

## Screening of Fungi from Natural Sources in Thailand for Degradation of Polychlorinated Hydrocarbons

<sup>1</sup>Premjet Siripong, <sup>1</sup>Bunthong Oraphin, <sup>2</sup>Tachibana Sanro and <sup>3</sup>Premjet Duanporn

<sup>1</sup>Department of Biology, Faculty of Science, Naresuan University, Pitsanulok, 65000 Thailand

<sup>2</sup>Department of Bioresources, Faculty of Agriculture, Ehime University,  
3-5-7 Tarumi Matsuyama, Ehime 790-8566, Japan

<sup>3</sup>Department of Agricultural Science, Faculty of Agriculture, Natural Resources and Environment,  
Naresuan University, Pitsanulok, 65000 Thailand

**Abstract:** Fungal isolates were collected from the Phitsanulok and Pichit provinces in the lower northern region of Thailand. All of the fungal cultures were tested for their ability to decolorize the dye Remazol brilliant blue R. It was observed that only 47 of the 296 fungal isolates formed clear areas with various diameters on agar plates when grown for 15 days. The fungal isolates E15, E109, I19, F33 and U11 produced the largest decolorization zone (Ø 90 mm). Subsequently, these five isolates were tested for their ability to degrade 2,8-DCDD (2,8-dichlorodibenzo-p-dioxin) and DDT (Dichloro-Diphenyl-Trichloroethane) in liquid culture medium. It was observed that 2,8-DCDD and DDT were eliminated by all tested isolates with different kinetics. The results demonstrated that the degradation of 2,8-DCDD by all tested isolates ranged from  $74.68 \pm 1.2\%$  to  $87.20 \pm 1.5\%$  during 15 days incubation. The degradation increased up to  $91.40 \pm 1.1\%$  to 100% when the isolates were cultured for 30 days. In contrast, degradation of DDT by all five tested isolates was less than 50%. Of these five isolates, F33 exhibited the greatest ability to degrade 2,8-DCDD during a 30 day incubation. The fungal isolates E15, E109 and I19 were identified as *Trametes* sp., *Polyporus* sp. and *Nigroporus* sp. respectively. However, the F33 and U11 isolates were not identified.

**Key words:** Fungi, Biodegradation, 2,8-DCDD, DDT and Remazol brilliant blue R

### INTRODUCTION

White rot fungi belong to the order *Basidiomycetes* that participates in the biodegradation of lignin in nature, which is essential for global carbon recycling [1,2,3]. Previous studies indicate that most of white-rot fungi produce and secrete various extracellular enzymes that degrade lignin. These are peroxidase or ligninolytic enzymes, predominantly lignin peroxidase (LiP), manganese peroxidase (MnP) and laccase. They are non specific enzyme system, enabling fungi to degrade natural complex aromatic polymers of lignin as well as complex aromatic polymers that share structure with lignin, such as pesticides, polyaromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs) and dyes [4]. Most researchers have utilized *Phanerochaete chrysosporium* as a model for studying the biodegradation of a wide variety of pollutants present in both liquid and soil cultures. Bumpus *et al.* [5] used *P. chrysosporium* as a

model for studying the degradation of DDT, 3,4,3M,4M-tetrachlorobiphenyl, 2,4,5,2M,4M,5M-hexachlorobiphenyl, 2,3,7,8-tetrachlorodibenzo-p-dioxin, lindane and benzo(a)pyrene. They stated that *P. chrysosporium* oxidized these organopollutants to carbon-dioxide using its lignin-degrading enzyme system under nitrogen-deficient conditions. Yadav *et al.* [6] observed that *P. chrysosporium* degraded polychlorinated biphenyl mixtures of Aroclors 1242, 1254 and 1260 by 60.9, 30.5 and 17.6%, respectively. The relatively high degradation of Aroclors 1242 and 1254 occurred in defined media under both high and low N<sub>2</sub> conditions. Tachibana *et al.* [7] screened 129 isolates of natural wood-rotting fungi and found that 3 were extremely effective to degrade the dye Remazol brilliant blue R. One of these isolates exhibited a greater rate of 2,7-DCDD degradation than *P. chrysosporium*. Itoh *et al.* [8] observed that the rate of 2,7-DCDD degradation in culture medium correlated with accumulation of LiP, but not MnP and Laccase.

Tekere *et al.* [9] isolated a variety of white rot fungi based on their ability to degrade various polymeric dyes present in tropical forests. They found that white rot fungi, *T. cingulata*, *T. versicolor*, *T. pocas*, *Datronia concentrica*, *Pycnoporus sanguineus* in addition to unidentified isolate as DSMP95. They exhibited the greatest efficacy in degrading the polymeric dye Poly R478 in liquid culture.

It well known that polychlorinated hydrocarbons, including Polychlorinated dibenzodioxin (DCDD) and Dichloro-Diphenyl-Trichloroethane (DDT), are mutagenic and toxic to humans and animals and are therefore important pollutants present in soil and sedimentation. These compounds have long existence period, so they remain in the environment for a long time. The objective of this study was to identify novel fungi that are able to degrade the polychlorinated hydrocarbons DCDD and DDT in culture media.

## MATERIALS AND METHODS

**Sample Collection:** A total of 370 samples were collected from two ecological sites in Thailand. In a natural community forest preservation in the Phisanulok Province, 148 fruiting body and 100 rotten wood samples were collected. In a natural community forest preservation in Phisanulok Province, 70 fruiting body and 52 rotten wood samples were collected. The white rot fungus, *P. chrysosporium* IFO 31249 (ATCC 34541), was purchased from the Institute for Fermentation in Osaka, Japan and used as a control for studying degradation of model compounds.

**Chemical Reagents:** In this experiment, 2,8-DCDD [Dichlorodibenzo-p-dioxin (DCDD)] and 1,1-bis (4-chlorophenyl)-2,2,2-trichloro ethane [Dichloro-Diphenyl-Trichloroethane (DDT)] were chosen as the model compounds for the biodegradation studies. The standard DDT (Aldrich) and Remazol Brilliant Blue R (Sigma) were purchased from a company in Thailand. The Department of Applied Bioscience, Faculty of Agriculture, at Ehime University in Japan provided 2,8-DCDD.

### Culture Media and Solutions:

C Potato dextrose agar (PDA) was prepared by boiling 200 g of scrubbed potatoes in 500 ml of distilled water and the liquid was strained through gauze. Then 20 g of glucose and 20 g of agar were added to the solution, followed by an additional 500 ml of distilled water. The pH was adjusted to 5.5.

- C Screening medium was composed of upper medium: malt extracts 5 g lG<sup>1</sup>, Remazol Brilliant Blue R 0.1 g lG<sup>1</sup> adjusted to pH 4.0 and agar 10 g lG<sup>1</sup> and lower medium: saccharose 10 g lG<sup>1</sup>, K<sub>2</sub>HPO<sub>4</sub> 1 g lG<sup>1</sup>, MgSO<sub>4</sub>.7H<sub>2</sub>O 0.5g lG<sup>1</sup>, KCl 0.5g lG<sup>1</sup>, NaNO<sub>3</sub> 2 g lG<sup>1</sup>, FeSO<sub>4</sub>.7H<sub>2</sub>O 0.01 g lG<sup>1</sup>, adjusted to pH 4.5 and agar 20 g lG<sup>1</sup>.
- C Vitamin solution consisted of Biotin 20 mg lG<sup>1</sup>, folic acid 20 mg lG<sup>1</sup>, thiamine hydrochloride 50 mg lG<sup>1</sup>, riboflavin 50 mg lG<sup>1</sup>, pyridoxal hydrochloride 100 mg lG<sup>1</sup>, cyanocobalamin 1 mg lG<sup>1</sup>, nicotinic acid 50 mg lG<sup>1</sup>, calcium panthothenate 50 mg lG<sup>1</sup>, P-aminobenzoic acid 50 mg lG<sup>1</sup> and DL-<sup>-</sup>-lipoic acid 50 mg lG<sup>1</sup>.
- C Mineral solution consisted of nitrilotriacetic acid 1.5 g lG<sup>1</sup>, MgSO<sub>4</sub>.7H<sub>2</sub>O 3 g lG<sup>1</sup>, Mn SO<sub>4</sub>.4H<sub>2</sub>O 0.713 g lG<sup>1</sup>, NaCl g lG<sup>1</sup>, FeSO<sub>4</sub>.7H<sub>2</sub>O 0.1 g lG<sup>1</sup>, CoSO<sub>4</sub>.7H<sub>2</sub>O 0.181 g lG<sup>1</sup>, CaCl<sub>2</sub>.2H<sub>2</sub>O 0.109 g lG<sup>1</sup>, ZnSO<sub>4</sub>.7H<sub>2</sub>O 0.178 g lG<sup>1</sup>, CaSO<sub>4</sub>.7H<sub>2</sub>O 0.01 g lG<sup>1</sup>, AlK(SO<sub>4</sub>)<sub>2</sub>.12H<sub>2</sub>O 0.018 g lG<sup>1</sup>, H<sub>3</sub>BO<sub>3</sub> 0.01 g lG<sup>1</sup> and Na<sub>2</sub>MoO<sub>4</sub>.2H<sub>2</sub>O 0.012 g lG<sup>1</sup>.
- C Basal stock solution consisted of K<sub>2</sub>HPO<sub>4</sub>.6H<sub>2</sub>O 2 g lG<sup>1</sup>, CaCl<sub>2</sub>.2H<sub>2</sub>O 0.5 g lG<sup>1</sup> and MgSO<sub>4</sub>.7H<sub>2</sub>O 0.5 g lG<sup>1</sup>. Mineral solution (10 ml) and vitamin solution (0.5 ml) were added and then distilled water was added to obtain a final volume of 1 L.
- C Basal solution medium consisted of 10 ml of basal stock solution, NaC<sub>4</sub>H<sub>9</sub>O<sub>4</sub>.6H<sub>2</sub>O 0.45 g, C<sub>4</sub>H<sub>12</sub>N<sub>2</sub>O<sub>6</sub> 0.0221 g, glucose 2 g and tween-80 0.1 g and distilled water was added to obtain a final volume of 100 ml.

All media were sterilized by autoclaving prior to use.

**Isolation of Fungal from Natural Sources:** The fresh fruiting body was collected from the woody substrate and soil surface. The exterior surface of the fruiting body was sterilized by wiping with alcohol. The fruiting body was aseptically torn to expose the interior tissue and a small piece of tissue was quickly cut using a sterile scalpel. The tissue was inoculated onto a PDA agar plate and a small piece of rotten wood was directly inoculated onto a PDA agar plate after surface sterilization. The plates were incubated at 25°C and monitored every day until mycelium developed. The tip of the mycelium was picked and transferred to a new PDA agar plate. This technique was repeated three times to obtain a purified culture. The purified cultured mycelium was cut into 5 mm (Ø) disks and transferred to slant agar for further analysis.

**Screening of Peroxidase Activity:** Primary screening of peroxidase activity for fungal isolates was performed using the reagent, dye Remazol brilliant blue R (RBBR) [10-12]. The functional output was measuring the

amount of dye decolorization on agar plates. The purified cultures of fungal strains derived from stock cultures were inoculated on PDA agar plates and incubated at 25°C. After 48 hr, 5 mm (Ø) agar disks were acquired from the growing margins of test strains and these were inoculated onto a screening medium agar plate and incubated at 25 °C for 15 days. Three replicates were used for each tested fungus. The diameters of clear zones were measured and recorded every day. The control plate was not inoculated.

**Culture Conditions for Biodegradation Studies:** The fungus isolate that produced the most rapid and greatest diameter of clear zone was selected for studying biodegradation. The mycelia of these fungal strains were transferred onto a PDA agar plate and incubated at 25°C for further analysis. After 48 hr, three 5 mm (Ø) disks of growing margin from test strains were inoculated into 10 ml of basal solution medium in a 125 ml Erlenmeyer flask with silicon stoppers. The cultures were incubated again at 25 °C under static conditions for 6 days. Then 2,8-DCDD and DDT diluted in DMF (100 µl) were added to the cultures to obtain a final concentration of 0.25 mM and incubated at 25 °C for 15 and 30 days under static conditions. The medium with 0.25 mM of each compound was inoculated with *P. chrysosporium* IFO 31249, to provide a positive control and the negative control was medium with 0.25 mM of diluents only. Three flasks were set up for each fungal isolate and all culture flasks were flushed with filter-sterilized O<sub>2</sub> for 5 min at a flow rate of 50 ml/min every day.

**Extraction of 2,8-dcdd from Cultures:** The test and control cultures were extracted after 15 and 30 days of incubation by adding 20 ml of concentrated H<sub>2</sub>SO<sub>4</sub> and 15 ml of hexane to the flasks. The flasks were incubated at room temperature until the solution separated into aqueous and residual layers. First, the aqueous solution was collected and transferred to a new flask (A). The aqueous solution was re-extracted with 15 ml of hexane. After gentle agitation, the hexane extraction was collected and transferred to a new flask (B). Second, 10 ml of acetone was added to the flask containing the residual layer and the flasks were sonicated in a bath for 5 min. The acetone extract was combined with the hexane extraction in flask (B). The residual layer was re-extracted twice with a 10 ml mixture of hexane and acetone (1:1), followed by 10 ml of hexane. This hexane extraction was combined with the previous hexane extraction in flask (B). The hexane extraction in flask (B) was evaporated using a Rota evaporator and re-dissolved in 5 ml of hexane. The hexane extraction was subjected to silica

gel chromatography (silica gel 5 g: eluted with 100 ml of hexane) to remove polar compounds. Finally, the hexane extraction was concentrated again using a Rota evaporator until 5 ml of extract remained. This concentrated extract was evaluated using GC/MS analysis.

**Extraction of DDT from Cultures:** Control and live cultures were extracted after 15 and 30 days of incubation. Hexane (30 ml) was added to the cultures. The flasks were sealed with silicon stoppers and agitated using a rotary shaker at 200 rpm/min for 24 hr in the dark at room temperature. The aqueous phase was discarded and the hexane extraction was evaluated using GC/MS analysis.

**Analysis of 2,8-DCDD and DDT Levels:** Analysis was performed using a Hewlett Packard 6890N series gas chromatograph with a 7683 series auto-sample and a 5973 series mass selective detector. Separation of 2,8-DCDD and DDT was achieved using a DB-5MS column (30 m x 0.25 mm i.d. x 25 µm). The carrier was 99.99 % helium gas at 32 cm/sec measured at 45°C in constant flow mode. The oven temperature program was 45°C for 1 min, 45-130°C at 30°C/min, 130°C for 3 min, 130-180°C at 12°C/min, 180-240°C at 7°C/min, 240-325°C at 12°C/min and 325°C for 5 min. The injector was set to splitless mode with a 1.0 purge activation time and 300°C focus liner. The detector was set at 5973 MSD, 325°C transfer line full scan at m/z 45-450. For injection, 1 µl of each of sample was used.

The percent degradation was calculated as follows: (b/a) x 100, where a is equal to the 0.25 mM of substrate that was added to the culture medium and b is the residual substrate.

## RESULTS AND DISCUSSION

**Screening of Fungi:** From the 370 samples collected at the two different sites in Thailand, 296 cultures were obtained. Of these cultures, those collected from the Phisanulok province consisted of 108 fruiting body and 99 rotten woods and those collected from the Pichit Province consisted of 62 fruiting body and 27 rotten wood. Subsequently, all purified cultures of fungal strains were screened for peroxidase activity using medium supplemented with RBBR dye that was carried out on Petri dishes (90 mm diameter). A positive result was characterized by a positive decolorization test, which resulted from oxidation of the dark green color of RBBR dye leading to a 90 mm wide clear zone on the agar plated in Petri dishes. Results acquired using this method indicated that only 47 of the purified cultures collected

from the fruiting body population produced various sized diameters of clear zone during 15 days of culture. The fungal isolates E15 and E109 exhibited the largest decolorization zone ( $\varnothing$  90 mm) between 2-11 days. Isolates I19, F33 and U11 exhibited large clear zones between 2-12 days, 1-13 days and 5-12 days, respectively. The decolorization zone of the control strain *P. chrysosporium* IFO 31249 was visible between 3-14 days, exhibiting a clear zone with a 90 mm diameter. In summary, the five isolates E15, E109, F33, I19 and U11 all effectively discolored RBBR, which resulted in a clear zone with a large diameter as compared to the control strain. Shin *et al.* [10] previously verified that the mode of action of Lip was distinct from RBBR decolorizing peroxidase. Borokhov and Rothenburger [11] demonstrated that the decolorization zone occurring on agar was a result of a redox reaction between RBBR dye and an extracellular oxidative enzyme secreted by fungi. Kiiskinen *et al.* [12] used RBBR dye as a substrate for screening laccase-producing fungi and found that the decolorization of RBBR by different basidiomycete strains was due to MnP and laccase activity. They observed that there is no RBBR decolorization by fungal strains exhibiting peroxidase activity. Although, some fungal strains showed increased MnP and laccase activity during RBBR decolorization, only laccase activity correlated with RBBR decolorization [13]. Subsequently, it was determined that the RBBR decolorizing enzyme in culture medium was laccase, which is synthesized by *F. trogii* [14] and *P. ostreatus* [15].

In order to evaluate the ability of the five test fungi strains to degrade recalcitrant pollutants, all strains were cultured in liquid culture medium with the two structurally diverse, differentially chlorinated model compounds, 2,8-DCDD and DDT. These pollutants were not degraded in flasks that had not been inoculated, demonstrating the requirement for fungal-derived enzymes.

**Degradation of 2,8-DCDD:** The degradation percentages of 2,8-DCDD by isolates I19, F33, U11 and the control *P. chrysosporium* IFO 31249 were all greater than 80% ( $87.20 \pm 1.5\%$ ,  $85.26 \pm 0.76\%$ ,  $84.00 \pm 1.3\%$  and  $81.60 \pm 1.8\%$ , respectively). However, isolates E15 and E19 showed slightly reduced degradation of 2,8-DCDD ( $74.68 \pm 1.2\%$  and  $76.80 \pm 1.9\%$ , respectively) compared to the other fungal test strains when incubated for 15 days (Fig. 1). By 30 days, the efficiency degradation of 2,8-DCDD by all five test strains was improved. The F33 isolate degraded 100% of 2,8-DCDD, which was greater than the E15, I19, U11 and E19 isolates, which exhibited degradation percentages of  $96.80 \pm 1.1\%$ ,  $93.20 \pm 1.8\%$ ,  $91.40 \pm 1.1\%$  and  $90.80 \pm 1.5\%$ , respectively. Furthermore, all five test strains showed greater degradation of

2,8-DCDD than the control *P. chrysosporium* IFO 31249 ( $88.80 \pm 1.5\%$ ). Bumpus *et al.* [5] determined that 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin was oxidized by the lignin-degrading enzyme of *P. chrysosporium*. Hammel *et al.* [16] observed that the activity of LiP played a role in catalyzing dibenzo-p-dioxin to produce a dibenzo-p-dioxin cation radical. The degradation pathways of 2,7-dichlorodibenzo-p-dioxin were investigated further and it was determined that only LiP from *P. chrysosporium* exhibited significant oxidation of 2,7-dichlorodibenzo-p-dioxin resulting in accumulation of 2-chloro-1,2-benzoquinone and 2-hydroxy-1,4-benzoquinone, as well as its essential intermediate 1,2,4-trihydroxybenzene [17]. In contrast, it was reported that only the activity of MnP in *P. sordida* YK-624 directly correlated with degradation of the mixture of tetra- to octachlorodibenzo-p-dioxins (TCDD and OCDD, respectively). Additionally, it was observed that the important metabolites 4,5-dichlorocatechol and tetrachlorocatechol accumulated during degradation of 2,3,7,8-TCDD and OCDD by *P. sordida* YK-624 [18]. Furthermore, the other white rot fungi and wood rot fungi, both identified and unidentified, were reported to have a high propensity for degrading dioxin [19-22]. The degradation time course for 2,8-DCDD indicated that high rates of degradation of 2,8-DCDD by five test strains occurred during the first 15 days of incubation. However, the highest degradation of 2,8-DCDD (100%) was achieved by F33 after a 30-day incubation. Moreover, the efficiency of fungal isolates E15 and F33 was higher than the efficiency of those fungi reported on in a study published by Miyoshi *et al.* [22].

**Degradation of DDT:** The amount of degradation of DDT by all test strains and *P. chrysosporium* IFO 31249 was variable (Fig. 2). During 15 days incubation, *P. chrysosporium* IFO 31249 could degrade DDT by  $25.68 \pm 1.2\%$ . F33 and I19 exhibited slightly lower rates of DDT degradation of  $20.76 \pm 0.9\%$  and  $21.60 \pm 0.05\%$ , respectively. DDT was degraded to less than 20% by E19 ( $16.00 \pm 1.1\%$ ), U11 ( $4.60 \pm 0.5\%$ ) and E15 ( $0.16 \pm 0.3\%$ ). The degradation of DDT was increased when all five test strains and *P. chrysosporium* IFO 31249 were cultured for 30 days. The greatest degradation of DDT ( $P < 0.05$ ) was achieved by isolate F33 ( $35.96 \pm 1.2\%$ ) and *P. chrysosporium* IFO 31249 ( $36.96 \pm 1.4\%$ ). Isolates I19, E19, E15 and U11 were unable to degrade DDT to levels lower than those of the control culture. Therefore, only isolate F33 could effectively degrade DDT to levels similar to *P. chrysosporium* IFO 31249 when cultivated for 30 days. We noted that all five test strains and the control culture were able to degrade DDT to a level of less than 50%. However, it has been previously reported that 50%

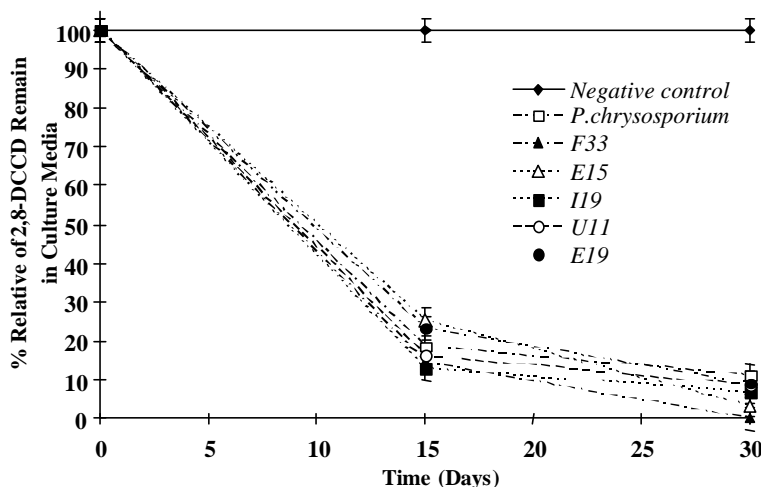


Fig. 1: Time Course of 2,8-DCCD Disappearance from Culture Media by the Five Test Strains. (Values represent the mean, n = 3, ± SE, P < 0.05)

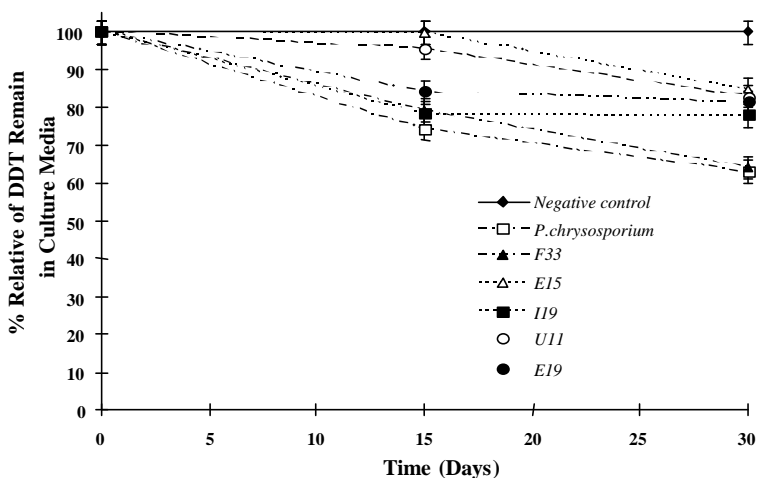


Fig. 2: Time Course of DDT Disappearance from Culture Media by the Five Test Strains. (Values represent the mean n = 3, ± SE, P < 0.05)

of DDT was degraded during a 30 day incubation period by *P. chrysosporium* in culture medium under low nitrogen conditions via the lignin-degrading enzyme system. DDT mineralization is not limited to *P. chrysosporium*; it also occurs in other white rot fungi, including *Pleurotus ostreatus*, *Phellinus weirii* and *Polyporus versicolor*. In addition, the metabolites dicofol, DDD, FW-152 and DBP were produced during the biodegradation of DDT [5, 23]. In contrast, Köhler *et al.* [24] demonstrated that ligninase activity secreted from *P. chrysosporium* had no direct role in removing DDT from culture medium. Instead, they reported that laccase, produced by *Trametes versicolor* ATCC 200801, directly affected the dechlorination of DDT [25]. Recently, the DDT degradation ability of various species of brown rot

fungi was examined and it was determined that 87%, 84% and 81% of DDT were removed by *Gloeophllum trabeum*, *Fomitopsis pinicola* and *Daedalea dickinsii*, respectively. The metabolite that was not produced by *P. chrysosporium* was identified as DDE [26].

The pure culture of five tested isolates were identified on the basis of cultural and morphological features including spore forming with the help of suitable literature [27-33]. The fungal test strains E15, E109 and I19 were identified as *Trametes* sp., *Polyporus* sp. and *Nigroporus* sp, respectively. However, the strains F33 and U11 remain unidentified.

Several authors have proposed that the biodegradability of organic pollutants depends on chlorine content, structure and fungal species [4, 34, 35].

Although the enzymes that degrade 2,8-DCDD and DDT utilized by the five test strains examined in this study have not been elucidated, the degradation of these organic pollutants is assumed to occur through similar mechanisms employed by other white rot fungi. Currently, the enzymes that are responsible for degrading these recalcitrant pollutants are being investigated.

In conclusion, our results determined that all five fungal isolates could degrade 2,8-DCDD and DDT with different efficiencies. They extensively degraded 2,8-DCDD with ranges from  $90.80 \pm 1.5\%$  to 100% within 30 days. Additionally, they degraded 2,8-DCDD to a greater extent than the control strain, *P. chrysosporium* IFO 31249. Among the five optimal strains identified, the F33 isolate had the greatest efficiency, degrading 100% of 2,8-DCDD. Additionally, it was observed that degradation of 2,8-DCDD correlated with an ability to decolorize the RBBR dye. Therefore, the RBBR decolorization assay was a robust screening tool to identify 2,8-DCDD degrading fungi.

#### ACKNOWLEDGEMENTS

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