DEGRADATION OF DIOXINS USING ENZYMES AND STERILIZATION OF *Pseudallescheria boydii*

Kazuei Ishii, Tohru Furuichi and Yumi Matsuda

Division of Environment Resource Engineering, Graduate School of Engineering, Hokkaido University, W8, N13, Sapporo, 060-8628 Japan

Introduction

In Japan, soil contamination caused by dioxins in and around incinerators for municipal solid waste (MSW) is a national problem. For example, wastewater containing concentrated dioxins at an MSW incinerator facility in Nose, Osaka, unfortunately caused serious soil and surface water contamination. The concentration of dioxins in the soil was about 8,000 pg-TEQ/g. Other possible contamination sites can include soils on which fly ash has been stored improperly (or even placed directly) and landfill sites which have received bottom and fly ash over a long period. Some countermeasures should be carried out immediately at these dioxin-contaminated sites.

We have developed bioreactor systems for dioxin-contaminated water and soil[1-3] because biological methods are inexpensive and have a little potential to produce toxic by-products. We showed that a fungus, *Pseudallescheria boydii* (*P. boydii*), isolated from activated sludge treating wastewater that contained dioxins would degrade highly chlorinated dioxins[1].

Nakamiya et al.[2] showed that dioxins in contaminated soil sampled from Nose could be effectively degraded by this fungus. When the water content was 70% and the temperature was 35 °C, the degradation ratio was nearly 80%. In addition, Ishii et al.[3] carried out 5L-bioreactor experiments and showed that 50% of the dioxins in the soil were degraded in 48 hour at 30 °C. This fact suggested that development of a bioreactor system was possible. However, *P. boydii* was found to be a weakly pathogenic fungus, known to cause opportunistic infection. We need to develop a method of sterilizing *P. boydii* in the treated soils to allow us to apply the bioreactor system to real contaminated sites. If living *P. boydii* is impossible to use in the bioreactor system, we would have to consider producing a large amount of dioxin-degrading enzyme from *P. boydii*.

The objectives of this study are 1) to develop a method of sterilizing *P. boydii* in soil, and 2) to identify the dioxin-degrading enzymes from *P. boydii*.

Materials and Methods

Soils: Surface soil within the Hokkaido University grounds was collected, dried at 100 °C for two hours and passed through a 2 mm mesh sieve. This soil was not contaminated by dioxins.

Media: The medium contained glucose, 1.0 g; lignin, 0.1 g; $(NH_4)_2SO_4$, 0.2 g; NaCl, 0.2 g; K_2HPO_4, 0.1 g; $MgSO_4 \cdot 7H_2O$, 0.1 g; $CaCO_3$, 0.2 g and 0.1 mL of a trace element solution ($FeSO_4 \cdot 7H_2O$, 0.01 g; $MnCl_2 \cdot 4H_2O$, 0.01 g and $ZnSO_4 \cdot 7H_2O$, 0.01 g per 10 mL of distilled water) per 100 mL of distilled water.

Sterilizing experiment: We tried to sterilize *P. boydii* by heat and/or ethanol. Heating is one of the

simplest and the most general sterilizing methods. Ethanol might extract remaining dioxins in the soils[4]. Since the sterilizing effect of ethanol depends on the amount of organic matter in the sample, we tested the following three cases: 1) only *P. boydii*, 2) *P. boydii* in the medium, and 3) P. boydii in the soils. In case 1), 48 hour-incubated *P. boydii* was separated from the mixture of cell and medium and sterilized. In the other cases, 48 hour-incubated *P. boydii* was sterilized directly, which means that, for example, the mixture of *P. boydii*, medium and soil was sterilized by heat and/or ethanol. Temperature conditions were 25, 29, 50, 54, 60 or 74°C for two hours and net concentrations of ethanol in the mixture were 0, 70, 80, or 87.5%.

Growing test: To confirm whether the sterilization was completely effective, samples sterilized by the above methods were incubated for seven days at 30°C in the medium described above. If no growth of *P. boydii* was observed, a part of the medium was incubated for a further five days using the Petri-plate method. If there had been no growth of *P. boydii* after this time, we determined that *P. boydii* had been sterilized completely.

Preparation of enzyme from *P. boydii*: We tried to confirm which membrane enzyme or external cell enzyme was able to degrade dioxins, especially, octachlorinated dibenzo-p-dioxins (OCDD). The membrane enzyme mixture was prepared using the method described by Ishii[5]. The external cell enzyme mixture was prepared by filtering the culture using 0.2 μm of membrane filter.

Dioxins degradation tests: After 1 μL of OCDD was injected into 5 mL test tube, 0.1 mL of the prepared membrane enzyme mixture and 0.9 mL of citric acid – trisodium citrate buffer (pH 3.5) containing 1 mM NAD and 1 mM Mn, or 1 mL of the external cell enzyme mixture were added to the test tube. The test tube was then shaken slowly at 30 °C for 48 hours. After labeled OCDD and NaOH or H_2NO_3 and H_2SO_4 were added to the tube, OCDD was extracted by shaking three times with 0.3 mL of toluene. These alkalis and acid were used to extract dioxins absorbed to organic matter in the samples as far as possible.

Analysis of dioxins: The toluene phase was evaporated and its contents were taken up into hexane. The hexane phase was applied to a multi-layer silica gel column which was filled from the bottom to top, in order, with 0.5 g of silica gel, 3.0 g of 2% potassium hydroxide-impregnated silica gel, 0.5 g of silica gel, 4.5 g of 44% sulfuric acid-impregnated silica gel, 6.0 g of 22% sulfuric acid-impregnated silica gel, 0.5 g of silica gel, 3.0 g of 10% silver nitrate-impregnated silica gel, 0.5 g of silica gel and 3.0 g of sodium sulfate. The dioxins in the column were eluted with 150 mL of hexane, evaporated, and taken up into 0.1 mL of toluene. Analysis of dioxins was carried out with a GC-MS/MS apparatus (ThermoQuest GCQ plus ion trap mass spectrometer and TRACE GC 2000 gas chromatograph) in accordance with the method of Kemmochi and Arikawa[6].

Chemicals: For calibration and cleanup, a PCDD/PCDF standard mixture, EDF-4931 (CIL, Inc.), and isotopically-labeled chlorodioxin standard ED-900 (Wellington Lab.) were used, respectively. OCDD was DD-12346789-S (Wellington Lab.). The other chemicals were all laboratory grade.

Results and Discussion

Sterilizing conditions for *P. boydii*

In the above mentioned case 1), *P. boydii* was sterilized by 70% ethanol at 25°C. However, *P. boydii* in the medium was not sterilized at the same conditions as shown in Fig. 1, because of the organic

matter in the medium. It was found that P. boydii in the medium was sterilized completely at temperatures of over 60 °C, or 54 °C when the concentrations of ethanol are 70 and 80%.
On the other hand, *P. boydii* in soil was not sterilized completely at 60 °C, but required temperatures of over 70 °C, as shown in Fig. 2. When the concentration of ethanol was 80%, *P. boydii* found to be sterilized at 54 °C. Since the effect of ethanol on sterilization of *P. boydii* was small, it is concluded that the sterilizing conditions of our *P. boydii* in soils are temperatures of over 70 °C for two hours. Further study is needed to reduce the required time for sterilizing.

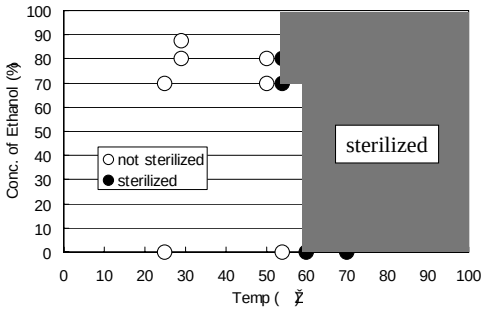

Fig. 1 Results of Sterilizing P. boydii in the medium (Case 2)

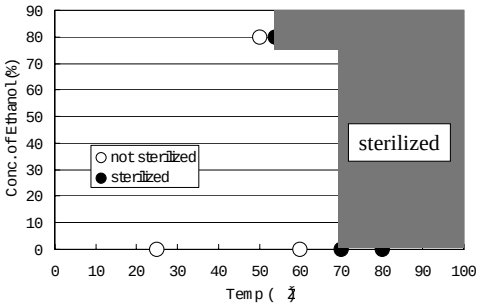

Fig. 2 Results of Sterilizing P. boydii in the soil (Case 3)

Proposal of post-treatment system

From the view of development of a bioreactor system for treatment of dioxins, safety issues related with *P. boydii* have restricted its application to real contaminated sites. *P. boydii* is a weakly pathogenic fungus, but which is ranked as the lowest level in Japanese guidelines. In addition, we confirmed that *P. boydii* was completely sterilized with the above methods. Therefore, we could take one step forward in the development of a bioreactor system, as shown in Fig. 3.

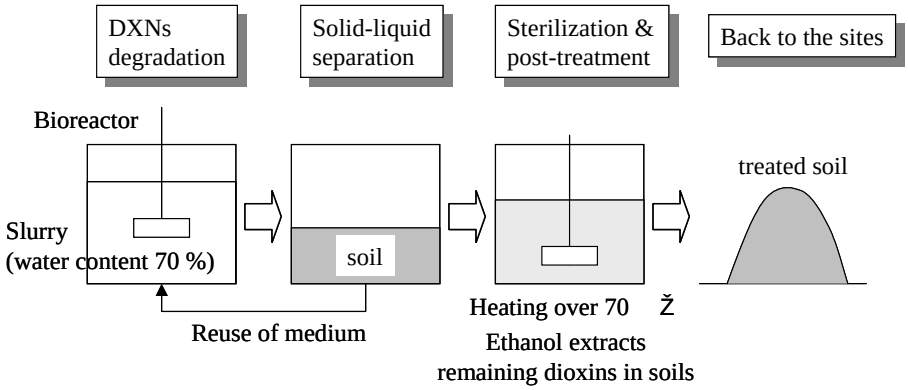

Fig. 3 Post-treatment system for bioreactor system

Degradation of OCDD by enzyme of *P. boydii*

Fig. 4 shows that the membrane enzyme mixture of *P. boydii* could degrade about 50% of OCDD within 24 hours. Since it was found that the membrane enzyme mixture, extracted by the same method as Ishii[5], could also degrade dibenzo-*p*-dioxin (DD), the membrane enzyme mixture might contain the enzymes that are related to degradation of OCDD and DD.

On the other hand, from results shown in Fig. 5, the external cell enzyme mixture can be considered to also degrade OCDD, although the degradation ratio was low. We are carrying out further studies to decide which enzyme is effective for degradation of OCDD.

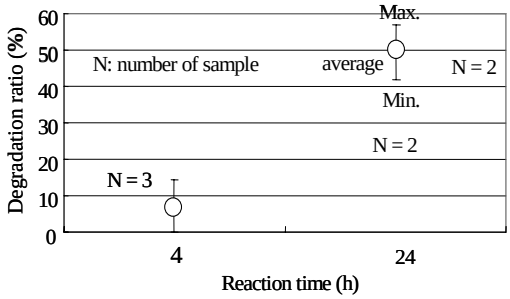

Fig. 4 Degradation ratio of OCDD by membrane enzyme mixture

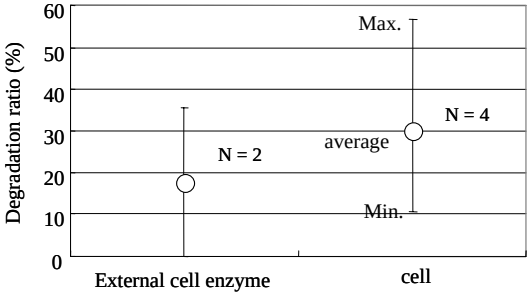

Fig. 5 Degradation ratio of OCDD by external cell enzyme mixture

Acknowledgments

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