Biodegradation optimization of 2,4,8-trichlorodibenzofuran by ligninolytic fungus

Ajeng Arum Sari1*, Kazutaka Itoh2, Sanro Tachibana2

1Research Center for Chemistry, Indonesian Institute of Sciences, Kawasan Puspiptek Serpong, Tangerang Selatan, Banten 15314 Indonesia
2Department of Applied Bioscience, Faculty of Agriculture, Ehime University, 3-5-7 Tarumi, Matsuyama, Ehime 790-8566 Japan

*Corresponding author: ajen001@lipi.go.id; ajeng_as@yahoo.co.id

Abstract

Incineration does have its critical problem if chlorine presence in the solid waste because it may lead to emissions containing highly toxic dioxins and furans. Dioxin is known to be toxic and carcinogenic compounds with long half-lives, and is among the most problematic environmental pollutants. Recently, the biological methods are receiving more attention since they are considered as an environmental friendly method. This study was focused on the biodegradation of 2,4,8-trichlorodibenzofuran in liquid medium and soil by Trametes versicolor U80 for 15 and 30 days. The optimization of 2,4,8-TCDF degradation was conducted by adding glucose as carbon source, surfactant Tween 80, and agitation. In liquid medium, this strain degraded 27 ppm 2,4,8-trichlorodibenzofuran 46.52% at 30 days. Adding glucose 2.5% and 2% Tween 80 were able to improve degradation of 2,4,8-trichlorodibenzofuran as approximately 59%. Wood meal has the highest ability used as lignocellulosic supports for the growth of T.versicolor U80 to degrade 2,4,8-trichlorodibenzofuran in soil. In soil, this strain pre-grown wood meal degraded 10 ppm 2,4,8-trichlorodibenzofuran 46.06% at 30 days.

Keywords: 2,4,8-trichlorodibenzofuran; biodegradation; ligninolytic enzymes; Trametes versicolor

Introduction

2,4,8-trichlorodibenzofuran (2,4,8-TCDF) that belongs to polychlorinated dibenzofurans (PCDFs) is formed as a result of incineration processes and as by-products of chemical reactions such as chlorine bleaching of paper and pulp, and from the manufacture of some chlorinated pesticides, herbicides, fungicides, wood preservatives, and textile dyes (Evans and Dellinger, 2005; Gilpin, Wagel and Solich, 2003; Krizanec et al., 2005) (Fig. 1). PCDFs are a harmful chemical that poses a risk of long-term contamination in soil or bottom sediment because they have a chemically stable structure and accumulates in the environment over a long time. They also pose to the health of humans and animals based on standard toxicity test performed on animal revealed differential sensitivity to PCDFs exposure (Kamei, Suhara and Kondo, 2009). The primary human health concerns associated with PCDFs are immunotoxicity, developmental toxicity, neurotoxicity, carcinogenicity, skin,
liver, and gastrointestinal toxicity (Kogevinas, 1999; Geusau et al., 2001). Because of their toxicity, how to remove them from polluted environments is one of the most challenging problems in environmental technology. Some physicochemical techniques for detoxifying and degrading dioxins such as thermal remediation, photodegradation, supercritical water with a metal catalyst, have been developed and considered for application. However, physicochemical methods are not feasible to remedy large areas of polluted soils and sediments from both ecological and economic viewpoints (Hiraishi, 2003).

![Image of 2,4,8-trichlorodibenzofuran](image)

**Figure 1.** Structure of 2,4,8-trichlorodibenzofuran

Recently, the biological methods are receiving more attention since they are considered as an environmental friendly way. Using defined microorganisms with the ability to degrade dioxins to eliminate pollution in the environment could have huge advantages over physicochemical methods. Bioremediation uses the microorganisms to degrade environmental pollution into the less toxic compound. The extent of biodegradation is highly dependent on the toxicity and initial concentrations of the contaminants, their biodegradability, the properties of the contaminated soil and the type of microorganism selected (Kulkarni, Crespo and Afonso, 2008). Bioremediation can take place under aerobic and anaerobic conditions. Dioxin degradation has been characterized in aerobic bacteria containing aromatic ring hydrolating dioxygenase enzymes, anaerobic bacteria through reductive chlorination under anaerobic conditions and fungi producing extracellular oxidative enzymes (Chang, 2008).

In recent years, some studies of the dioxin using microorganisms, as well as some of their less highly derivatives, have been performed (Hiraishi, 2003). The only evidence for the degradation of chlorinated dioxins by fungi is limited to wood-, or litter-degrading white-rot fungi (Field and Sierra-Alvarez, 2008). The white-rot fungi constitute the most important group of organisms responsible for the degradation of nature’s most complex polymer, lignin. The white rot fungi such as *Phanerochaete chrysosporium*, *P. Sordida*, *Bjerkandera*, *Phlebia* and *Pleurotus* showed the potential ability to degrade dioxin from various environments such as contaminated soils, temperate and tropical forests (Chang, 2008). They use oxidative enzymes to initiate the attack of a variety of xenobiotic pollutants, including chlorinated dioxins. *Phanerochaete chrysosporium*, *P. sordida*, *Phlebia lindtneri*, *Phlebia sp.* MG-60, unidentified strain MZ-227, *Phlebia* strains (BMC3014, BMC9152 and BMC9160) and PL1 have able to degrade PCDDs and PCDFs in several concentrations (Valli, Wariishi and Gold, 1992; Takada et al., 1996; Mori and Kondo, 2002a; Mori and Kondo, 2002b; Kamei, Suhara and Kondo, 2005; Tachibana, Kiyota and Koga, 2005).

In general, several factors can influence biodegradation by fungi, such as the fungal growth conditions and the chemical structure of the compounds. The fungal growth conditions are affected by carbon source, surfactant, and agitation. Ligninolytic fungi are not able to use lignin as their sole source of energy and carbon. They depend on the more digestible polysaccharides in lignocellulosic substrates. The induction of the chemical compounds degradation, when adding glucose as readily metabolizable carbon source, probably occurs as a result of increasing cell biomass cause faster degradation of that compound. Studies have demonstrated synthetic surfactant such as Tween 80 at low concentrations may be useful for bioremediation of sites contaminated with hydrophobic pollutants. Wong et al., (2004) reported that *Pseudomonas aeruginosa* when combined with Tween 80 effective enhanced the solubility and degradation of phenantherene. By increasing the chemical compound, Tween 80 facilitates the transport of it to microbial cells and improves the metabolism of this hydrocarbon (Hadibarata and Tachibana, 2009). Some research studies have also shown ligninase production in agitated pelleted cultures. Stirring could increase the contact between the substrate, oxygen, and biomass. It will enhance mass transfer and finally affect to degradation rate.
Compared to bacteria, some white rot species are better able to colonize soil and to compete with microflora. Some fungi were reported to release the target pollutants as they decompose the macromolecular organic matter with which the target pollutants have combined. Moreover, the ability of fungal hyphae to reach the pollutant by penetrating contaminated soil, coupled with the production of extracellular oxidases, gives fungi a significant advantage over bacteria (Pointing, 2001). However, the degradation experiment for PCDFs was carried out commonly under liquid culture conditions and provided little information about the treatment of soil that was contaminated with PCDFs.

This study was focused on the bioremediation of 2,4,8-trichlorodibenzo-furan (2,4,8-TCDF) by a white-rot fungus, *Trametes versicolor* U80. Five enzymes (lignin peroxidase, manganese peroxidase, laccase, 1,2-dioxygenase, and 2,3-dioxygenase) were also measured to know enzyme activities of the fungus. The several treatments were also conducted to obtain the optimum condition during 2,4,8-TCDF degradation in liquid and soil media.

**Material and Methods**

**Chemical reagents**

2,4,8-trichlorodibenzo-furan (2,4,8-TCDF) was purchased from Tokyo Chemical Industry, Ltd (Tokyo, Japan). Agar, glucose, and other chemicals were purchased from Wako Co. Ltd (Osaka, Japan). Malt extract and polypeptone were purchased from Difco.

**Liquid culture condition**

*T. versicolor* U80 was tested in a liquid medium to know its ability in degrading 2,4,8-TCDF. Liquid cultures experiments were conducted in 100 mL Erlenmeyer flasks containing 20 ml of malt extract liquid medium (Sari, Tachibana and Itoh 2012). As much as 27 ppm 2,4,8-TCDF was eluted in N, N-Dimethylformamide, Tween 80, and distilled water. The medium was sterilized for 20 min at 121 °C, and then three plugs of each fungus were inoculated into the Erlenmeyer flask and pre-incubated for 7 days. Each of inoculated flasks was supplemented with 27 ppm of 2,4,8-TCDF, and then incubated for 15 and 30 days in dark condition at 25 °C. Positive control was used to support this experiment to quantify the adsorption losses of pollutant caused mycelium in liquid medium. The positive control was sterilized by using autoclave before addition of 2,4,8-TCDF. The optimization of 2,4,8-TCDF degradation was conducted by adding glucose as carbon source (2-10%), surfactant Tween 80 (0.25-2%), and agitation (80 and 120 rpm).

**Soil culture preparation**

Soil was collected from a land farming area in Faculty of Agriculture, Ehime University, Japan. It was obtained from the surface layer (0-20 cm) with pH 6.16 (in distilled water ratio 1:5). The soil was air-dried, passed through a 3 mm sieve and homogenized. Before its use, the soil was autoclaved 121°C for 180 min to eliminate exist of microorganisms and spiked with 10 ppm 2,4,8-TCDF dissolved in distilled water containing DMF and Tween 80. The water soil content was adjusted 30% with addition distilled water. The several types for inoculation were conducted (Table 1). All treatments were added with 10% shiitake no sato and 10% glucose. The container culture was incubated for 15 and 30 days after adding pollutant at 25°C in the dark condition.

**Table 1.** Experimental design for pre-incubation condition in soil medium

<table>
<thead>
<tr>
<th>No.</th>
<th>Inoculum type</th>
<th>Nutrients</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Fungi: pre-incubation in wood meal</td>
<td>Glucose 10%, shiitake no sato 10%, wood meal (5 and 10%)</td>
</tr>
<tr>
<td>2.</td>
<td>Fungi: pre-incubation in paper sludge</td>
<td>Glucose 10%, shiitake no sato 10%, paper sludge (5 and 10%)</td>
</tr>
<tr>
<td>3.</td>
<td>Fungi: pre-incubation in liquid medium</td>
<td>Glucose 10% and shiitake no sato 10%</td>
</tr>
</tbody>
</table>

**Analytical methods**

After culture period, the samples in liquid medium were extracted with ethyl acetate for three times and then purified by column chromatography (5 g silica gel C200 and anhydrous Na₂SO₄) elute with hexane: dichloromethane (3:1). The concentrated samples were analyzed using GC-MS Shimadzu QP-5000. The capillary column used was TC-5 (length: 30 m, diameter 0.24 mm. The carrier gas was helium at constant flow rate 1.5 mL/min with column pressure 100 kPa and interface temperature 260°C. The temperature program was started at 80°C, 20°C/min to 150°C, 25°C/min to 300°C which maintained for 10 min to allow eluting peak to exit the column. The injection volume was 1 µL, and the injector was maintained at 260°C.
After culture period, the samples in soil medium were extracted using a soxhlet apparatus for 16 hours with dichloromethane, purified using column chromatography (5 g C200 silica gel and anhydrous Na2SO4) eluted with hexane: dichloromethane (3:1), and then analyzed using GC-MS as described above.

**Enzyme assays**

The samples in liquid medium were filtered through a 0.2 µm membrane filter to determine the enzyme activities (laccase, manganese peroxide, lignin peroxidase, 1,2-dioxygenase, and 2,3-dioxygenase) using Spectrophotometer Shimadzu UV-1600. The enzyme assays were following methods from Sari et al. (2012).

**Results and Discussion**

**2,4,8-trichlorodibenzofuran degradation by T. versicolor U80 in liquid medium**

*T. versicolor* U80 degraded 2,4,8-TCDF in exponential phase up to 15 d, and it was followed by a slower rate during the remaining period of incubation. *T. versicolor* U80 degraded 2,4,8-TCDF 34.69%±3.98 on 15 days and 46.52%±4.71 on 30 days (Fig. 2). The loss of 2,4,8-TCDF approximately 23-24% on 15 and 30 days were may caused by adsorption process in mycelia of fungus during incubation time.

![Figure 2: Degradation rate of 2,4,8-TCDF (27 ppm) by T. versicolor U80 in malt extract liquid medium](image)

White rot fungi capable of degrading lignin are usually capable of degrading a wide variety of aliphatic and aromatic xenobiotics, including PCDD/Fs. A subsequent study by Takada et al., (1996) revealed that *P. sordida* YK-624 degraded 45-70% of PCDFs (TCDF-HxCDFs) in Kirk's medium. The ability of fungi to degrade PCDD/Fs depends on the configuration of the isomers and increasing the degree of chlorination of the aromatic rings (Hiraishi, 2003). The rapid PCDF reduction by fungi is occurred when one aromatic ring has free of chlorine substitution or position 1 does not chlorinated. PCDF congeners substituted in position 1 could also degrade rapidly only if the position 4 and 6 are free of chlorine substitution (Schreiner et al., 1997).

Enzyme activities of *T. versicolor* U80 were measured when cultivating fungi with and without the addition of 2,4,8-TCDF. The high enzyme activities of *T. versicolor* U80 during 2,4,8-TCDF degradation were obtained by 1,2-dioxygenase (80.0±13.6 U l⁻¹) and LiP (89.8±25.5 U l⁻¹) (Fig. 3). On the contrary, the activity of laccase and MnP did not show the constant tendency during the incubation. From these results, it was suggested that the 1,2-dioxygenase and LiP were responsible for the degradation of 2,4,8-TCDF. This result was in line with result from Tachibana, Kiyota and Koga (2005) that LiP and MnP played a role for degradation of 2,4,8-TCDF. Laccase amount during addition of 2,4,8-TCDF was higher than absence of 2,4,8-TCDF. However, laccase did not play a role for degradation because this enzyme has only able to oxidize phenolic compounds (Farnet et al. 2010).

![Figure 3: Enzyme activities of T. versicolor U80 during the incubation with or without addition of 2,4,8-TCDF](image)

The redox potential of LiP is higher than for most other peroxidases (Cameron, Timofeevski and Aust, 2000). It allows for the oxidation of chemicals that are not easily oxidized. LiP can oxidize series of pollutant which has some similarity structure to the lignin model compounds. The dioxygenase enzyme plays a crucial role in the degradation of xenobiotic compounds. By catalyzing the incorporation of two hydroxyl groups into the aromatic ring, these enzymes increase the reactivity of these...
compounds, making them susceptible to enzymatic ring fission reactions (Erickson and Mondello, 1993). On the other hand, Cordyceps militaris KS-92 has been shown to degrade 2,4,8-TCDF 25% using P-450 monooxygenase over 30 d in liquid medium (Mori et al. 2005).

Tachibana, Kiyota and Koga (2005) reported that 3,5-dichlorosalicylic acid and 5-chlorosalicylic acid were detected as metabolite products during degradation of 2,4,8-TCDF in soil. On the other hand, degradation of 2,4,8-TCDF using P-450 monooxygenase of C. militaris resulted hydroxyl-triCDFs as metabolite product (Mori et al. 2005). This enzyme was the responsible for the hydroxylation of chlorinated dioxins (up to tri-Cl) in the initial step of the metabolic pathway.

Evaluating the optimum condition in improving ability for 2,4,8-TCDF degradation in liquid medium by T. versicolor U80

Several treatments were conducted to obtain the optimum condition for 2,4,8-TCDF degradation by adding variation of glucose concentration, surfactant concentration and agitation. Variation of glucose concentration has effect for 2,4,8-TCDF degradation (Fig. 4).

![Figure 4](image-url) **Figure 4.** Effects of glucose on 2,4,8-TCDF degradation by T. versicolor U80 in the liquid medium

The maximum degradation 59% was obtained during the adding 2.5% glucose concentration. The ability to degrade lignocellulose efficiently is associated with a mycellial growth and ability fungus to transport nutrients into the nutrient-poor lignocellulosic substrate (Hammel 1997). Otherwise, ligninolytic fungi are not able to use lignin as their sole source of energy and carbon. They depend on the more digestible polysaccharides in lignocellulosic substrates. The adding glucose as readily metabolizable carbon source increased cell biomass, and then caused the higher degradation of 2,4,8 TCDF. Otherwise, the increasing of glucose concentration resulted in the inhibition of fungus growth and the decreasing of degradation rate. The fungus avoids synthesizing and secreting metabolically ligninolytic agents when substrates more accessible than lignocellulose (such as glucose) were available in medium (Hammel 1997).

Effect of surfactant using Tween 80 was investigated. Tween 80 has no adverse effect on the growth of T. versicolor U80. It was found that 2% concentration of Tween 80 increased 2.8 fold degradation rate at the end of 30 day incubation by T. versicolor U80, compare to the absence of surfactant (Fig. 5).

![Figure 5](image-url) **Figure 5.** Effect of Tween 80 on 2,4,8-TCDF degradation by T. versicolor U80 in the liquid medium

Surfactants, such as Tween 80, have been shown to enhance bioremediation processes because of the increasing the bioavailability of contaminant aqueous insolubility, reducing surface and interfacial tensions, and improving the contaminant accessibility in medium (Singh, Van Hamme and Ward, 2007). It has also been used as a ligninase inducer in cultures that protects the enzymes from denaturing and increase the production of ligninolytic enzymes (Zhou et al., 2007). Tween 80 promotes both uptake and exit of compounds from the cell through modification of plasma membrane permeability.

Billingsley, Backus and Ward, (1999) demonstrated that the presence of the anionic surfactant Hostapur SAS 60 increased 2.5 fold the extent of PCB degradation by Pseudomonas LB400. A surfactant, 6'-O-palmitoylmaltotriose, increased the apparent solubility from 140 to 305 µg/l and enhanced two folds biodegradation of Aroclor 1242 by Burkholderia cepacia LB400 (Ferrer, Golyshin and Timmis, 2003). Bautista et al., (2009) showed that experiments performed with Tween 80 for degradation PAHs reached
>90% in 15 days by bacteria. However, little information about the effects of Tween 80 and other surfactants to degrade PCDFs was provided.

Agitation only gave little improvement for biodegradation of 2,4,8-TCDF (Fig. 6). The oxygen concentration is dependent on the air flow rate. Stirring could increase the contact between substrate, oxygen, and biomass. It will enhance mass transfer and finally affect to degradation rate. Jager, Croan and Kirk (1985) showed that ligninase was produced in agitated pelleted cultures at 150 rpm by including detergents Tween 20, Tween 80 or 3--[[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate (CHAPS) in the culture medium.

![Figure 6. Effect of agitation on 2,4,8-TCDF degradaton by T. versicolor U80 in the liquid medium](image)

Fungi need lignocellulosic substrates to grow and degrade pollutants in soil. The growth ability of T. versicolor U80 in several pre-grown sources were different. T. versicolor U80 grew well in the wood meal and paper sludge. These results are affected for degradation of 2,4,8-TCDF (Fig. 7).

Investigating of the 2,4,8-TCDF degradation by T. versicolor U80 in soil medium

The result showed that 2,4,8-TCDF degradation by T. versicolor U80 pre-grown in 10% wood meal was higher than others. Fungi pre-grown in wood meal could more secreted ligninolytic enzymes thus enhanced 2,4,8-TCDF degradation. On the other hand, paper sludge only contains cellulose and hemicellulose that could not enhanced 2,4,8-TCDF degradation. To increase the levels of degradation in soil, inoculated polluted soils with various fungal species on different lignocellulosic supports (e.g., wood meal, wood chips, corn cobs and wheat straw) was developed (Quintero, et al., 2008).

![Figure 7. Degradation rate of 10 ppm 2,4,8-TCDF in soil by bioremediation with T. versicolor U80](image)

Plant residues are a plentiful source of nutrients that favor fungal growth and soil colonization. Additionally, plant residues elicit production of adaptive ligninolytic enzymes (Castillo et al., 2001; Fujian, Hongzang and Zuohu, 2001). However, the lower remediation values obtained in soil than liquid medium because the pollutant is retained in the soil pores thereby impeding transfer to the fungi (Quintero et al., 2005). Further, the structural changes from higher to lower chlorinated congeners was occurred based on support of fungal metabolism.

Conclusion

In this report, the ability of T. versicolor U80 to degrade 2,4,8-TCDF was studied. In the liquid medium, this strain degraded 46.52% at 30 days. This degradation rate can be enhanced became approximately 59% after addition of glucose 2.5% and 2% Tween 80. Wood meal can be used as lignocellulosic supports for the growth of T. versicolor U80 to degrade 2,4,8-TCDF in soil. T. versicolor U80 degraded 2,4,8-TCDF 46.06% at 30 days in soil. Further investigation of the metabolite pathway involved in 2,4,8-TCDF degradation is still required.

References


