

ENZYMATIC BIOREMEDIATION OF ENDOSULFAN IN SOIL USING LIGNINOLYTIC EXTRACT OF SPENT MUSHROOM COMPOST OF *PLEUROTUS OSTREATUS*

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Abstract. Endosulfan is known for its non target toxicity in various compartments of ecosystem. Its presence in hotspots and agricultural fields is an environmental concern. In this study spent mushroom compost extract of *Pleurotus ostreatus* (SMCE) was evaluated for its potential in bioremediation against Endosulfan in different microcosms. Activities of Laccase and MnP were also monitored to observe their possible role in this process. The SMCE alone (M₁) and SMCE with H₂O₂(M₂) exhibited no significant difference in reduction of α - and β -Endosulfan. This suggested that stimulation of ligninolytic enzymes, Laccase and Manganese peroxidase, after addition of H₂O₂ as observed by monitoring their activity had no effect on bioremediation of this pesticide. In soil microcosms however, there was a significantly higher reduction in M₄(H₂O₂ + SMCE) than M₃(SMCE) suggesting that SCME not only served as source of complex enzymes but also as carbon resulting in enhanced soil indigenous microbial communities and enzymes. Under the effect of H₂O₂, certain enzyme complexes either already present in soil or introduced after SMCE addition worked in symbiotic way and resulted in higher reduction. Endosulfan sulfate was detected in all microcosms expect Endosulfan Sulfate while Endosulfan lactone was only formed in M₃ and M₄.

Keywords: *enzymatic bioremediation, spent mushroom compost, Endosulfan, soil, microcosms*

Introduction

In the past few decades use of agrochemical residues increased in the ecosystem, causing momentous contamination in many terrestrial regions resulting in poisoning of human foods (Carvalho, 2017). Organochlorine pesticides (OCPs), group of such agrochemicals are also known for high persistence and non target toxicity within ecosystem at each level of food chain (Jayaraj et al., 2016). Because of their bio accumulative nature (Zhang et al., 2015) these are among the priority pollutants according to the Stockholm convention (Tsai, 2010). Despite the ban, indiscriminate use of OCPs for farm activities is still in practice (Yadav et al., 2017). Majority of these

pesticides are located in overpopulated tropic and sub tropic areas (Wang et al., 2016; Buah-Kwofie and Humphries, 2017) but due to atmospheric circulation, they could be transported to areas where there was no history of their usage and production (Hageman et al., 2015).

Endosulfan, an important OCP, was also banned but its residues are still a bio concern in the ecosystem (Hu et al., 2014; Lal et al., 2010; Barcelo-Quintal et al., 2008; Wang et al., 2016) and because of their widespread nature they are considered nowadays a “global pollutant” and a challenge for land management agencies. The conventional methods for remediation of contaminated soil are costly and not ecofriendly (Ojuederie and Babalola, 2017), an alternative effective approach for detoxification of Endosulfan in the soil is an environmental responsibility. Few such methods include bio-augmentation, intrinsic bioremediation and phytoremediation (Singh and Singh, 2017) was investigated in the past and found the best because of their effectiveness in decontaminations (Uqab et al., 2016) and considered as the major pathway for Endosulfan degradation in soil (Ozidal et al., 2017).

One of the Endosulfan bioremediation approach is exploiting white rot fungi (WRF) for its treatment (Kamei et al., 2011). However, direct application of them in the soil under actual treatment is not a viable option because WRF are not stable in the soil under physiological stress due to extreme environmental conditions (Hatakka, 1994; Crawford and Ramachandra, 1993). The resultant end product of certain WRF is spent mushroom compost (SMC) which is left after production of fruiting bodies. Application of farmyard waste (Fogarty and Tuovinen, 1991) and other solid composts have got attention in the past for biodegradation of various OCPs including Endosulfan (Mukherjee, 2012; Al-Hassan et al., 2004). Application of organic wastes (solid and liquid form) alter the physicochemical behavior of pesticides in the soils (adsorption-desorption, mobility, degradation, etc.) and also has positive effects on soil quality (Marín-Benito et al., 2016). However, relatively long microbial lag phases tend to increase contaminant sorption within soil microspores (Manilal and Alexander, 1991).

In the past SMC of *Pleurotus* was used for treatment of polycyclic aromatic hydrocarbon (Li et al., 2010), biocide/fungicide (Juárez et al., 2011), synthetic and textile dyes (Papinutti and Forchiassin, 2010) and many other pollutants (Cole, 1998; Marín-Benito et al., 2016) because of its ability to tolerate and/or detoxify pesticides of complex nature (Ellouze and Sayadi, 2016) at high concentrations. SMC Extract (SMCE) contains variety of immobilized enzymes including Cellulose-degrading enzymes, xylin-degrading enzymes and lignin degrading enzymes (Nakajima et al., 2018) which were reported to be involved in process of bioremediation (Lim et al., 2013; Ko et al., 2005; Karigar and Rao, 2011). These enzymes are viable under stress condition which make SMC the best candidate to treat the contaminants under field conditions. Application of WRF directly at field level has not had much success due to the difficulties in growth to adequate biomass in soil (Chirnside et al., 2011). For bioremediation of certain pollutants in soil, production of sufficient biomass and acclimatization of that particular fungus is essential (Husaini, 2014). Extracellular ligninolytic enzymes of these WRF fungi do not lose their identity in soil and can be successfully used as bio remedial agent for treatment of herbicides Application of WRF directly at field level has not had much success due to the difficulties in growth to adequate biomass in soil (Chirnside et al., 2011). For bioremediation of certain pollutants in soil, production of sufficient biomass and acclimatization of that particular fungus is essential (Husaini, 2014). Extracellular ligninolytic enzymes of these WRF

fungi do not lose their identity in soil and can be successfully used as bio remedial agent for treatment of herbicides (Chirnside et al., 2011).

This showed that there is a clear gap in knowledge regarding role of ligninolytic enzymes in effluent treatment. In the present study we sought to determine the role of ligninolytic enzyme extract obtained from SMC of *Pleurotus ostreatus* in the treatment of Endosulfan. For this purpose, we tried to address this gap by extracting and concentrating two ligninolytic enzymes from SMC of this fungus, Laccase and Manganese peroxidase (MnP) and studied their potential for detoxification of this pesticide under incubation studies of soils using biometric flasks.

Materials and methods

Chemicals/gases

Endosulfan (analytical grade) was purchased from Sigma Aldrich (St. Louis, Missouri) (99.99%). Pestanal® grade organic solvents including n-hexane, acetonitrile and acetic acid were used. The salts used for extraction of Endosulfan from samples e.g., magnesium Sulfate (anhydrous) and sodium acetate trihydrate ($\text{NaCH}_3\text{COO}\cdot 3\text{H}_2\text{O}$) were also of Analytical grade. Gases used for GC-MS (helium) and for GC- μECD (nitrogen) were 99.999% pure. All the other chemicals used to monitor the activity of enzymes, Carbon dioxide, physicochemical properties of soil and SMC were of laboratory grade. Millipore water was used for measuring physicochemical properties of soil and SMC and extraction of pesticides.

Physicochemical properties of soil

Fine loamy Soil obtained from Organic Farming Orchard, National Agriculture Research Centre, Islamabad, Pakistan (73.127855°E , and latitude 33.666042°N) with no history of OCPs (established by GC- μECD analysis) was used for this study. The physical and chemical parameters e.g., particle size distribution (sand, silt and clay), pH, electrical conductivity (EC), total organic carbon, total nitrogen, Calcium Carbonate content (CaCO_3) were measured by using standard procedures (Sparks et al., 1996; Dane et al., 2002) and results are given in *Table 1*. The basic characterization of soil and incubation studies were carried out in Soil Environment laboratory, Land resources research Institute, National Agricultural research center, Islamabad, Pakistan.

Table 1. Physicochemical properties of soil used in experiment ($n = 5$)

Properties (units) of soil	
pH	8.04
EC ($\mu\text{S}/\text{cm}$)	2.49
Organic Matter(%)	0.901
Total CaCO_3 (%)	3.15
Texture class (sandy clay loam)	
Clay (%)	25.32
Silt (%)	22.55
Sand (%)	52.53

Concentrated SMC extract (SMCE)

Culture of *P. ostreatus* (WC-814) were shipped from Mushroom Research Program, Pennsylvania State University.

(i) Preparation of Spawn: Spawn was prepared by placing mycelium of above mentioned fungus onto steam-sterilized wheat grains (Cooled) at 22 °C. When the mycelium was completely grown through all the grain, the grain/mycelium mixture (known as spawn) was used to “seed” mushroom compost. (ii) Preparation of SMC: It was prepared by using 2 kg well chopped wheat straw, ½ kg of wheat bran and small quantity of calcium hydroxide Ca(OH)₂ in polyethylene bag. The bags were entirely sterilized by autoclaving at 121 °C and 15 psi for 50 min. On cooling the spawn (10 g) was added in each bag and was allowed to yield fruiting bodies. At the end of generation of fruiting bodies, the product leftover was called as SMC. (iii) Preparation of SMCE: 3 g fresh SMC Fresh (ground and sieve through 10 mm) was taken in an Erlenmeyer flask and then 30 mL sodium tartrate buffer (pH = 5.2) was added. The suspension was shaken at 22 °C and 150 rpm for 2 h as described by Lang et al. (1998) with slight modifications. Then, contents were extracted by squeezing manually using mira cloth and fluids was centrifuged (make and model) at the rate of 10,000 × g for 30 min (Márquez Araque et al., 2007). (iv) Concentration of SMCE: It was concentrated using Vivaspin 20 (10 kDa cutoff) – a disposable ultra-filtration device with twin vertical membrane for unparalleled speed in Centrifuge at the speed of 2800 × g.

Physicochemical properties of SMC

Physicochemical properties of concentrated extracellular SMCE e.g., pH (707 soil/compost pH meters), total organic carbon (TOC) (Tandon, 2005), protein and C: N ratio were determined using standard methods already used by Marín-Benito et al. (2016). Total N in SMC was determined by Kjeldahl method (Helrich, 1995) (Table 2).

Table 2. Physicochemical properties of concentrated spent mushroom compost extract used in experiment (n = 5)

Sr. No	Properties (units)	
1	pH	7.25
2	Total carbon (%)	16.62
3	Total nitrogen (%)	1.036
4	Total protein (%)	8.19
5	Organic matter (%)	30.66
6	C:N ratio	15:1

Ligninolytic enzymes activity of SMCE

UV-VIS (Perkin-Elmer-Lambda-25) at different wavelengths was used to monitor ligninolytic enzymes activities of concentrated extract with/without additions of hydrogen peroxide (H₂O₂) periodically (Table 3). Activities of manganese peroxidase (MnP) were determined by O-dianisidine (C₁₄H₁₆N₂O₂) oxidation at 460 nm (molar extinction coefficient = 29,400 M⁻¹ cm⁻¹) (Paszczyński et al., 1988). Similarly, Laccase activities were monitored using 2, 2'-azino-di-[3-ethyl-benzo-thiazolin-sulphonate (ABTS) as a substrate while activities of Lignin Peroxidase (LiP) were measured by

oxidation of Veratryl Alcohol (C₉H₁₂O₃) to veratraldehyde (C₉H₁₀O₃) at 310 nm (Tien and Kirk, 1988).

Table 3. Lignolytic enzyme activities of various microcosms during four-week incubation studies (n = 3)

Days	Microcosms containing Endosulfan					
	M ₁ ** (SMCE*+H ₂ O ₂)		M ₂ ** (SMCE only)		Control 1 and 2	
	Laccase	MnP	Lac	MnP	Lac	MnP
0	32±2.3	18±1.4	22±1.4	8±1.1	ND	ND
7	25±1.3	12±3.5	17±2.2	5±3.1	ND	ND
14	16±2.4	7±2.4	13±3.1	2±1.5	ND	ND
21	9±2.0	4±1.3	7±2.2	0±0	ND	ND
28	3±1.5	1±2.9	1±1.7	0±0	ND	ND

*SMCE: spent mushroom compost extract, M₁ and M₂ mean microcosm 1 and 2

Experimental set up for biodegradation of OCPs

Using concentrated aqueous SMC extract (SMCE)

Concentrated SPME was used as bio remedial to treat Endosulfan. Erlenmeyer flasks (250 ml) were autoclaved twice and were spiked with Endosulfan at the final concentration of 25 mgL⁻¹. Microcosms were treated in the following manner

- Microcosm 1(M₁): 10 mL of the collected concentrated SMCE
- Microcosm 2(M₂): 10 mL of the SMCE and 800 µl of H₂O₂ (0.4 mM)
- Control 1(C₁): 10 mL of boiled SMCE (to denature all the enzymes present in it). This control was used as associated control of M₁
- Control 2(C₂): 10 mL of boiled SMCE and 800 µL of H₂O₂ (0.4 mM). This control was used as associated control of M₂.

Application of concentrated SMCE in soil

Biometric flasks (250 mL) (Bellco, Glass Inc., Vineland, NJ) were used for preparation of soil microcosms while SMCE extract was used as bio remedial for Endosulfan decontamination. The main body of biometric flasks consisted of soil (25 g) spiked Endosulfan at final concentration of 25 mgkg⁻¹. During the experiment, side arm of flasks was filled with 50 ml solution of 0.4N NaOH [CO₂-free Double distilled water (DDW)] and was used as trap to monitor CO₂. Following five treatments were applied in main body of flasks already contaminated with mixture of Endosulfan isomers to access role of SMCE, derived from *P. ostreatus* in the process of bioremediation:

- Microcosm 3(M₃): Fresh soil (20 g) was amended with 10 mL of freshly prepared SMCE.
- Microcosm 4(M₄): 20 g fresh soil + 10 mL SMCE + 800 µL H₂O₂ (0.4 mM).
- Control 3(C₃): Associated control of M₃: 20 g soil+ denatured SMCE (10 ml).
- Control 4(C₄): Associated control of M₄: 20 g soil + denatured SMCE +H₂O₂(800µL H₂O₂ (0.4 mM) to monitor role of H₂O₂ activated sterilized SMCE in contaminated soil.

5. Microcosm 5(M₅) (abiotic control): 20 g double autoclaved soil + denatured SMCE to find role of other soil inhabitant microbes in biodegradation.

During both the experiment flasks were incubated at 22 °C for 28 days. During the experiment, moisture level (60%) was maintained by taping DDW. The flasks were weighed and corrected for evaporative water loss by addition of sterile water prior to sampling at each time series. At the end of experiment, the samples were immediately put in boiling water and subsequently mixing with 10 mL acetonitrile to stop enzymes activity. All the experiment was conducted with three replicate flasks to improve the confidence of results. Required number of microcosms were taken off (sampled as whole) after each seven days till the end of experiment.

Measurement of carbon dioxide (CO₂)

Sodium hydroxide (NaOH) present in trap of biometric flasks was used to monitor CO₂ production using traditional acid titration method (Paul et al., 1999). Acid/base titration was done (at the same time intervals) for measurement of total carbon dioxide evolved. Samples were taken at day 0, 7, 14, 21 and 28 and production of CO₂ was monitored. The total amount of CO₂ evolved from the treatment bio meters was used as measure of rate of mineralization.

Extraction of OCPs from microcosms and analysis

Extraction from SPME microcosm

SMCE microcosms contents were transferred to centrifuge tubes and thoroughly mixed for 1 min using vortex mixer (Velp scientific centre, Bohemia, NY). After addition of 10 mL of acetone: water (1:3, v/v), the content was transferred to separating funnel (Pyrex[®] Squibb, Corning, New York), acetonitrile (2 mL) added and shaken vigorously for 5 min manually followed by addition of petroleum ether (2.0 mL). Aqueous portion was discarded and organic layer was dried with 1 g Sodium sulfate (Na₂SO₄) (anhydrous) and gauged at 2 mL using rotary evaporator (Buchi-Rotavapour.R-210) (Hernandez-Rodriguez et al., 2006)

Extraction from soil microcosm

Quick, easy, cheap, effective, rugged, and safe (QuEChERS) extraction method (with cleanup) (Boes et al., 2015) was used for extraction of selected OCP from soil with slight modifications. After hydration of samples (10 g) for 30 min using water (10 mL) and acidified acetonitrile (10 mL) (99:1 v/v) in 50 mL tube, the contents were mixed for 5 min using vortex mixer (Velp scientific centre, Bohemia, NY) and dried and buffered by adding 4.0 g magnesium sulfate (anhydrous)(MgSO₄), 1.0 g sodium chloride (NaCl), 1.0 g trisodium citrate dehydrate (C₆H₅Na₃O₇·2H₂O) and 0.5 g disodium hydrogen citrate sesquihydrate (C₆H₈Na₂O₈). Centrifugation was done at 5000 × g for 5 min. After separation of acetonitrile layer, it was concentrated to 5 ml and was later mixed with water (2 mL) and n-hexane (10 mL) and swirled for 1 min. After 2 min, an aliquot of 9 ml of the upper n-hexane layer was collected and reduced again to 1.4 mL in an amber glass vials and stored at -20 °C. Analysis of Endosulfan in extracted samples was done using Gas Chromatography (7890B Agilent) equipped with micro-electron capture detector (G3440B). HP-5(30 m × 0.25 µm I.D × 0.320 mm) capillary column was used.

Gas chromatography (GC- 7890B Agilent) mass spectrometry (MSD-5977A Agilent) with column DB-5 Ultra inert (30 m × 0.25 mm i.d × 0.25 μm). Summary of conditions is shown in *Table 4*. Matching library used was Retention time lock Pesticides and endocrine disruptor MS Library (RTLPEST3.L). Recovery of Endosulfan (*Table 5*) was in accordance with acceptability criteria set in SANCO's (2017).

Table 4. Conditions for gas chromatography analysis using electron capture detector and mass spectrometer detector

Conditions		
	GC-MSD	GC-μECD
Column	DB-5 Ultra inert (-60 °C to 325 °C (350 °C) (30 m × 0.25 mm i.d × 0.25 μm)	HP-5 (30 m × 0.25 μm i.d × 0.320 mm)
Injector temperature	250 °C	225 °C
Carrier gas/purity/flow rate	Helium gas/99.999%/2 ml min ⁻¹	Nitrogen/99.999%/2 ml min ⁻¹
Detector temperature	280 °C	280 °C
Oven temperature ramping	Initial oven temperature 60 °C (0.5 min) was increased at 20 °C min ⁻¹ to 170 °C (3 min hold). Later on, it was increased at rate of 5 °C min ⁻¹ to 295 °C (44 min) to allow all the metabolites to elute from column	Initial oven temperature 80 °C (0.5 min) increased at 10 °C min ⁻¹ to 180 °C (held for 10), then increased at 15 °C min ⁻¹ to 250 °C and finally held for 10 min

Table 5. Recovery of Endosulfan isomers by selected extraction method (n = 5)

Compound	SMCE		Soil spiked with SMCE	
	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
α-Endosulfan	91.00	2.19	88.59	3.18
B-Endosulfan	99.22	0.59	96.73	0.78

Kinetic studies and statistical analysis

Simple first-order kinetics (SFO) was used found the most fitted kinetic equation for the set of experiments. The reduction in extractable amount of Endosulfan from the artificially spiked soil was calculated using *Equation 1* (Yang et al., 2014)

$$\ln C = a + K_1 t \quad (\text{Eq.1})$$

where $\ln C$ is natural logarithm of concentration, t is time given to compound for degradation and K_1 is first order rate constant.

Half-life (DT_{50}) was calculated by *Equation 2*:

$$DT_{50} = t_{1/2} = \frac{0.693}{k_1} \quad (\text{Eq.2})$$

For DT_{50} and DT_{90} calculations, a computer program R (version 3.0.3) with the kinfit software package was used. The best fitted SFO (simple first-order kinetics) was used. The data was statistically analyzed (analysis of variance) using complete randomized

design with two factors (treatments and times) at $\alpha = 0.05$ by Statistix 8.1 (Analytical Software, USA) computer program.

Results and discussion

This study was designed to find the potential of ligninolytic enzymes extract obtained from SMC to treat this pesticide in soil. In order to make the enzymatic effect clear, concentration of the enzyme and treatment of the microcosms with or without H_2O_2 was done. SMC has many immobilized enzymes that could serve as bio remedial agent (Valentin et al., 2010). Therefore, some researchers applied SMC directly in soil to treat contaminants (Marín-Benito et al., 2016). Direct application is beneficial because it acts as the substrate to change the bioavailability and immobilization of pesticides in the soil. However, while studying the role of ligninolytic enzymes in bioremediation, direct use of SMC is also a big problem because of adsorption of pesticides onto fungal biomass and compost which hinders true revelations. Therefore, extraction of enzymes from SMC and their application in soil is another option.

Bioremediation of Endosulfan using only SMCE

After application of Endosulfan in flask, microcosms were treated with SMCE and bioremediation of both the isomers of this pesticide was studied. In M_1 where H_2O_2 was added in microcosm to stimulate the effect of enzyme in SMCE, 24.99% of applied α -Endosulfan was reduced at the end of first week of incubation. At the end of 4th week, 58% of applied extractable α -Endosulfan was removed. However, 56.85% of removal was observed in M_2 , where no H_2O_2 was added. It showed addition of H_2O_2 did not play any substantial role in reduction of applied α -Endosulfan as there was no significant difference ($\alpha = 0.05$, $p < 0.01$) in both above mentioned microcosms, M_1 and M_2 (Table 6).

Table 6. Bioremediation of Endosulfan under different microcosms to access the role of extracellular enzyme extract, where M_1 and M_2 are Microcosm 1 and 2, while C_1 and C_2 are their associated controls ($\alpha = 0.05$; $p < 0.01$)

Days	Relative reduction (%) of applied concentration									
	α -Endosulfan					β -Endosulfan				
	M_1	M_2	C_1	C_2	Means	M_1	M_2	C_1	C_2	Means
0	0.00	0.00	0.00	0.00	0 ^e	0	0.00	0.00	0.00	0 ^e
7	24.99	17.73	6.68	3.20	13.15 ^d	7.64	6.85	2.85	1.25	4.65 ^d
14	40.22	43.37	8.35	6.61	24.64 ^c	14.65	10.57	3.64	2.99	7.96 ^c
21	48.25	50.05	10.93	9.01	29.56 ^b	18.42	16.76	4.96	3.46	10.9 ^b
28	58.00	56.85	12.36	10.25	34.37 ^a	20.87	21.49	5.49	4.59	13.11 ^a
Means	34.29 ^a	33.6 ^a	7.66 ^b	5.184 ^b	20.34	12.32 ^a	11.13 ^a	3.39 ^b	2.46 ^b	7.324

α -Endosulfan: LSD for Days 2.69; LSD for treatments 3.21

β -Endosulfan: LSD for Days 1.22; LSD for treatments 1.46

The calculated rate of dissipation using SFO kinetics was not substantially different in both the microcosms which resulted in DT_{50} equal to 22 days and 21 days for M_1 and M_2 respectively (Table 7). C_1 and C_2 which were the associated control of M_1 and M_2 ,

showed the reduction of only 12.36% and 10.25% of applied α -Endosulfan, respectively. Ultimate DT_{50} calculated for C_1 was 154 days ($K_1 = 0.0045$) while under C_2 it was 173 days ($K_1 = 0.004$). However, there were a significant difference between each microcosm and their associated controls (*Table 6*). In the past, it was reported that presence of H_2O_2 in ligninolytic system is responsible for faster bioremediation or decolonization (Vyas and Molitoris, 1995; Eichlerová et al., 2006). However, in this case, addition of H_2O_2 did not pose any effect on reduction of M_2 than M_1 where no H_2O_2 was added.

Table 7. Kinetic of dissipation of Endosulfan in spent mushroom compost extract using simple first order kinetic model

Experimental microcosm	α -Endosulfan				
	Kinetic equation	K_1	DT_{50}	DT_{90}	R^2
M_1	$LnC=4.56-0.0318t$	0.0318	21.80	72.40	0.99
M_2	$LnC=4.58-0.033t$	0.033	21.00	69.80	0.97
C_1	$LnC=4.59-0.0045t$	0.0045	154.03	511.69	0.91
C_2	$LnC=4.60-0.004t$	0.004	173.29	575.65	0.98
Experimental microcosm	β -Endosulfan				
	Kinetic equation	K_1	DT_{50}	DT_{90}	R^2
M_1	$LnC=4.59-0.0087t$	0.0087	79.67209	264.665	0.97
M_2	$LnC=4.60-0.0085t$	0.0085	81.54673	270.8924	0.99
C_1	$LnC=4.60-0.00194t$	0.00194	357.2924	1186.9	0.91
C_2	$LnC=4.60-0.00167t$	0.00167	415.0582	1378.793	0.97

The other isomer of Endosulfan, β -Endosulfan showed the similar reduction pattern (*Table 6*) but the rate of this reduction was about $\frac{1}{3}$ of that observed in case of α -Endosulfan. Total removal of this isomer, during incubation period, in M_1 and M_2 was 20.87% and 21.49% respectively showing there was no significant difference ($\alpha = 0.05$, $p < 0.01$) in bioremediation efficacy of SMCE with or without H_2O_2 (*Table 6*). It shows that stimulation of enzymes by adding H_2O_2 as were mentioned in another study (Chirnside et al., 2011) had no role in reduction of β -Endosulfan. DT_{50} , calculated for M_1 and M_2 were 80 days and 82 days respectively. It also showed autoclaving to denature the SMCE was responsible for the slow rate of removal (*Table 7*) because it eliminated biotic factor including enzymes and microbes in associated controls.

SMCE of *P. ostreatus* was monitored for two main ligninolytic enzymes MnP and Laccase enzyme activities in all the microcosms (*Table 3*). Immediately after addition of H_2O_2 on day zero, the activities of both the enzymes, Laccase and MnP, were higher showing stimulatory effect of H_2O_2 on M_1 (*Table 3*). All the activities had downward trend at each sampling time which shows that with the passage of incubation time, the enzymes activities were declined. The activities of MnP and Laccase in M_2 where no H_2O_2 was added were lower than M_1 at each time interval (*Table 3*). M_1 showed significantly more activities of Laccase and MnP than M_2 but there was no significant difference in reduction of both the isomers of Endosulfan. For example, a study conducted by Ulčnik et al. (2013) showed that purified laccase obtained from this fungus and other related ligninolytic enzymes were found to play no role in the process of bioremediation. Another study conducted by Purnomo et al. (2010) also showed that

ligninolytic enzyme are not involved in the bioremediation of OCPs. Same is proved in our case.

During the course of study, oxidation occurred and only one dechlorination metabolite, Endosulfan sulfate was formed in flasks from day 7 and no other metabolite was detected on GC-MSD.

Bioremediation of Endosulfan using SMCE in Soil

For preparation of soil microcosms, biometric flasks containing soils treated with SMCE were incubated for 28 days at 22 °C. NaOH solution was added in the side arm of biometric flask and served as trap of CO₂. Application of SMCE in fresh soil (M₃) resulted in total reduction of 41.62% of applied concentration of α -Endosulfan at the rate 0.013 days⁻¹. Resultantly, calculated DT₅₀ was 51 days ($r^2 = 0.98$). Addition of H₂O₂ along with SMCE in soil (M₄) resulted in total reduction up to 47.16% while under M₅ (abiotic microcosm), the reduction of 7.42% at the rate $K_1 = 0.013$ days⁻¹ was observed which is less than both M₃ and M₄. Denaturation of all the biotic factors present in soil and SMCE (M₅) resulted in least reduction of applied α -Endosulfan (Fig. 1). There was a significant difference in reduction between M₃ and M₄ ($\alpha = 0.05$, $P < 0.01$). Since there are various other constituents in the soil that could be stimulated by addition of H₂O₂ (not necessarily enzymes from added extract) which resulted in significant difference in amount of reduction of α -Endosulfan in M₄ than M₃. The role of addition of SMCE in M₃ and M₄ in bioremediation was assessed by running associated controls (C₃ and C₄). The reduction of α -Endosulfan under M₃ was significantly different from its associated control (C₃). Same was true for M₄ and associated control.

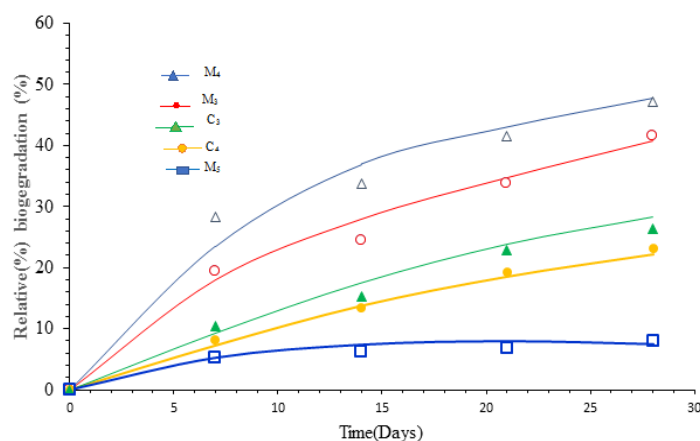


Figure 1. Relative reduction of α -Endosulfan in soil using spent mushroom compost extract in different microcosms

Similarly, recorded reduction for β -Endosulfan under M₃ was 32.40% at the rate of 0.016 day⁻¹ while its associated control C₃ exhibited reduction equals to 20.05% at the rate of 0.0078 day⁻¹ (Fig. 2; Table 8). Similar pattern was observed in M₄ and its associated control C₄. Removal of Endosulfan isomers in C₃ and C₄ revealed the role of denatured SMCE as carbon source (Kodjo-Wayo, 2006) (Fig. 2). In case of abiotic control (M₅), total of 2.84% of applied β -Endosulfan was reduced. Detailed DT₅₀ calculated based on equation 2 and rates of all the treatments are given in Table 8.

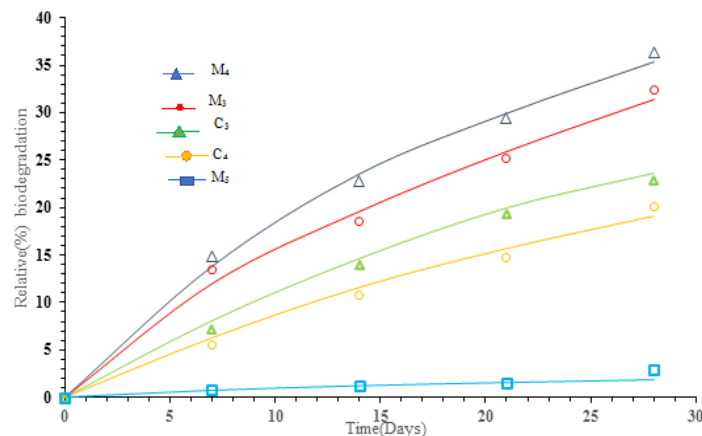


Figure 2. Relative reduction of β -Endosulfan in soil using spent mushroom compost extract

Table 8. Dissipation kinetic of Endosulfan in soil treated with SMCE using SFO model

Kinetic studies of Endosulfan in soil using SMCE						
Isomers	Treatments	Kinetic equation	K ₁ (day ⁻¹)	DT ₅₀	DT ₉₀	r ²
α -Endosulfan	M ₃	4.58-0.0133t	0.013	51.41	170.77	0.98
	M ₄	4.52-0.0211t	0.023	29.65	98.48	0.91
	C ₃	4.60-0.009t	0.009	73.38	243.75	0.99
	C ₄	4.59-0.011t	0.011	62.45	207.461	0.98
	M ₅	4.59-0.002t	0.002	259.71	862.74	0.86
β -Endosulfan	M ₃	4.58-0.02t	0.016	43.2	143.49	0.98
	M ₄	4.57-0.0186t	0.0186	37.08	123.17	0.97
	C ₃	4.60-0.007846t	0.0078	88.33	293.46	0.97
	C ₄	4.58-0.0095t	0.0096	72.48	240.79	0.99
	M ₅	4.61-0.00094t	0.0009	737.75	2450.76	0.95

Addition of SMCE initiates oxidative pathways and produces free radicals in soil which are removed by soil constituents and cause less reduction of toxic compounds in soil bio meters (Chirnside et al., 2011) than flasks which contained only SMCE (M₁ and M₂). This was evident in this finding where SCME alone reduced more Endosulfan than after addition in soil. The overall reduction of both the isomers was higher in SMCE microcosms without soil (M₁ and M₂) than the microcosms where SMCE was added in soil (M₃ and M₄). Presence of total organic carbon (TOC) in denatured SMCE resulted in decontamination of Endosulfan in soil because of its role in increasing the indigenous population of soil microbes (Guerin, 1999; Özyer et al., 2016). In our case, SMCE was rich in organic carbon (Table 2), which further helped to enhance the population of indigenous microbes (Zhang et al., 2013) in the soil. This increase in Endosulfan removal in H₂O₂ amended microcosm of soil M₄ indicated that addition of H₂O₂ in soil amended with SMCE stimulated certain soil enzymes and microbes which were not present in sole SMCE. Addition of SMCE within the soil involves couple of reactions whose nature is very difficult to explore. The less degradation of β -Endosulfan by application of SMCE in soil than α -Endosulfan could be due to inter-conversion of the

α -isomer to β -Endosulfan (Rice et al., 1997; Schmidt et al., 2001) and also microbial species prefer α -Endosulfan for degradation over β -Endosulfan (Siddique et al., 2003). The bioremediation pathways for Endosulfan after application of SMCE were built based on the time of appearance of peaks, their position and centre of mass of concentration profiles of the metabolites detected by GC-MS. In all associated controls and treatments of M₁ and M₂, only Endosulfan sulfate was formed (Fig. 3). Endosulfan lactone was only formed in M₃ and M₄ soil microcosms. Under M₅, there was no appearance of any metabolites. This is possibly because of absence of process of oxidation because of unavailability of microbes or enzymes. Formation of Endosulfan lactone is also in line with the findings of (Kataoka and Takagi, 2013). Other expected bioremediation products could be Endosulfan diol, Endosulfan ether, Endosulfan hydroxyether and Endosulfan monoaldehyde (Kullman and Matsumura, 1996) which were not detected during the course of this study. However, formation of Endosulfan lactone is evident for the formation of other above mentioned intermediate products and the fact that hydrolysis also occurred in the soil treated with SMCE.

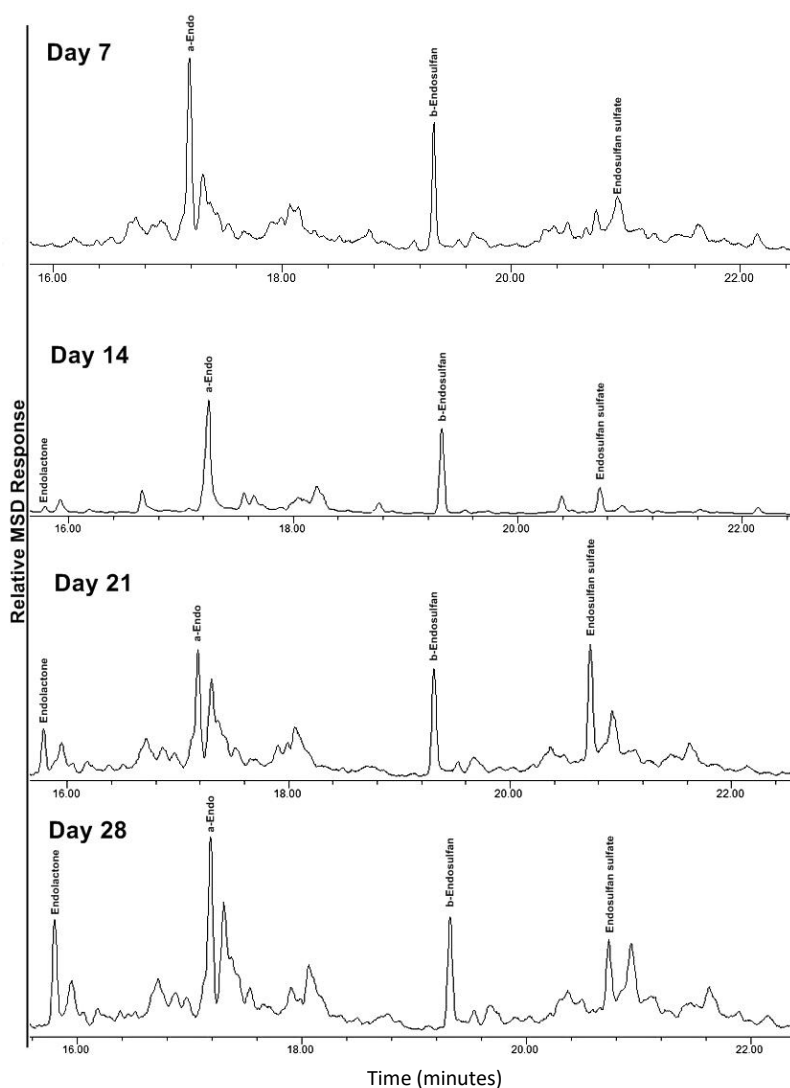


Figure 3. The metabolic products of Endosulfan detected at GC-MS after incubation of soil with spent mushroom compost extract. Formation of Endosulfan sulfate indicate oxidation while generation of Endosulfan lactone is the result of hydrolytic pathways

Emission of carbon dioxide is a bioremediation indicator and contaminant depletion can be estimated from the amount of carbon dioxide emitted from the system (Schoefs et al., 2004). Amount of CO₂ produced in spiked bio-meter flask with M₃ and M₄ was much more than associated controls of containing denatured SMCE (Fig. 4). Evolution of CO₂ was increased exponentially as experiment progressed. The evolution of CO₂ from M₅ flask was the slowest of all the microcosms. The highest amount of CO₂ generated was in M₄. It is observed that the emission and measurement of CO₂ was good qualitative indicator of bio mineralization. This similarity also existed for abiotic controls where the CO₂ production stayed very low. It can be assumed that since OCPs are very persistent and complex compounds therefore free radicals did not play any role in their oxidation. Nevertheless, the inactivated extract, used as control, showed significantly less Endosulfan decrease than the activated SCME extract. This suggested that both wash-off and volatilization had no impact on OCPs loss. This is in accordance with the studies conducted by Juárez et al. (2011). Since it is proved that Laccase and MnP have no role in bioremediation here, there could be another enzyme or enzymatic complex which is responsible for the degradation of Endosulfan. Although role of laccase in biodegradation of many pollutants has been widely studied (Karigar and Rao, 2011) but there is no direct evidence which connect the effect of presence or quantity of this enzyme in biodegradation of Endosulfan.

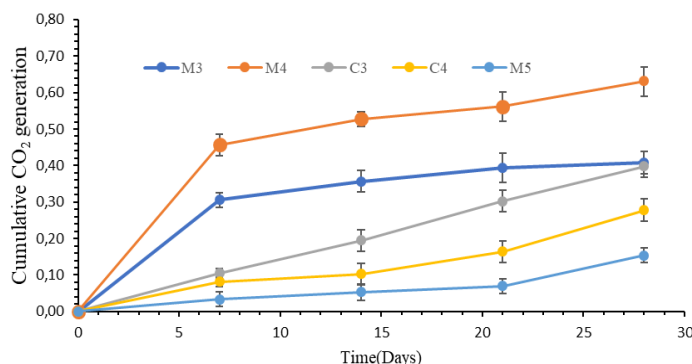


Figure 4. Evolution of CO₂ during the incubation studies of the Endosulfan bioremediation by application SMCE treatments in soil, where M₁ is microcosm having fresh soil and SMCE and M₂ is microcosm that contain H₂O₂ along with while C₁ and C₂ are the associated controls of microcosms 1 and 2. M₅ is the abiotic control microcosm

Conclusion and recommendation

In order to avoid the problem of adsorption, instead of its direct application spent mushroom compost extract was used to evaluate the role of enzyme complex in bioremediation of Endosulfan. Five different microcosms along with associated controls were used to demonstrate the role of SMCE. This study revealed that lignolytic enzyme stimulation after addition of H₂O₂ does not affect the bioremediation efficacy of SMCE, rather it has certain other extracellular enzymes that could possibly be involved in process of decontamination. This was evident from almost equal reduction of this pesticide in the presence and absence of H₂O₂. In soil, this extract showed some effect of addition of H₂O₂. There was significantly more reduction of both isomers in the soil microcosm containing SMCE spiked with H₂O₂ than the other where H₂O₂ was not spiked. Associated controls showed if SCME was denatured after sterilization, there

was less reduction in both isomers of Endosulfan. It also indicated the role of stimulation of indigenous soil microbes/enzymes in the presence of H₂O₂ and the role of SMCE as carbon source to enhance the microbial population of soil. All these results provide a basis for the development of new bioremediation strategy for decontamination of Endosulfan in soil. However, there is a strong need of understanding the complex nature of reactions involved in symbiotic interaction between SMCE and soil constituents which were responsible for more reduction of Endosulfan in soil.

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