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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.
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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,

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