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# Biological removal of dibenzothiophene from soil and changes to soil Sulfate by White-Rot Fungus *Phanerochaete chrysosporium*

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## ABSTRACT

This study investigated biodegradation of dibenzothiophene (DBT) in marsh soil spiked by white-rot fungus *Phanerochaete chrysosporium*. Soil samples were spiked with 100 ppm DBT and incubated at 30˚C in a dark chamber for 30 days. Samples were evaluated for pH, Mn-peroxidase activity, sulfate ion concentration and growth during the tests. Results showed maximum levels of pH, Mn-peroxidase and sulfate concentrations at 8.19, 66 U $L^{-1}$ and 31.21 mg $kg^{-1}$ respectively. Colony forming unit assay determinations for contaminated soil samples showed that the fungus was able to grow and use dibenzothiophene as a source of carbon and energy. GC analysis of contaminated samples compared to control samples, demonstrated degradation of DBT by *Phanerochaete chrysosporium*. There was no significantly improved effect on degradation when the treatment was performed in presence of soil micro flora. The system attained a high rate of DBT degradation at approximately 83.34%, the 30 day test operation under optimal conditions. After 20 days, the sulfate ion concentration in soil samples had almost doubled.

**Keywords:** biodegradation, dibenzothiophene, *Phanerochaete chrysosporium*, soil.

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## Introduction

Polycyclic aromatic hydrocarbons (PAHs) cover a large group of organic pollutants that are produced and circulated from incomplete combustion of organic materials. The most relevant of which are anthropogenic sources such as road traffic, fossil fuel combustion, oil spills; other sources of organic pollutants are naturally occurring such as forest fires (1). PAHs contaminate soil, water, and sediment and low water solubility means that they persist in ecosystems; furthermore PAHs present a significant risk to the environment and to human health (2). Since dibenzothiophene (DBT) is a typical recalcitrant organic sulfur compound in petroleum, biodegradation of DBT has been studied as a model organo- sulfur compound in fossil fuels (3).

Microorganisms are able to degrade and thereby detoxify organic contamination. So biodegradation of hydrocarbon-contaminated soil exploits this ability and is already established as an efficient, economic, versatile and environmentally compatible treatment of PAHs (4). The rate of biodegradation of hydrocarbon in contaminated soils is critically dependent on three factors: a) the creation of optimal environmental conditions to stimulate biodegradation activity; b) the predominant petroleum hydrocarbon types in the contaminated matrix and c) bioavailability of contaminants to microorganisms. Additionally, petroleum hydrocarbon degradation is affected by molecular composition of the hydrocarbons (5). A small number of bacterial genera, *Arthrobacter*, *Bacillus*, *Corynebacterium*, *Rhodococcus*, and *Mycobacterium* are known to be effective for DBT degradation (6). Other research has presented ligninolytic fungi as a possible alternative for degradation of PAH compounds (7). The lignin-degrading white rot fungus *Phanerochaete chrysosporium,* has the ability to degrade a wide variety of organo-pollutants due to its non-specific extracellular enzymes (8). *Phanerochaete chrysosporium* typically secretes two principal ligninolytic enzymes; lignin peroxidase (LiP, E.C. 1.11.1.14) and Mn- peroxidase (MnP, E.C. 1.11.1.13) but not laccase (LAC, E.C. 1.10.3.2) (9). Lignin peroxidase is a heme-protein isozyme secreted by the ligninolytic fungus *Phanerochaete chrysosporium* during secondary metabolism. This isozyme catalyzes oxidation of a large number of aromatic compounds by $H_2O_2$. The catalytic mechanism for lignin peroxidase is similar to that for other peroxidases. The resting enzyme is oxidized by $H_2O_2$ to form a two-electron oxidized intermediate, compound I. Compound I then oxidizes a substrate molecule by one electron, forming a one-electron oxidized intermediate, compound II. Compound II returns to resting enzyme by oxidizing another substrate molecule via one electron (10). Manganese peroxidase (MnP) oxidizes phenolic compounds in the presence of $H_2O_2$ and manganese. This enzyme oxidizes Mn (II) to Mn (III), and in turn oxidizes monomeric phenol, phenolic lignin dimers and synthetic lignin via formation of phenoxy radicals (11).

Increased solubilization of poly aromatics in aqueous media would enhance the bioavailability of a pollutant and affect the potential level of degradation of such compounds (1). Soil slurry systems can be applied to facilitate PAH desorption from soil and thus, to increase PAH bioavailability for degradation.

The aim of this study was to evaluate biological removal of DBT by the fungus *Phanerochaete chrysosporium* in soil with poor levels of microorganism activity

## Materials and Methods

### Chemicals

Dibenzothiophene (DBT) was purchased from the local representative of Merck Company in Iran to tests fungal degradation of the complex sulfur-containing compound in terms of selectively. All other chemicals used in the tests were of analytical grade and commercially available.

## Microorganism

The fungal strain *Phanerochate chrysosporium* RP78 was considered in these tests, samples were kindly gifted from Dr. D. Cullen (forest products laboratory, medicine, WI, USA). Samples of the fungus were transferred from slant culture tubes (maintained at 4˚C and transferred every 6 months) to malt extract agar plates (per liter, 15g agar, 20g malt extract, 1 g peptone and 20g glucose) and kept at 30˚C for 5–7 days before being used for inoculum preparations.

### Preparation of the Fungal Inoculum

15ml Kirk media (12) were added to Erlenmeyer flasks. They were inoculated with three malt agar plugs of active mycelium and incubated at 30˚C. Carbon source for the culture medium was 10 g glucose and nitrogen source was 2g peptone per liter (pH 4.5). After autoclaving, a 1.5 ml dose of filter-sterilized thiamine was added to each flask from a stock solution (10 mg $l^{-1}$). After 7 days, fungal cultures from the flasks were homogenized in a sterilized blender for 1 min and used as inoculum, referred to as free mycelia.

### Soil Preparation

Uncontaminated soil was obtained from the research field of the Agriculture College of Tehran University in Karaj (Iran). The soil sample was analyzed with GC and results showed no trace of contamination. Soil was air-dried and all particles larger than 2 mm were removed to attain soil homogeneity. Soil characteristics were determined according to standard methods (13). Prior to use, soil was autoclaved (121˚C for 40 min for three consecutive days) and spiked with a solution of 1000 mg $l^{-1}$ of DBT, in acetone. Doses of 4 ml of this solution were added to soil samples of 40 grams to constitute final individual DBT concentrations of 100 mg $kg^{-1}$ soil. Then soil samples were placed in 500 ml flasks, shaken and kept under a fume hood for 2 h to allow evaporation of the solvent.

### Biodegradation Experiments

For consideration of DBT degradation in aqueous phase, the fungus *Phanerochaete chrysosporium* was cultured on malt extract agar plates (MEA) for 5-7 days. Then tubes containing 4 ml Kirk media (12) were inoculated with one malt agar plug of fresh mycelium and kept at 30˚C for 7 days. 250 ml Erlenmeyer flasks with 40 ml working volume were selected that contained 10-20 g $l^{-1}$ glucose, 2 g $l^{-1}$ peptone, 100 ml $l^{-1}$ basal III medium, 200 ml $l^{-1}$ fungal inoculum and 50 ppm DBT (1000 mg $l^{-1}_{acetone}$). Samples were incubated on a shaker at 120 rpm at 30˚C for 28 days. They were extracted with n-hexane/acetone (1:1) for analysis by gas chromatography. Fungal biomass, sulfate ion concentration and pH were measured on days 0, 7, 14, 21 and 28.

500 ml Erlenmeyer flasks with 200 ml working volume were selected for consideration of DBT degradation in the soil slurry phase. The soil was treated batch wise as a slurry phase with the soil/water ratio of 1:4. 40 gram samples of spiked soil were each put in to an Erlenmeyer flask and supplemented with 25 g glucose and 100 ml basal III medium in per kilogram soil. Fungal

Inoculums of 15 ml were added to samples treated with the fungus then contents of flasks were adjusted on 200 ml with sterile distilled water. The slurry flasks were incubated on a shaker at 120 rpm at 30˚C in dark chamber for 30 days. For consideration of microorganism activity as fungus, microflora and combinations of both cultures under different experimental conditions were assayed according to the following: (experiments were repeated three times at two time periods).

The experiment was arranged according to a completely random design with four treatment combinations and three replications.

a) Sterile soil + DBT (abiotic control)

b) Sterile soil + DBT + *Phanerochate chrysosporium* (pure culture)

c) Unsterile soil + DBT (endogenous culture)

d) Unsterile soil + DBT + *Phanerochate chrysosporium* (mixed culture)

First, DBT degradation was considered in aqueous phase to determine fungal growth of *P. chrysosporium* and degradation of DBT in an aqueous medium: Preparation of fungal inoculums was made according to the following procedure; fungus *Phanerochaete chrysosporium* was cultured on malt extract agar plates (MEA) for 5-7 days. Then a one-plug amount was taken from this medium and added to each tube containing 4 ml Kirk media and kept at 30 ˚C for 7 days. For consideration of DBT degradation in aqueous phase, 250 ml Erlenmeyer flasks with 40 ml working volume were selected that contained 10-20 g/l glucose, 2 g/l peptone, 100 ml/l basal III medium, 200 ml/l fungal inoculums and 50 ppm DBT. Erlenmeyer flasks were incubated on a shaker with 120 rpm at 30 ˚C for 28 days. On days 7, 14, 21 and 28 extracts were taken from samples. Measurement of fungal biomass in the presence of contaminant, sulfate ion and pH, indicated fungal growth and sulfur oxidation. Gas chromatography analysis of samples treated with DBT, showed biodegradation of DBT and were compared to control samples that showed none (experiments were done in three replications).

**Extraction and GC-MS Analyses**

Shaking extraction was conducted as follows: 8 ml of soil suspension were poured in a 20 ml glass vial, and then an 8 ml dose of n-hexane/acetone (1/1) solution was added. After agitation for 10 min in a tube shaker, soil particles were separated from the solvent by centrifugation at 3000 rpm for 10 min and the supernatant was transferred to another vial, then 1 g of anhydrous sodium sulfate was added and the extract was placed in an amber vial and the solvent allowed to evaporate under an argon stream until it reached the state of near dryness. Then, 1 ml of n-hexane was added to each sample that was then analyzed using GC-MS to determine residual levels of DBT concentration in soil samples.

The gas chromatograph (Agilent Technology, 6890N) was equipped with a capillary column HP-5% phenyl methyl Siloxane (30 m×0.2 mm×0.25 μm). The oven temperature was programmed from 90˚C to 280˚C with an increasing rate of 5˚C $min^{-1}$, and held at 280˚C for 32 min. The injector was set at the temperature of 280˚C.

**Enumeration of Soil Microorganisms**

Agar plates (containing per liter: 10 g glucose, 15 g agar, 10 g malt extract, 100 ml BIII mineral medium, 8 drops Chloramphenicol sterile eye drops) were inoculated with samples from flasks to monitor the most dominant microorganisms growing in the flasks. An aliquot of 0.1 ml from $10^{-5}$ dilution was added and incubated at 30˚C (typically 6–7 days for pure fungal cultures).

**Mn-Peroxidase Activity Assay**

Manganese-peroxidase (MnP, EC1.11.1.13) activity was determined by the phenol red oxidation (14): 0.5 ml of culture supernatant was added to a 1-l cuvette containing 0.25 mol $l^{-1}$ of sodium lactate (0.1 ml), 2 mmol $l^{-1}$ $MgSO_4$ (0.05 ml), 5% bovine serum albumin (0.2 ml), 2 mmol $l^{-1}$ $H_2O_2$ in 0.2 mol $l^{-1}$ of succinate buffer pH 4.5 (0.05 ml), and 1% phenol red (0.1 ml). After 5 min incubation at 30˚C, 2 M NaOH (40 ml) was added to cease the reaction and absorbance was measured at 610 nm.

**Sulfate Assay**

10 g samples of air-dried sieved soil were weighed into a 50 ml Erlenmeyer flask. 25 ml of mono calcium phosphate extracting solution (15) were added and shaken at 200 rpm for 30 minutes. 0.25 g of charcoal was added to each sample and shaken for an additional 3 minutes. Samples were filtered through sulfate-free filter paper (Whatman No. 42 or equivalent). 10 ml of filtrate was selected from the extraction process and standard solutions (15) were pipetted into a 50 ml Erlenmeyer flask, to which 1 ml dose of acid "seed" solution (15) was added. The solution was swirled, and then a 0.5 g dose of $BaCl_2$-$2H_2O$ crystals was added to each. The mixture was allowed to stand for one minute and then the solution was swirled frequently in the flask until the crystals had dissolved. Within the time interval of 3 to 8 minutes, measurement of transmittance or optical density was read using a spectrophotometer (6705 UV / Vis. JENWAY) at the wavelength of 420 nm. Evaluations for percentage transmittance reading vs. concentration were plotted on semi-log paper. The concentration of sulfate concentration was determined for each sample from the standard curve. Calculations were made according to a 10 g sample of soil, 25 ml of extracting solution, and a 10 ml aliquot:

$$\text{mg } SO_4\text{-S/kg of soil} = \frac{\text{mgS/l} \times 0.025\ \text{l}}{0.010\ \text{kg soil}} = \text{mg S /l} \times 2.5$$

Analysis of variance was performed for all traits by SAS software. Means of values were compared using analysis of variance and Duncan's procedure.

**Table 1. Physical-chemical characteristics of the farming soil used for experimentation**

| Character | Farming soil |
|---|---|
| pH | 7.5 |
| EC (dS/m) | 2.29 |
| Soil texture | loam |
| F.C (%) | 18.84 |
| Nitrogen concentration (%) | 0.10 |
| Phosphorus concentration (mg/Kg) | 21.2 |
| Organic matter (%) | 1.58 |
| $Mg^{+2}$ (meq/L) | 7.2 |
| $Na^{+}$ (meq/L) | 4.5 |
| $Ca^{+2}$ (meq/L) | 16.2 |
| $K^{+}$ (meq/L) | 0.19 |
| Fe (mg/kg) | 3.36 |
| Mn (mg/kg) | 2.23 |
| Zn (mg/kg) | 0.69 |
| Cu (mg/kg) | 1.21 |
| $SO_4^{-2}$ (mg/Kg) | 15.33 |

## Results

### Soil Properties

The soil was loam and constituted 26.6% clay, 37.6% silt, and 35.8% sand. Contents of organic matter and total nitrogen were 1.58% and 0.10%, respectively. Physical and chemical characteristics of the soil used for tests are shown in Table 1.

### Growth and Ligninolytic Activity of *P.chrysosporium*

In this study degradation of DBT was performed in a soil slurry system. Operational parameters such as pH, CFU and Mn-Peroxidase activity were monitored during fermentation (Figure 1 a, b and c).

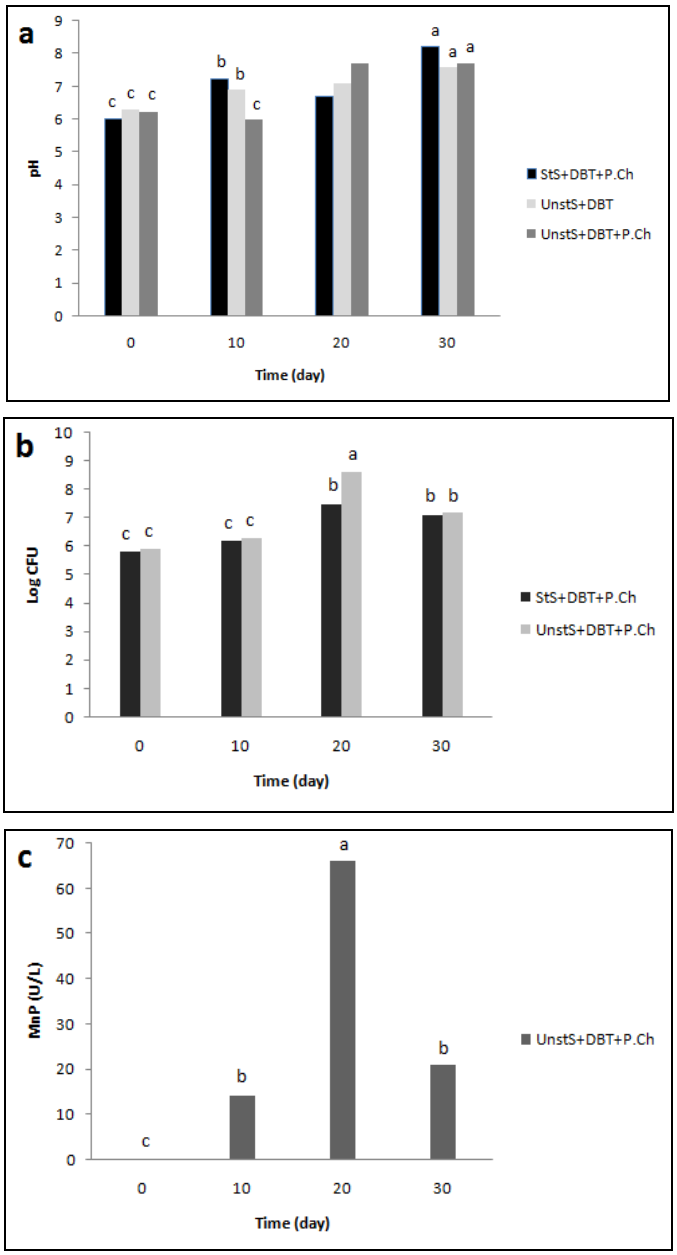


**Figure 1. Evolution of pH (a), CFU (b) and MnP activity (c) in soil slurry system. UnstS: Unsterile soil, P.Ch: *Phanerochaete chrysosporium*, StS: sterile soil, DBT: dibenzothiophene**

Results showed that soil microorganism communities did not change significantly during the first 10 days. This result can be attributed to the operational conditions imposed by tests to maintain a soil slurry system (soil water ratio 1:4 and 120 rpm) that were somewhat extreme for the fungal growth.

The maximal MnP was produced in day 20 as a secondary metabolite (16) that was only 66U $l^{-1}$. This lower MnP production could be associated to stress conditions in the soil slurry system (17, 18). DBT degradation may not only be associated with MnP production by the white-rot fungi because in similar fermentations without soil, this value was as much as 10–25-fold higher (19).

Initially there was little evidence of microorganism growth in either pure culture or mixed culture. Figure 1 b shows increased microorganism communities in both treatments after 20 days. This result determines that microorganisms were compatible with the contaminated soil and used DBT as a carbon and energy source. Glucose that had been added to soil at the initial stage and the indigenous microorganism community became inactive during early days of the tests so the fungus had to use DBT as a carbon source.

**Influence of Fungal Inoculum and Mixed Cultures on DBT Degradation**

In the artificially contaminated soil, abiotic processes such as volatilization and adsorption on soil colloids (clay minerals and humus particles) played an important role in decontamination of petroleum hydrocarbons (20). In this study, soil slurry fermentation was performed in presence of the pollutant in sterile soil to demonstrate that a part of DBT added to the soil remained undetectable (data not shown). Results of DBT degradation with the fungal inoculum and mixed culture are presented in Figure 2. Gas chromatographic analysis demonstrated biological decontamination of DBT in sterile soil with fungal inoculums and mixed culture (unsterile soil) were 83.34% and 94.34% respectively within 30 days. Results showed that additions of inoculum to soil stimulated bioremediation of DBT. The most DBT was removed in the first 20 days. Fungal degradation appears to be the main process responsible for DBT removal from soil.

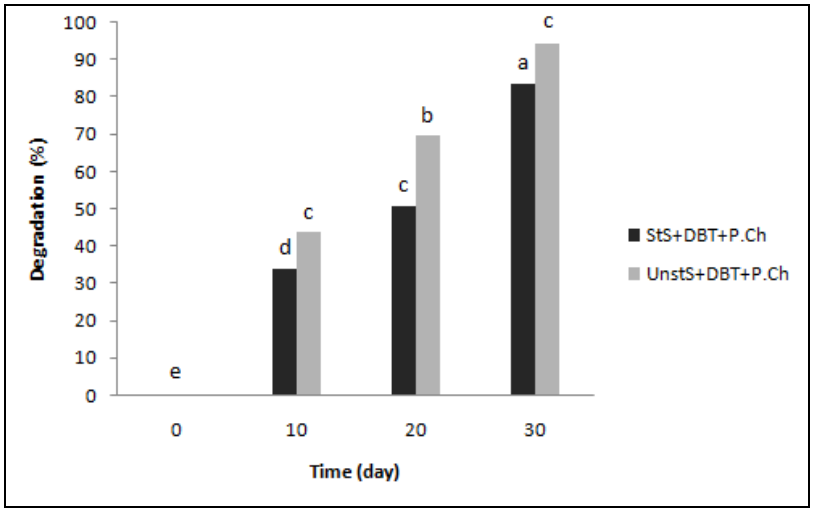


**Figure 2. Degradation profile of dibenzothiophene in the soil slurry system. UnstS: unsterile soil, P.Ch: *Phanerochaete chrysosporium*, StS: sterile soil, DBT: dibenzothiophene**

Oxidation of PAHs by white rot fungus *P. chrysosporium* by Zheng and Obbard (8) was investigated in a soil-slurry. Results showed that *P. chrysosporium* acted synergistically with soil the indigenous microorganisms in the process of oxidation of low molecular weight PAH (i.e. acenaphthene, fluorene, phenanthrene, fluoranthene and pyrene) in soil-slurry, where the rate of oxidation was enhanced by up to 43% in the presence of fungus. Degradation of four different PAHs by white-rot fungus *Bjerkandera adusta* in spiked marsh soil was evaluated in a slurry system by Valentin *et al*., (1). Degradation rate of B. *adusta* was reported at around 30 mg DBT/kg soil, after 30 days under optimal conditions. There was no significant improvement of on rate of degradation when the treatment was performed in presence of soil micro flora.

### Changes of Sulfate Concentration in Soil

Changes in sulfate ion in pure culture and mixed culture showed that this ion increased in different days. As Because of sulfur was have been released from DBT as $H_2S$ or sulfur oxidized to $SO_3$ And sulfate spontaneously (Equivalent mineralization) (Figure 3).

At the end of the experiment, concentrations of sulfate in soil samples had increased from 15.33 to 23.22, 19.55 and 31.21 mg $kg^{-1}$ in the treatments of sterile soil + fungus, unsterile soil and unsterile soil + fungus respectively.

## Discussion

In order to monitor growth of microorganisms during the slurry phase fermentation, agar plates containing glucose and malt extract were inoculated with samples taken from flasks under sterilized conditions. This monitoring was conducted for fungal and mixed cultures and the results are presented in Figure 2. Results concluded that DBT degradation occurred during pure and mixed cultures and that this was attributed to fungal action of the fungus *P. chrysosporium*. A few studies have considered white-rot fungi in soil slurry systems for bioremediation processes. In one of these, the ligninolytic fungus *Bjerkandera adusta* was used to degrade polycyclic aromatic hydrocarbons (dibenzothiophene, fluoranthene, pyrene and chrysene) with degradation 30 mg PAH kg $soil^{-1}$ after 30 days of incubation. PAH degradation from soil by white-rot fungi and bacteria has been previously been demonstrated in other studies (6, 21).

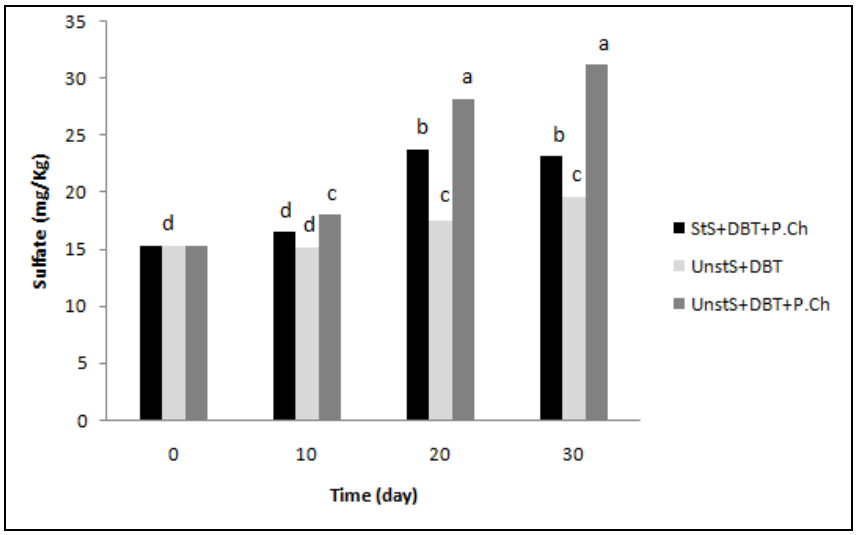


**Figure 3. Mineralization of DBT by *P. chrysosporium* and endogenous microorganisms in soil slurry system. UnstS: unsterile soil, P.Ch: *Phanerochaete chrysosporium*, StS: sterile soil, DBT: dibenzothiophene**

In the present study, fungal inoculum for removal of DBT achieved better results than those previously cited (22, 23, 24, 25, 26, 27 and 28).

Figure 3 demonstrates that the effect of fungus on increasing the level of sulfate was much higher than levels in soil with indigenous microorganisms. So it can be concluded that the effect of fungus in DBT degradation was much more than that of interaction of soil with indigenous microorganisms. This figure shows that *P. chrysosporium* released more sulfate, affected by DBT degradation.

Smith and Kelly (29) proposed a pathway for aerobic biodegradation of dimethyl dandisulfide by the *Thiobacillus thioparus* strain E6 that began with reductive cleavage of the disulfide to give two molecules of methanethiol. These authors postulated that an oxygen-requiring reaction released $H_2S$ from methanethiol and that the $H_2S$ was rapidly oxidized to sulfate. Although there was stoichiometric conversion of the disulfide sulfur to sulfate, Smith and Kelly (30) could not detect $H_2S$ in their cultures. Similarly (3), sulfate was released when the *Variovorax sp*. strain RM6 degraded 2, 2-dithiodibenzoic acid. Rozlyn *et al*., (3) were unable to detect $H_2S$ in these cultures. They expressed strain RM6 mineralized 2, 2-dithiodibenzoic acid and released 88% of the sulfur as sulfate.

Finally, results show that *P. chrysosporium* was a suitable candidate for cleaning-up soil contaminated with DBT in slurry phase batches because it is capable of robust growth and efficient extracellular enzyme production in soil. *P. chrysosporium* was able to degrade around 83.34% DBT after 30 days of operation. Moreover, results of DBT degradation obtained from fermentation with fungus and a mixed culture (white-rot fungi + endogenous microorganisms) indicate that DBT degradation was mainly attributed to white-rot fungal activity. Due to mineralization of DBT, finally amounts of sulfate had almost doubled.

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