Bioremediation of Fungicides by Spent Mushroom Substrate and Its Associated Microflora

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Abstract Experiments were conducted both under in vitro and in situ conditions to determine the biodegradation potential of button mushroom spent substrate (SMS) and its dominating microbes (fungi and bacteria) for carbendazim and mancozeb, the commonly used agricultural fungicides. During 6 days of incubation at $30 \pm 2^\circ\text{C}$ under broth culture conditions, highest degradation of carbendazim (17.45%) was recorded with B-1 bacterial isolate, while highest degradation of mancozeb (18.05%) was recorded with *Trichoderma* sp. In fungicide pre-mixed sterilized SMS, highest degradation of carbendazim ($100.00\text{--}66.50 \mu\text{g g}^{-1}$) was recorded with mixed inoculum of *Trichoderma* sp. and *Aspergillus* sp., whereas highest degradation of mancozeb ($100.00\text{--}50.50 \mu\text{g g}^{-1}$) was with mixed inoculum of *Trichoderma* sp., *Aspergillus* sp. and B-I bacterial isolate in 15 days of incubation at $30 \pm 2^\circ\text{C}$. All these microbes both individually as well as in different combinations grew well and produced extracellular lignolytic enzymes on SMS, which helped in fungicides degradation. Under in situ conditions, among three different proportions of SMS (10, 20 and 30%, w/w) mixed with fungicide pre-mixed soil ($100 \mu\text{g g}^{-1}$ of soil), the degradation of carbendazim was highest in 30% SMS

treatment, while for mancozeb it was in 20% SMS treatment. The residue levels of both fungicides decreased to half of their initial concentration after 1 month of SMS mixing.

Keywords Bioremediation · Spent mushroom substrate (SMS) · Carbendazim · Mancozeb · Spectrophotometer

Introduction

Environmental pollutants of diverse chemical nature are released into the environment through various industrial and agricultural activities. Upon entering into the biosphere, these chemicals are degraded at varied rates and it depends upon the physico-chemical properties of the medium and the population dynamics of potential microorganisms it harbours [1]. If these chemicals are not degraded instantaneously, they get immobilized on soil particles and become part of many interlinking food chains. In a country like India, where feeding to an increasing population has remained a challenging task; pesticides and chemical fertilizers had played a pivotal role in boosting the overall food production of the country. These chemicals have also become major contributors of the environmental pollutants and have affected a good proportion of the arable land. Basically, two types of processes [chemical degradation or biological (microbial) transformation] are involved in removal of the chemical pollutants from a particular system and working efficiency of these processes depends upon the prevailing environmental conditions. Among these two, microbial transformation is considered as environment friendly, as it does not add further chemicals in the environment. It can occur in any of the five different ways, which includes co-metabolic degradation, detoxification,

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polymerization and binding with natural compounds, extra-cellular or intracellular accumulation and mineralization [2].

The spent mushroom substrate (SMS) released after button mushroom cultivation contains all the essential nutrients needed for raising a healthy field crop in addition to harbouring fungal biomass and large population of heterotrophic microbes [3]. SMS also has the ability to chemically adsorb the organic and inorganic pollutants, while the diverse category of microbes it harbours have the capability of biological breakdown of the organic xenobiotic compounds present in soil and water [4–6]. The previous studies have also revealed several advantages of SMS on being used as an organic fertilizer, which includes its use as manure for nutritionally poor soil [7–10], amending acidic soils and in some cases for improving polluted sites [1, 11]. The present study was thus aimed at to study the role of SMS in biodegradation of carbendazim and mancozeb, the commonly used agricultural fungicides.

Materials and Methods

In Vitro Studies

Spent substrate of button mushroom was used for the isolation of dominant fungi and bacteria with their ability to biodegrade the two fungicides (carbendazim and mancozeb). The dominating fungi and bacteria were isolated by the method of serial dilution and plating on malt extract agar (MEA) and nutrient agar (NA) media, and incubation at $30 \pm 2^\circ\text{C}$. The dominating micro flora was selected based upon their colony forming units on respective media. The purified fungi were evaluated for their fungicide degradation potential by growing in ME broth with $100 \mu\text{g ml}^{-1}$ concentration of the respective fungicide at $30 \pm 2^\circ\text{C}$ for 6 days. The degradation of fungicides was measured by determining the changes in concentration of two fungicides by the method mentioned under the heading residue assay.

In second stage experiment, fungi (3) and bacterial isolates (2) exhibiting higher fungicide degradation potential under broth culture conditions were inoculated in different permutations and combinations in sterilized button mushroom spent substrate premixed with respective fungicide at $100 \mu\text{g g}^{-1}$ of SMS. The inoculated SMS was incubated at $30 \pm 2^\circ\text{C}$ for next 15 days and the fungicide degradation was recorded at an interval of 3 days each by measuring the changes in fungicides residue by the method mentioned under the heading residue assay.

In Vivo Studies

The study was carried out by mixing different proportions of the recomposted button mushroom spent substrate in soil

pre-mixed with 100 ppm concentration of the respective fungicide. The crops of tomato and pea were also raised on fungicide mixed and spent mushroom substrate amended soil to study the biodegradation of fungicide under natural field conditions.

Residue Assay

- (a) *Reagents* Reagents of sodium sulphate (anhydrous), sodium bicarbonate, ammonia, ethyl acetate, hydrochloric acid, potassium hydroxide, carbon disulfide, lead (II) acetate (trihydrate), stannous chloride, sulphuric acid, methanol, carbendazim (50% WP, BASF) and mancozeb (75%, Indofil) were prepared with analytical grade chemicals.
- (b) Preparation of standards and working solutions
 - (i) *Potassium hydroxide (0.5 M)* 7.0 g of potassium hydroxide (KOH) was dissolved in methanol in 250 ml volumetric flask and allowed to cool down at room temperature and made up to the mark with methanol. Potassium hydroxide (secondary standard) was standardized by titrating it with 0.5 M oxalic acid (primary standard) to make it exactly 0.5 M.
 - (ii) *Carbon disulfide ($10 \mu\text{g ml}^{-1}$)* 0.5 mg of carbon disulfide was taken in 50 ml volumetric flask and made up to the mark with methanol.
 - (iii) *Lead acetate (30%)* 30 gm lead acetate (trihydrate) was taken in 100 ml volumetric flask and final volume was made with distilled water.
 - (iv) *Stannous chloride (40% in HCl)* 40 g of stannous chloride was taken in 100 ml volumetric flask and final volume was made with concentrated hydrochloric acid.
- (c) *Mancozeb residue assay* Mancozeb residue was assayed as per the method of Dubey and Stan [12]. The apparatus was set up as per the protocol described in the manual of pesticide residue analysis [13] with modified adsorption tubes. The first absorption tube was poured with 25 ml of 30% lead acetate, while the second absorption tube with 15 ml of concentrated sulphuric acid. The third tube was kept in an ice-water bath ($0-4^\circ\text{C}$) and 15 ml of 0.5 M methanolic KOH was added in it. A known weight (50 g) of sample was poured into the flask and 150 ml water was added followed by heating for 15 min. To this 15 ml concentrated HCl + 20 ml stannous chloride was added and the sample was digested for 30 min. The decomposition was carried out following DFGSIS method of CS_2 determination. The third tube containing methanolic KOH along with xanthate was allowed to stand for 20 min at room temp after removal from

ice-water bath. Residue assay was carried out by recording the absorbance at 302 nm using UV–Visible spectrophotometer (Chemito 1500).

- (d) *Apparatus* Modified dithiocarbamate decomposition and distillation apparatus was used for hot acid decomposition of the sample.
- (e) *Carbendazim residue assay* Carbendazim residue was estimated as per the method of Nath et al. [14]. A 50 g sample with equal amount of anhydrous sodium sulphate and 2.5 ml of concentrated ammonia were blended at a high speed for 3 min with 100 ml of ethyl acetate. Sodium sulphate was used as an anti-emulsifying agent during blending. The upper organic layer was decanted and filtered over anhydrous sodium sulphate. The left over residue was again extracted with 100 ml ethyl acetate and filtered in a Buchner funnel under vacuum. The filtrates were mixed together and final volume was reduced to about 80 ml under vacuum at 40–45°C. 20 ml filtrate was taken in a separating funnel and was partitioned twice with 10 ml of 0.1 N HCl.

The lower aqueous phase was pooled in another separatory funnel followed by addition of 40 ml of ethyl acetate. To this about 30 ml of saturated sodium bicarbonate solution (80%) was added with vigorous shaking for 2 min. The lower aqueous phase was rejected. Organic phase was washed with water and then partitioned with 10 ml of 0.1 N freshly saturated HCl. Absorbance of 0.1 N HCL was recorded at 280 nm using UV–Visible spectrophotometer (Chemito 1500).

Results and Discussion

In Vitro Study

The data depicted in Figs. 1 and 2 reveal the fungicides biodegradation potential of 6 SMS dominating fungi and

Fig. 1 Biodegradation of carbendazim by SMS dominating microbes under broth culture conditions

Fig. 2 Biodegradation of mancozeb by SMS dominating microbes under broth culture conditions

bacteria under in vitro conditions. Out of six dominating microbes, highest degradation of carbendazim was recorded with B–I bacterial isolate during 6 days ($100\text{--}82.55\text{ }\mu\text{g mL}^{-1}$) of incubation at $30 \pm 2^\circ\text{C}$, whereas highest degradation of mancozeb ($100\text{--}81.95\text{ }\mu\text{g mL}^{-1}$) was recorded with *Trichoderma* sp. The degradation of two fungicides in control was gradual and far less as compared to dominating fungi and bacterial isolates. In control, the degradation of mancozeb was almost negligible ($100\text{--}93.75\text{ }\mu\text{g mL}^{-1}$). Very scanty information is available on fungicide biodegradation potential of SMS and microbes inhabiting in it. However, role of white rot fungi particularly, *Phanerochaete chrysosporium* and a putative peroxidase mutant has been studied for co-mineralization of a mixture of pesticides including 2,4-D and 2,4,5-T under nutrient rich broth conditions, wherein the mycelial fractions were recorded to contain only 5% of the pesticides [3, 4].

The fungicide degradation potential of SMS dominating fungi and bacterial isolates was also studied for 15 days at $30 \pm 2^\circ\text{C}$ by inoculating them in different permutations and combinations in sterilized fungicide mixed button mushroom SMS (at $100\text{ }\mu\text{g g}^{-1}$ of SMS). Carbendazim was degraded from lowest of 11.90% ($100\text{--}88.10\text{ }\mu\text{g g}^{-1}$) in control to highest of 33.50% ($100\text{--}66.50\text{ }\mu\text{g g}^{-1}$) in mixed inoculum of *Trichoderma* sp. and *Aspergillus* sp. It was followed by 33.00% degradation in mixed inoculum of *Trichoderma* sp., *Aspergillus* sp. and B–IV bacterial isolate (Table 1). The degradation of mancozeb was higher than carbendazim and after 15 days of incubation, it was highest of 49.50% ($100\text{--}50.50\text{ }\mu\text{g g}^{-1}$) in mixed inoculum of *Trichoderma* sp., *Aspergillus* sp. and B–I bacterial isolate, followed by 41.60% in mixed inoculum of *Trichoderma* sp. and *Aspergillus* sp. (Table 2). In control, the degradation of mancozeb was lower than carbendazim and it was only

Table 1 Biodegradation of carbendazim by SMS dominating microbes under in vitro conditions, inoculated individually and in different combinations in SMS

Treatment	Initial concentration ($\mu\text{g g}^{-1}$)	Carbendazim residue in ($\mu\text{g g}^{-1}$) spent substrate at different interval (days)					
		0	3rd	6th	9th	12th	15th
<i>Trichoderma</i> sp.	100	96.70	95.20	87.30	83.60	80.40	75.20
<i>Mucor</i> sp.	100	96.50	94.10	89.80	86.20	83.10	79.60
<i>Trichoderma</i> sp. + <i>Mucor</i> sp.	100	97.00	94.50	90.10	87.50	81.90	76.40
<i>Trichoderma</i> sp. + <i>Aspergillus</i> sp.	100	97.10	92.50	84.40	76.35	70.10	66.50
<i>Trichoderma</i> sp. + <i>Mucor</i> sp. + <i>Aspergillus</i> sp.	100	96.90	93.00	88.20	80.00	75.90	68.00
<i>Mucor</i> sp. + <i>Aspergillus</i> sp.	100	96.90	93.40	88.10	85.60	82.30	78.80
B-I (bacterial isolate)	100	97.00	93.00	86.70	84.20	80.50	74.30
<i>Trichoderma</i> sp. + <i>Aspergillus</i> sp. + B-I	100	97.40	92.50	87.50	76.80	72.50	68.90
B-IV (bacterial isolate)	100	97.30	90.20	84.30	78.90	75.40	70.50
<i>Trichoderma</i> sp. + <i>Aspergillus</i> sp. + B-IV	100	97.00	93.40	88.90	83.40	71.60	67.00
B-I + B-IV	100	97.10	92.70	85.20	79.40	76.10	70.00
<i>Aspergillus</i> sp.	100	96.98	94.20	86.50	80.10	78.60	72.40
Control	100	97.00	96.40	95.33	92.60	90.44	88.10

CD_(0.05): Treatment 1.82, Interval 7.73, Treatment $\times$ Interval 2.25**Table 2** Biodegradation of mancozeb by SMS dominating microbes under in vitro conditions, inoculated individually and in different combinations in SMS

Treatment	Initial concentration ($\mu\text{g g}^{-1}$)	Mancozeb residue in ($\mu\text{g g}^{-1}$) spent substrate at different interval (days)					
		0	3rd	6th	9th	12th	15th
<i>Trichoderma</i> sp.	100	98.50	89.7	82.00	75.10	70.30	65.80
<i>Mucor</i> sp.	100	97.60	94.50	89.00	80.60	74.10	70.60
<i>Trichoderma</i> sp. + <i>Mucor</i> sp.	100	98.10	92.50	86.50	74.20	70.70	66.80
<i>Trichoderma</i> sp. + <i>Aspergillus</i> sp.	100	98.00	88.00	81.60	70.80	66.90	58.40
<i>Trichoderma</i> sp. + <i>Mucor</i> sp. + <i>Aspergillus</i> sp.	100	98.20	91.50	82.00	72.20	65.00	59.10
<i>Mucor</i> sp. + <i>Aspergillus</i> sp.	100	98.10	95.70	90.30	82.40	78.10	72.90
B-I (bacterial isolate)	100	98.00	92.20	85.10	80.00	76.70	71.60
<i>Trichoderma</i> sp. + <i>Aspergillus</i> sp. + B-I	100	98.40	89.20	80.30	71.60	63.00	50.50
B-IV (bacterial isolate)	100	98.50	95.60	88.90	81.00	79.20	74.50
<i>Trichoderma</i> sp. + <i>Aspergillus</i> sp. + B-IV	100	98.20	92.80	87.40	75.50	67.60	62.10
B-I + B-IV	100	98.20	91.7	85.20	77.40	70.10	64.20
<i>Aspergillus</i> sp.	100	98.15	93.40	87.44	72.50	69.30	66.00
Control	100	98.00	96.75	94.15	92.80	91.40	90.00

CD_(0.05): Treatment 2.55, Interval 10.37, Treatment $\times$ Interval 2.72

10.00% in comparison to 11.90% for carbendazim. In earlier studies, the SMS has been recorded to stimulate the microbial population (10^8 – 10^9) in soil, which it maintains for 28 days. The stimulated microbial population of *Aspergillus* sp., *Penicillium* sp., *Trichoderma* sp., *Aeromonas* sp. and *Bacillus* sp., has been reported to degrade alachlor within 7 days of incubation under in vitro conditions [15, 16].

In Situ Study

The fungicide degradation potential of button mushroom spent substrate was also evaluated under in situ conditions by mixing different proportions of SMS in fungicide mixed soil and the residue level of two fungicides was assayed at a regular interval of 1 month for next 6 months. Among different proportions of SMS, the 20% (w/w) and 30% (w/

w) SMS gave results at par with each other and very low level of carbendazim residue was detected after 6 months of SMS mixing. At different intervals of residue assay, the level of carbendazim degradation in 30% SMS was 2–3 folds higher than the control (Fig. 3). In soil carbendazim gets degraded at varied rates depending upon the nutrient status of the soil and the type of vegetation growing on it. On bare soil its DT_{50} is 6–12 months [17]. In line with the in vitro studies, the degradation of mancozeb under in situ conditions was also higher than carbendazim. After second and third month of SMS mixing, the degradation of mancozeb in 20 and 30% SMS treatments was 3–5 folds higher than the control (Fig. 4). The mixing of SMS at 20 and 30% (w/w) has been found to stimulate the degradation of two fungicides and 6 months time was recorded to be sufficient for complete degradation of the fungicides. As

Fig. 3 Effect of SMS mixed in different proportions in soil on degradation of carbendazim under in situ conditions

Fig. 4 Effect of SMS mixed in different proportions in soil on degradation of mancozeb under in situ conditions

the previous studies have clearly revealed the growth and yield stimulatory role of SMS in different vegetables [7–10], the present findings further support the all-round role of SMS in raising organic field crops. In several earlier studies, the spent substrate from different mushrooms have been used to decontaminate the soils contaminated with PCP [4], 3-ring and 4-ring compounds [18], hazardous industrial wastes [19], and chlorophenols (PCP), polycyclic aromatic hydrocarbons (PAHS) and aromatic monomers [20].

Earlier in a similar study, Busswel [19] studied the bioremediation stimulating catabolic activities of indigenous microflora by optimizing their in situ growth conditions. However, Tekere et al. [21] reported very high rate of (about 82%) degradation of the pesticide, lindane by *P. chrysosporium* with very low initial concentrations of the pesticide (5 and 10 mg l⁻¹). Faster degradation of fungicides in microflora inoculated treatments is attributed to the involvement of lignin degrading enzymes system. Some other findings have also revealed the role of laccase, Mn-dependant peroxidase and high carbon content in SMS in breakdown of organopollutants [22–24]. The SMS from *Pleurotus pulmonarius* has also been tried earlier to treat a PCP contaminated water system and after 2 days of incubation, 89% removal of PCP has been reported with 5% SMS [24]. This has been attributed to biodegradation through lignolytic enzymes (70%) and bio-sorption by the SMS (19%).

The present study provides sufficient evidences that SMS has great potential in bioremediation of contaminated sites. Results from field study are of great importance, as the contamination of soil from agricultural chemicals is continuously increasing and plenty of untimely deaths had been reported time to time from the affected areas of the state of Punjab. The multi-facet utilities of SMS on using as manure in field crops will not only curtail the additional burden of chemical fertilizers on poor farmers and will also improve the affected soil for field crop raising.

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