optimization parameters (responses), namely lag time, propagation velocity, biomass growth rate, biodegradation rate, biodegradation/biomass, biomass/propagation and biomass content were analyzed. The optima of the responses of the adequate models were found to be the following: propagation velocity 4.25 mm/day, biomass growth rate 408 mg/day, biodegradation/biomass 56.9 μg/g, biomass/propagation 250 mg/mm and fungal biomass content in solid mixture 260 mg/cm$^3$. The most important response for bioremediation purposes is biodegradation/biomass which is maximized at the factors levels: temperature 17.3 °C, moisture 58%, straw content 45%, lindane content 13 ppm and nitrogen content 8.2 ppm.


*Keywords:* Bioremediation; Contaminated soil; Ligninolytic fungi; *Ganoderma australe*; Central composite design

## 1. Introduction

One of the major problems facing the industrialized world today is the contamination of soils, ground water, sediments, surface water, and air with hazardous and toxic chemicals. While regulatory steps have been implemented to reduce or eliminate the production and release to the environment of these chemicals, significant environmental contamination has occurred in the past and will probably continue to occur in the future [1]. Isomers of 1,2,3,4,5,6-hexachlorocyclohexane (HCH) have been the most extensively used broad-spectrum organochlorine pesticides against a wide range of soil-dwelling and plant-eating (phytophagous) insects. A number of publications depict the health effects of HCH isomers on animals and humans and the occurrence of residues in soil, water, air, plants, plant products, animals, and food commodities. Although the widespread use of lindane and technical-grade HCH has been discontinued for a long time, the problem of their residues, due to the lengthy persistence of these chemicals in many soils, exists. Adverse health effects associated with HCH isomers include neurological problems and immunosuppression in humans and liver cancer in rats and mice [2].

Among fungi, white rot species show high efficiency in degradation of a wide range of lignocellulosic substances, this ability being of great interest for the development of environmentally friendly biotechnological processes to be applied in food production, medical application and bioremediation purposes [3–7]. Application of fungal technology for the cleanup of pollutants depends upon the ability of white rot fungi like *Phanerochaete*, Trametes, *Bjerkandera* and *Pleurotus* to degrade lignin (and lignin-like substances) through production and secretion of a group of highly potent, non-specific extracellular enzymes [8,9]. The production and activity of these enzymes in contaminated soil under field conditions are two prerequisites for successful application of white rot fungi in soil bioremediation [10].

Beyond introduction of white rot fungi in natural soil, enhanced degradation of pesticide molecules requires effective growth and competition with indigenous microorganisms [11].

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Since the fungal bioremediation process depends on the extent to which the fungal inoculant succeeds in colonizing the contaminated soils, an interesting approach is to assess fungal growth in soil. However, this is difficult, because of the problems of quantification which occur when trying to measure the growth of filamentous fungi in such heterogeneous environments. Therefore, in studies with wood rot fungi, indirect methods are often used [12].

A number of methods has been described to estimate fungal biomass in soil including the use of specific biochemical components of fungal cells (chitin, ergosterol, phospholipids-fatty acids), measurement of metabolic activity (selective respiratory inhibition), viable counts and direct microscopic counting [13]. Previous studies have shown that enzymatic activity and ergosterol amount vary during fungal growth. Nutrient consumption can only be applied in axenic conditions. On the other hand, the measurement of the cell wall constituent glucosamine is an indicator well adapted to the estimation of fungal development [14].

The ability of the fungi to degrade a wide variety of compounds has been attributed, at least in part, to the action of ligninolytic enzymes. The primary carbon source of the fungi is trees and other plants cellulose, which is protected by the complex biopolymer known as lignin. The lignin degrading enzymes (lignin peroxidases, manganese peroxidases and laccases) are thus essential for the fungal survival. Nevertheless, enhancing fungal growth and generation of the ligninolytic enzymes in the soil environment has proven to be difficult [15].

There are many studies on degradation of organochlorine, organophosphorus and pyrethroid insecticides by ligninolytic fungi [8,12,16]. Early experiments have demonstrated the ability of white rot fungi and particular *Phenerochaete chrysosporium* to degrade the organochlorine insecticide DDT [8]. In an other work, the ability of this fungus to degrade six insecticides dieldrin, aldrin, heptachlor, chlordane, lindane and mirex has been investigated in both liquid culture and soil corncob matrices. Among the insecticides, only lindane and chlordane underwent significant biodegradation [17]. Recently [12] also showed that these species were able to degrade DDT, lindane and atrazine. Most of the studies on the degradation of organopollutants by white rot fungi have been concentrated on *Phanerochaete chrysosporium*, *Pleurotus ostreatus* and a few other fungi, which represent only a small part of many hundreds of similar species existing in nature [18].

Fungal bioremediation is subject to the prevailing temperature, moisture and soil conditions [19]. The soil pH, water availability, nutritional status and oxygen levels vary and may not always are optimal growth of white rot fungi [20,21] or extracellular enzyme production for pollutant transformation [12]. Thus, the kinetics of pesticides degradation in the soil is commonly biphasic with a very rapid degradation rate in the beginning followed by a very slow prolonged dissipation. The remaining residues are often quite resistant to degradation [22]. Among environmental parameters, the availability of water in soil may be a very important factor affecting the success of bioremediation, since water availability affects oxygen supply and thus fungal growth and enzyme production [23,24]. Besides affecting microbial behaviour, water availability affects pesticide binding and distribution in the soil. The behaviour of organic compounds in water plays a very significant role in their availability for microbial utilization in the environment [25]. Other factors that can contribute to pesticide degradation in soils include chemical nature, concentration of the pesticide, soil type and amount of soil organic matter and microbial community structure and activity [26]. Degradation of a diverse group of organopollutants has been reported to be dependent on the non-specific and non-stereoselective ligninase, which is produced under substrate limiting growth conditions, yet not induced specifically by the pollutants [27].

The bracket-like polypore fungus, *Ganoderma australe* has been previously studied for its potential to degrade lindane in liquid agitated cultures [28]. However, the potential of this fungus to degrade various organopollutants in soil systems has not been extensively studied yet. One of the important aspects of *Ganoderma* spp. relates to the use of its ligninolytic potential. Studies of ligninolytic enzymes with this fungus are still not completely known. The enzymes produced by these fungi are lignin peroxidase, manganese peroxidase, and laccases, which are frequently referred to as lignin-modifying enzymes (LMEs), presence of wheat bran induced a high production of the enzymes. Generally, laccases and MnP are more widely distributed among white rot fungi than LiP recent work detected genes intimately related to the LiP in two *Ganoderma* species: *G. applanatum* and *G. australe* [29].

Thus, the purpose of this work is to contribute to the study of bioremediation of lindane by *G. australe* in contaminated soils utilizing a multi-parametric design of experiments.

## 2. Materials and methods

### 2.1. Isolation and culture conditions

*G. australe* usually grows flat against a woody substrate, forming a skin-like upside crust. The fungal strain used in this work was isolated from a *Pinus pinea* stump in Athens/Greece and has been described for its ligninolytic potential previously [30]. The stock cultures were grown on potato dextrose agar at 4 °C, with periodical sub-culturing. Cultures were aseptically maintained at 25 °C on potato dextrose agar (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 100 mL Erlenmeyer flasks containing 50 mL malt extract broth (MEB) and incubated at 25 °C under stationary conditions. After 6 days of growth, the culture was homogenized for 30 s and further used as inoculum in the soil-straw environment for the estimation of lindane degradation and biomass level in the solid-state system.

### 2.2. Media and growth conditions

The growth medium used was silicious sand and milled wheat straw, both of particle size <1 mm, at a total quantity of 10 g. The mixture of soil and straw was put into large test tubes (diameter 3.5 cm, length 24 cm), sterilized in an autoclave (121 °C,

20 min) and inoculated with 150 mg homogenized inoculum. All cultures and controls in the study were run in duplicate including the biodegradation experiments. Ammonium nitrate was used as a nitrogen source. The insecticide lindane (γ-hexachlorocyclohexane), purity 99% (GC), was obtained from Sigma–Aldrich. Fungal growth was estimated by measuring the propagation of the fungus in the straw-soil columns (mm) and by the *N*-acetylglucosamine determination (in mg dry biomass).

### 2.3. Biomass determination

Direct quantitation of fungal biomass in solid substrate is not possible because of the difficulty in isolating fungal hyphae from the substrate [31]. Fungal chitin hydrolysis into *N*-acetylglucosamine was used to determine the biomass according to the method described by Scotti et al. [14]. Thus, 5 g of the solid-state system (soil-straw-mycelium) was treated at 25 °C with 5 mL of 72% $H_2SO_4$ on a rotary shaker at 130 rpm for 30 min. After dilution with 54 mL deionized water, the hydrolysis was carried out by autoclaving the mixture for 2 h at 121 °C. The hydrolyzate was neutralized to pH 7.0 with initially 10 M and then 0.5 M NaOH solution. Glucosamine was assayed with the following colorimetric method. After the sample neutralized to pH 7.0 a sample of 1.5 mL extracted and an equal volume of (1.5 mL) 5% (w/v) $NaNO_2$ and 5% $KHSO_4$ added. Then stirred the solution for 15 min and centrifuged at (1500 × *g*, 2 min, 2 °C), then two samples (1.5 mL) of the supernatant were removed. To each of them 0.5 mL of 12.5% $NH_4SO_3NH_2$ was added, shaking for 5 min and added to the solution 0.5 mL of 0.5% 3-methyl-2-benzothiazolone hydrazone hydrochloride (MBTH) (prepared daily). The mixture was heated in a boiling water bath for 3 min, cooled and 0.5 mL of 0.5% $FeCl_3$ (stored at 4 °C and discarded after 3 days) was added. After standing for 30 min, the absorbance at 650 nm was read. The blank was the medium just before inoculation.

### 2.4. Analysis of lindane in soil

The extraction of lindane was conducted according to the procedure given by Rigas et al. [7], modified for a solid-state system. Half of the soil-straw incubated mixture (5 g) was dried and added into a 100 mL volumetric flask. Hexane (80 mL) was added in the solid-state and agitated on a rotary shaker at 130 rpm for 30 min. After standing for 15 min to allow soil settlement, a 6 mL aliquot was removed and suspended with 4 mL deionized water, and cleaned from any straw residues. The upper phase was collected and stored at 4 °C. The same extraction procedure was repeated five times for each test tube.

Aldrin was used as an internal standard and the samples analyzed in 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 100 °C, respectively, and 1 μL sample was injected each time for analysis. For all the procedures the chemicals and solvents used were of the highest purity available.

Table 1
Coded and natural values of design factors (coded values are dimensionless and shown in bold)

| Effects | Design factors | −α | −1 | 0 | +1 | +α |
|---|---|---|---|---|---|---|
| A | $X_1$: temperature (°C) | 10.1 | 15 | 23 | 31 | 35.8 |
| B | $X_2$: moisture (% in total 10 g) | 50.4 | 58 | 70.5 | 83 | 90.5 |
| C | $X_3$: straw content (% in total 10 g) | 20.2 | 26 | 35.5 | 45 | 50.7 |
| D | $X_4$: lindane content (ppm in soil) | 5.1 | 7 | 10 | 13 | 14.8 |
| E | $X_5$: nitrogen content (ppm in soil) | 5.2 | 8 | 12.5 | 17 | 19.7 |

### 2.5. Statistical design of experiments

The orthogonal central composite design $2^{5-1}$ plus star with two central points was applied to study the effect of five *design factors*, namely temperature ($X_1$), moisture ($X_2$), straw ($X_3$), lindane content ($X_4$), and nitrogen content ($X_5$). The column of the effects interaction ABCD was used to create the column for factor $X_5$ plus the star points. The coded levels and the natural values of these factors set in the statistical experiment are shown in Table 1.

The design consisted of 28 trials and two replicates were performed for each trial giving 56 runs totally, as calculated by the following equation:

$$N = F + T = 2^{k-1} + 2k + n_c = 2^{5-1} + 2 \times 5 + 2 = 28 \quad (1)$$

where $N$ is the total number of runs, $F$ the number of factorial experiment, $T$ the number of the additional star points, $k$ the number of factors, and $n_c$ is the number of central points.

The distance of the axial points from the center of the design (star distance, $\alpha$) was calculated [32] by the equation:

$$\alpha = \left[\frac{(NF)^{1/2} - F}{2}\right]^{1/2} = \left[\frac{(28 \times 16)^{1/2} - 16}{2}\right]^{1/2} = 1.607 \quad (2)$$

The selected *optimization parameters* or *responses* are the following:

$Y_1$, lag time (days); $Y_2$, propagation velocity (mm/day); $Y_3$, biomass growth rate (mg/day); $Y_4$, biodegradation rate (μg/day); $Y_5$, biodegradation/biomass rates (μg/g); $Y_6$, biomass growth rate/propagation velocity (mg/mm).

Lag time is the time needed after inoculation of the soil system for the fungus to adapt in the new medium and start propagating in it. The propagation velocity of the fungal mycelium front in the soil was measured visually on the sides of the test tubes immediately after the lag time. Biomass growth rate and biodegradation rate were also determined after the lag time had elapsed. All values of the optimization parameters were determined for the total quantity of the soil-straw systems, which was always equal to 10 g of soil and wheat straw.

The results of the statistical experiment were treated by the software Design-Expert and StatGraphics to determine the various components of analysis of variance (ANOVA). The corresponding quadratic models for the above optimization parameters were first computed, from which the outliers

(statistically unacceptable measurements) were found and excluded from the subsequent calculations. The insignificant effects (factors and interactions) having $p$-values higher than 0.05 were then excluded, and those with $p$-values lower than 0.05 were further used being statistically 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 significance of models, *lack-of-fit test*, and adequate precision statistic.

## 3. Results and discussion

### 3.1. Regression models and statistical testing

After running the 56 trials of the central composite design, the results of the statistical experiment were treated with regard to the coded design matrix. The statistically distant values of the responses were excluded using the *outlier t*-test. This is a measure of how many standard deviations the actual value deviates from the value predicted after deleting the point in question. Abnormal runs were identified and excluded when found to deviate more than plus or minus 3.5 standard deviations.

The significant effects of the responses were then found testing the statistical significance of each effect by comparing the mean square against the estimate of the corresponding error. The significant effects having $p$-values less than 0.05, thus indicating that they are significantly different from zero at the 95% confidence level, are the following ranking from the more significant to the less significant effects:

| Response | Effects |
|---|---|
| $Y_1$ | AA, A, AB, E, BE, AD, DE, AE, C, BD, CE, BB, AC, B, D |
| $Y_2$ | A, C, AC, AA, DD, D |
| $Y_3$ | AA, CE, BD, D, BB, B, AD, E, A, C |
| $Y_4$ | AA, BB, DE, AC, E, AB, CE, AD, BC, CD, BE, CC, D, DD, EE, AE, BD, B, C, A |
| $Y_5$ | B, BB, CC, D, C |
| $Y_6$ | A, AA, BE, CD, AE, BB, B, E, AB, D, C |

The reduced second order regression models fitted to the data for the coded values of factors after having excluded the insignificant effects are the following:

$$Y_1 = 3.27 - 1.21X_1 - 0.17X_2 - 0.47X_3 + 0.08X_4 - 1.05X_5 + 1.05X_1^2 + 1.33X_1X_2 - 0.66X_1X_3 + 1.22X_1X_4 + 0.62X_1X_5 + 0.37X_2^2 + 0.56X_2X_4 - 1.27X_2X_5 + 0.84X_3X_5 - 1.03X_4X_5 \quad (3)$$

$$Y_2 = 2.76 + 1.01X_1 + 0.42X_3 + 0.13X_4 - 0.23X_1^2 + 0.28X_1X_3 - 0.23X_4^2 \quad (4)$$

$$Y_3 = 55.47 - 19.34X_1 + 26.90X_2 + 6.41X_3 + 38.46X_4 - 26.14X_5 + 71.23X_1^2 - 32.24X_1X_4 + 34.149X_2^2 + 45.79X_2X_4 - 50.52X_3X_5 \quad (5)$$

$$Y_4 = -0.42 - 0.01X_1 - 0.47X_2 - 0.08X_3 + 0.84X_4 - 1.63X_5 + 3.55X_1X_3 + 3.01X_1X_4 - 0.80X_1X_5 + 2.44X_2^2 + 1.75X_2X_3 - 0.61X_2X_4 - 1.54X_2X_5 + 0.90X_3^2 - 1.92X_3X_4 + 3.03X_3X_5 + 0.69X_4^2 - 4.44X_4X_5 + 0.50X_5^2 \quad (6)$$

$$Y_5 = 12.87 - 16.07X_2 - 0.63X_3 + 5.91X_4 + 11.64X_2^2 + 11.02X_3^2 \quad (7)$$

$$Y_6 = 22.42 - 52.67X_1 + 10.29X_2 - 0.56X_3 - 3.10X_4 + 10.09X_5 + 52.60X_1^2 - 8.74X_1X_2 - 16.89X_1X_5 + 12.22X_2^2 + 31.46X_2X_5 - 29.87X_3X_4 \quad (8)$$

The complete models (all factors included) as well the reduced models (those including only the significant effects) were tested with the statistics: *model significance*, *lack-of-fit and adequate precision*.

The *model significance test*, checks whether the model $F$-value occurs due to noise. To do this, the probability of sensing the observed $F$-value ($prob > F$-, or $p$-value) is used to test if the null hypothesis is true. The $p$-value is the proportion of the area under the curve of the $F$-distribution that lies beyond the observed $F$-value. Small $p$-values dictate rejection of the null hypothesis, meaning that if a $p$-value is less than 0.05 the model is significant at the 95% confidence level.

The *lack-of-fit test* is designed to determine whether the selected model is adequate to describe the observed data, or whether a more complicated model should be used. The test is performed by comparing the variability of the current model residuals to the variability between observations at replicate settings of the factors. A model is considered adequate (significant) at the 95% confidence level, if the $p$-value of the *lack-of-fit test* is higher than 0.05.

The *adequate precision test* measures the signal to noise ratio. It compares the range of the predicted values at the design points to the average prediction error. A ratio greater than 4 indicate adequate model discrimination and then the model can be used to navigate the design space.

The results shown in Table 2 indicate that all models comply with the desired values of the statistics *model significance* and *adequate precision*. Moreover, the reduced models give better test values for these statistics than the complete ones, due to the elimination of the insignificant factors. Yet, concerning the *lack-of-fit test* the models $Y_1$ and $Y_4$ prove to be inadequate.

More precisely, the model for *response* $Y_1$ (lag time) is not adequate, because the $p$-value of *lack-of-fit* is null for both, the complete and the reduced models. This may be explained by the indefinable factors that would influence the adaptation time of a living organism, till the moment it decides to colonize a new and hostile (due to lindane) medium. Thus, this model cannot further be used to investigate the factors space.

On the contrary, the model for *response* $Y_2$ (propagation velocity) is adequate for both the complete and the reduced models, because the *lack-of-fit test* gives $p$-values greater than 0.05.

Table 2
Statistics used to test the adequacy of the complete (all effects included) and reduced (only significant effects) models

| Response | *Model significance* (desired $p$-value < 0.05) | | *Lack-of-fit* (desired $p$-value > 0.05) | | *Adequate precision* (desired ratio > 4) | |
|---|---|---|---|---|---|---|
| | Complete | Reduced | Complete | Reduced | Complete | Reduced |
| $Y_1$ | 0.0033 | 0.0001 | <0.0001 | <0.0001 | 5.600 | 6.948 |
| $Y_2$ | <0.0001 | <0.0001 | 0.0037 | 0.0988 | 10.954 | 19.185 |
| $Y_3$ | 0.0005 | <0.0001 | 0.0680 | 0.3935 | 9.665 | 13.121 |
| $Y_4$ | <0.0001 | <0.0001 | <0.0001 | <0.0001 | 13.847 | 13.847 |
| $Y_5$ | 0.0059 | <0.0001 | 0.0792 | 0.2718 | 6.112 | 9.718 |
| $Y_6$ | <0.0001 | <0.0001 | 0.0313 | 0.2648 | 14.178 | 20.329 |

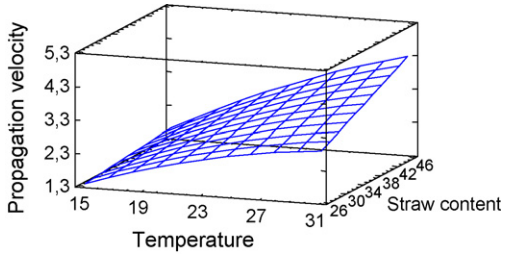


Fig. 1. The estimated response surface of propagation velocity ($Y_2$) in mm/day plotted against temperature ($X_1$) (°C) and straw content ($X_3$) (%) with the other factors kept at their zero levels (moisture 70.5%, lindane content 10 ppm, nitrogen content 12.5 ppm).

Thus, the reduced model can now be used to investigate the effects of the significant factors on propagation velocity starting from the end of the lag phase. Temperature, straw content and lindane content were the factors found to be significant in a quadratic model including the effects A, C, AC, AA, DD and D. Nevertheless, in a second order model the effects of factors on optimization parameters are not obvious. To visualize the effects of a quadratic model only two factors at a time can be plotted against one response. This is shown in Fig. 1, where the propagation velocity ($Y_2$) in mm/day is plotted against temperature (°C) and straw content (%) with the other factors kept at their zero levels (moisture 70.5%, lindane content 10 ppm, nitrogen content 12.5 ppm).

The complete as well the reduced model for *response* $Y_3$ (biomass growth rate) are adequate as tested with the *lack-of-fit test*. All factors appear in the reduced model as expected in a quadratic model including the effects AA, CE, BD, D, BB, B, AD, E, A, C. In Fig. 2, the estimated response surface of biomass growth rate ($Y_3$) in mg/day is plotted against moisture (%) and straw content (%) with the other factors kept at their zero levels (temperature 23 °C, lindane content 10 ppm, nitrogen content 12.5 ppm).

The complete as well as the reduced model of *response* $Y_4$ (biodegradation rate) are not adequate as is evident from the null $p$-values of their *lack-of-fit tests* shown in Table 2. In addition the effects AA, BB, DE, AC, E, AB, CE, AD, BC, CD, BE, CC, D, DD, EE, AE, BD, B, C, A are confounded. This inadequacy may be attributed to the differing biomass contents in the soil-straw system of the 56 experimental runs producing different quantities of ligninolytic enzymes. Consequently, while the biodegradation rate is inadequate the biomass growth rate would rectify the problem and this is attempted with the next transformed *response* $Y_5 = Y_4/Y_3$ (biodegradation/biomass), which is the rational function of biodegradation rate to the biomass growth rate.

Indeed, *response* $Y_5$ yields an adequate model for both the complete and the reduced model as concluded from the higher than 0.05 $p$-values of the *lack-of-fit test*. Thus, the reduced to biomass biodegradation can be used to navigate the design space. The significant factors determined are moisture ($X_2$), lindane content ($X_4$), straw content ($X_3$), in a quadratic model with significant effects B, BB, CC, D, and C. The effect of nitrogen content ($X_5$) is not significant and is excluded from the model. In Fig. 3, the estimated response surface of biodegradation/biomass ($Y_5$) in μg/g is plotted against moisture (%) and lindane content (ppm) with the other factors kept at their basic levels (temperature 23 °C, straw content 35.5%, nitrogen content 12.5 ppm).

Some researchers choose to determine the biomass produced in test tubes by measuring the propagation of mycelium front

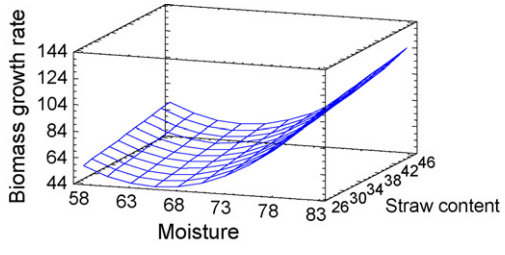


Fig. 2. The estimated response surface of biomass growth rate ($Y_3$) in mg/day plotted against moisture ($X_2$) (%) and straw content ($X_3$) (%) with the other factors kept at their zero levels (temperature 23 °C, lindane content 10 ppm, nitrogen content 12.5 ppm).

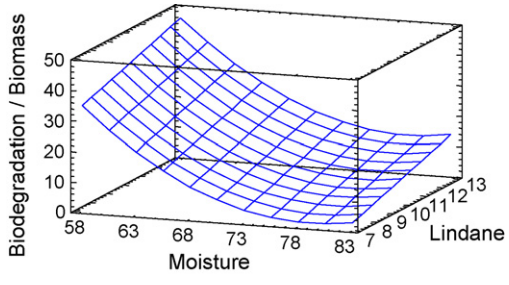


Fig. 3. The estimated response surface of biodegradation/biomass ($Y_5$) in μg/g plotted against moisture ($X_2$) (%) and lindane content ($X_4$) (ppm) with the other factors kept at their zero levels (temperature 23 °C, straw content 35.5%, nitrogen content 12.5 ppm).

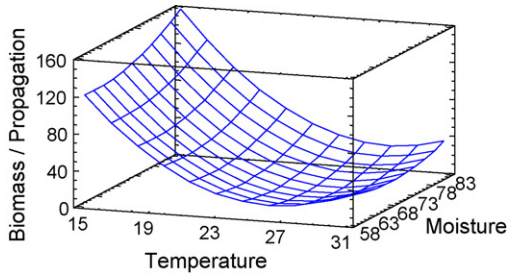


Fig. 4. The estimated response surface of biomass/propagation ($Y_6$) in mg/mm plotted against temperature ($X_1$) (°C) and moisture ($X_2$) (%) with the other factors kept at their zero levels (straw content 35.5%, nitrogen content 12.5 ppm and lindane content 10 ppm).

into the solid medium. This is an easy measurement to do, but it has to be proven first that the ratio biomass/propagation is independent from the principal variables effecting the biomass growth. This dependence can be explored now selecting as a new optimization parameter the transformed *response* $Y_6 = Y_3/Y_2$ (biomass/propagation), which is the rational function of biomass growth rate to the propagation velocity.

Indeed, *response* $Y_6$ yields an adequate model for both the complete and the reduced model as concluded from the higher than 0.05 *p*-values of the *lack-of-fit test*. Thus, this response can be used to clear up whether the ratio biomass/propagation is independent from other factors. It was revealed after the statistical treatment of data that all factors are significant in a quadratic model with significant effects A, AA, BE, CD, AE, BB, B, E, AB, D, and C. Thus, the indirect measurement of the mycelium front propagation velocity cannot be used in lieu of the direct determination of biomass with methods such as the glucosamine method. In Fig. 4 the estimated response surface of biomass/propagation ($Y_6$) in mg/mm plotted against temperature (°C) and moisture (%) with the other factors kept at their zero levels (straw content 35.5%, nitrogen content 12.5 ppm and lindane content 10 ppm).

It is interesting to notice that dividing the response $Y_6$, expressed in mg of fungal biomass per length of propagation, by the cross-section of the test tubes in which the fungus propagated, the *biomass content in the soil-straw system* ($Y_7$) arises with dimensions mass per volume. Taking into account that $Y_6$ is expressed in mg/mm and the diameter of the tubes is 3.5 cm the new transformed *response* $Y_7$ is then:

$$Y_7 = Y_6 \frac{(\text{mg/mm})\,(10\,\text{mm}/1\,\text{cm})}{\pi(3.5/2)^2\,(\text{cm}^2)} \quad (9)$$

where: $Y_7$ in mg/cm$^3$ (biomass/soil).whence:

$$Y_7 = 23.32 - 54.77X_1 + 10.71X_2 - 0.58X_3 - 3.44X_4 + 10.50X_5 + 54.70X_1^2 - 9.09X_1X_2 - 17.56X_1X_5 + 12.71X_2^2 + 32.71X_2X_5 - 31.06X_3X_4 \quad (10)$$

### 3.2. Optimization of responses

The optimal values of the responses examined in this work inside the experimentation space were calculated from their reduced models and are shown together with the corresponding natural values of factors in Table 3. It is worth mentioning that the optimal conditions are not the same for all responses. So, one has first to select the response of interest and then to accommodate the factors in order to obtain the desired optimum. Optima shown in Table 3 were obtained for the best values of all significant factors, whereas the response surfaces in figures refer only to two factors at a time. So, it is expected that calculated optima may be better than the values depicted with a limited number of factors.

For bioremediation purposes the most important response, after the rejection of biodegradation rate model $Y_4$, is biodegradation/biomass $Y_5$ which is maximized at the factors levels: temperature 17.3 °C, moisture 58%, straw content 45%, lindane content 13 ppm and nitrogen content 8.2 ppm. Nevertheless, one has also to take into account that biodegradation depends mainly on the fungal biomass ($Y_3$) producing the ligninolytic enzymes needed to degrade lindane, which is favored at different levels of the design factors, namely temperature 15 °C, moisture 83%, straw content 45%, lindane content 13 ppm and nitrogen content 8 ppm.

Nevertheless, the fitted value of response $Y_5$ for the levels of factors that maximize $Y_3$ is calculated to be equal to 24.7. This value is 2.3 times lower than the optimal estimated value of $Y_5$ (56.9 μg/g). On the other hand, the fitted value of response $Y_3$ for the levels of factors that maximize $Y_5$ is calculated to be equal to 210, a value which is 1.9 times lower than the optimal estimated value of $Y_3$ (408 mg/day). A solution to this problem would be the fungal biomass content of the soil-straw system to be used as a factor in a future experimental study, although this seems quite difficult to be accomplished experimentally.

There are different optimal values for temperature between the responses $Y_2$ (propagation velocity) and $Y_3$ (biomass growth rate). This may be attributed to two different mechanisms: propagation has to do with penetration of hyphae into the soil which is favored by a more fluffy material obtained at higher temperature

Table 3
Natural values of the design factors for the optima of responses of adequate models

| Design factors | Propagation velocity, $Y_2$ | Biomass growth rate, $Y_3$ | Biodegradation/biomass, $Y_5$ | Biomass/propagation, $Y_6$ | Biomass content, $Y_7$ |
|---|---|---|---|---|---|
| Optima | 4.25 mm/day | 408 mg/day | 56.9 μg/g | 250 mg/mm | 260 mg/cm$^3$ |
| $X_1$ (°C) | 31.0 | 15.0 | 17.3 | 15.0 | 15.0 |
| $X_2$ (%) | 58.2 | 83.0 | 58.0 | 83.0 | 83.0 |
| $X_3$ (%) | 45.0 | 45.0 | 45.0 | 45.0 | 45.0 |
| $X_4$ (ppm) | 10.8 | 13.0 | 13.0 | 7.0 | 7.0 |
| $X_5$ (ppm) | 8.0 | 8.0 | 8.2 | 17.0 | 17.0 |

and lower soil moisture, whereas biomass growth is favored by high moisture content and lower temperature at which this fungus grows in its natural environment. The different mechanisms of these two responses reflect in the inability to use propagation velocity instead of biomass growth rate, as analyzed above in the discussion of responses $Y_6$ (biomass/propagation) and $Y_7$ (biomass content in the soil-straw system).

## 4. Conclusion

The central composite design selected as a response surface methodology proved to be suitable for performing bioremediation studies in a complex solid-state system in which colonization and growth of *G. australe* in presence of the toxic compound lindane do not permit attainable straightforward studies.

Six response surface models were constructed from which four proved to be adequate. The *lag time model* was found to be inadequate due probably to indefinable variables. The significant factors of the adequate fungus *propagation rate model* are temperature, straw content and lindane content in a quadratic model. In the *biomass growth rate model* all factors appear in the reduced model as expected.

The *biodegradation rate model* proved to be inadequate and this was attributed to the differing biomass contents in the soil-straw system of the experimental runs, thus producing different quantities of ligninolytic enzymes. The reduction of biodegradation rate to the biomass growth rate rectified the problem after the construction of an adequate *biodegradation/biomass model*, which is the rational function of biodegradation rate to the biomass growth rate. The significant factors determined for this model are moisture, straw content and lindane content in a quadratic model.

The *biomass/propagation model* was found to depend on all factors. Therefore, the indirect measurement of the mycelium front propagation velocity cannot be used in experimental studies in lieu of the direct biomass determination.

The optima of the selected responses were determined utilizing the reduced adequate mathematical models as follows: propagation velocity 4.25 mm/day, biomass growth rate 408 mg/day, biodegradation/biomass 56.9 μg/g and biomass/propagation 250 mg/mm. In addition, the maximum fungal biomass content in solid mixture was found to be equal to 260 mg/cm$^3$.

An important future work is the investigation of remediation of pollutants such as lindane at in situ conditions. Field applications likely to be used involve solid phase approaches such as landfarming (spreading of polluted materials on soils usually combined with soil tillage and/or nutrient inputs), composting, or the use of biopiles (piles of polluted soils constructed to facilitate aeration and addition of nutrients).

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