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Estimation of the biodegradation rate of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin by using dioxin-degrading fungus, *Pseudallescheria boydii*

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ABSTRACT

We are developing a bioreactor system for treating dioxin-contaminated soil or water using the dioxin-degrading fungus, *Pseudallescheria boydii* (*P. boydii*). In order to design the bioreactor system, this study estimated the rate at which *P. boydii* degraded 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (2,3,7,8-TCDD), which is the most toxic of the dioxins. The experimental results showed that *P. boydii* degraded 2,3,7,8-TCDD during its logarithmic growth phase, using glucose as a carbon source for growth, and that the growth of *P. boydii* was not affected by 2,3,7,8-TCDD concentrations usually found at contaminated sites. These results were then used to apply successfully an existing mathematical model to the degradation of 2,3,7,8-TCDD by *P. boydii*. This allowed an estimation of the rate of degradation of 2,3,7,8-TCDD by *P. boydii* that can be used in the design of the bioreactor system.

1. Introduction

Soil contamination with polychlorinated dibenzo-*p*-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs) in and around municipal solid waste (MSW) incinerators is of great concern in Japan. For example, serious soil and surface water contamination was caused by mist containing high concentration of dioxins (PCDD/Fs) that was created by a wet gas scrubber at the MSW incinerator in Nose, Osaka. The concentration of dioxins in the soil was 8000 pg-TEQ/g. Other sites have been contaminated by the stockpiling or burying of fly ash containing dioxins around a MSW incinerator. In addition, an old landfill site that has been receiving non-treated fly ash is also considered one of the causes of contamination. Immediate and cost-effective countermeasures are therefore required at these dioxin-contaminated sites.

We are developing a bioreactor system using the dioxin-degrading fungus *Pseudallescheria boydii* (*P. boydii*), which was isolated by us from activated sludge collected from a leachate treatment facility at a MSW landfill site [1,2]. *P boydii* has the ability to decrease dioxin concentration from 7310 to 2860 pg-TEQ/g in 48 h using the bioreactor process [1]. Heptachlorodibenzo-*p*-dioxin (HpCDD) was detected in vitro as one of the by-products of octachlorodibenzo-*p*-dioxin (OCDD) degradation by *P. boydii* [2]. This means that a dechlorination process occurred. Nonchlorinated dibenzo-*p*-dioxin and dibenzofuran (DD and DF) were oxidized and transformed to hydroxydibenzo-*p*-dioxin and hydroxydibenzofuran, respectively, by *P. boydii* [3]. *P. boydii* has the abilities of both dechlorination and oxidation. However, the rate of degradation of dioxins by *P. boydii* has not yet been quantified sufficiently to allow the design of treatment processes. According to our previous studies, *P. boydii* uses glucose as a carbon source for growth [1]. However, the following three fundamental questions must still be answered in order to estimate the degradation rate of dioxins.

(1) Is a carbon source such as glucose really needed for *P. boydii* to degrade dioxins?
(2) During which phase of growth dose *P. boydii* degrade dioxins: the logarithmic growth phase or the stationary phase?
(3) Dose dioxins have a bad effect on the growth of *P. boydii*?

In order to design the bioreactor system, the above three fundamental questions must be answered. That is, the tripartite relationships among *P. boydii*, glucose and dioxins, in relation to the growth of *P. boydii* and the degradation of dioxins, must be clarified. The degradation rate of dioxins by *P. boydii* must be estimated, based on these fundamental characteristics, for the design of the bioreactor system.

Research on kinetic analysis of the biodegradation of dioxins using bacteria and fungi is very limited. Parsons and Storms [4] determined the pseudo-first order biodegradation rate constants of a mixture of chlorinated dibenzo-*p*-dioxins (1,3- and 2,8-

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dichlorodibenzo-*p*-dioxins and 1,2,4-trichlorodibenzo-*p*-dioxin) using the biphenyl-utilizing bacterium, *Alcaligenes* strain JB1. Hu and Bunce [5] conducted a degradation test of 1-chlorodibenzo-*p*-dioxin and 1,2,3,4-tetrachlorodibenzo-*p*-dioxin using rat CYP1 enzymes and found that the reactions followed first order kinetics. Mason and Safe [6] reported that 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (2,3,7,8-TCDD), which is the most toxic dioxin, was transformed into 2-hydroxy-3,7,8-trichlorodibenzo-*p*-dioxin and 2-hydroxy-1,3,7,8-tetrachlorodibenzo-*p*-dioxin by microsomes isolated from immature male Wistar rats. However, they did not determine the degradation rate of 2,3,7,8-TCDD. At the present time, the biodegradation rate of 2,3,7,8-TCDD has not been estimated.

Therefore, the objectives of this study were (1) to clarify the quantitative characteristics of 2,3,7,8-TCDD degradation by *P. boydii*, including the tripartite relationships among *P. boydii*, glucose and 2,3,7,8-TCDD in relation to the growth of *P. boydii* and the degradation of 2,3,7,8-TCDD, and (2) to apply an existing mathematical model to estimate the degradation rate of 2,3,7,8-TCDD by *P. boydii*.

2. Material and methods

2.1. Fungus

The fungus was isolated from the activated sludge that was collected from a leachate treatment facility associated with a MSW landfill sites in Japan [2]. The leachate contained dioxins and the fungus was identified as *P. boydii* based on 18S rDNA and morphological analysis [7].

2.2. Incubation conditions of P. boydii

P. boydii was incubated in 100 mL of medium containing glucose (0.5 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), and $CaCO_3$ (0.2 g). It also contained 0.1 mL of a trace element solution ($FeSO_4{\cdot}7H_2O$ (0.01 g), of $MnCl_2{\cdot}4H_2O$ (0.01 g), and $ZnSO_4{\cdot}7H_2O$ (0.01 g) per 10 mL of distilled water). We called this "normal medium". *P. boydii* was pre-cultivated in Erlenmeyer flasks for 48 h at 30 °C before commencing. We called the normal medium containing pre-cultivated *P. boydii* the "pre-cultivated solution".

2.3. Preparation of resting cells

The cells were separated from the medium by centrifugation (10 000 × *g* for 10 min) after 20 h cultivation and were rinsed with 25 mM phosphate buffer (pH 7.3 at 4 °C). The cells were then separated from the buffer by centrifugation (10 000 × *g* for 10 min) and resuspended in 50 mM phosphate buffer (pH 7.3 at 4 °C). We called the buffer containing the cells the "resting cell solution".

2.4. Degradation tests with 2,3,7,8-TCDD

A 0.5 mL volume of the pre-cultivated solution and 4.5 mL of the normal medium were injected into a 50 mL Erlenmeyer flask, to which 50 ng 2,3,7,8-TCDD was added. When using the resting cell, 5 mL of the resting-cell solution and 50 ng 2,3,7,8-TCDD were injected into the flask. The flasks were then shaken while being maintained at 30 °C. Triplicate samples were prepared for analyzing the concentrations of dioxins, *P. boydii* cells, and glucose.

2.5. Analytical methods

2.5.1. Concentration of P. boydii cells

The concentrations of *P. boydii* cells were determined by two methods. In the first method, the cells of *P. boydii* were separated from the 5 mL sample using 0.45 μm filter, which was then dried at 100 °C for 20 h. The weight of cells on the filter was measured to determine the concentration of *P. boydii* cells (drying method). The second method used a spectrophotometer to measure absorption (620 nm) of the sample. This method was particularly useful for samples containing 2,3,7,8-TCDD. The correlation coefficient for the relationship between the concentrations of cells determined by the drying method and those by absorption at 620 nm was 0.9532 ($n = 4$).

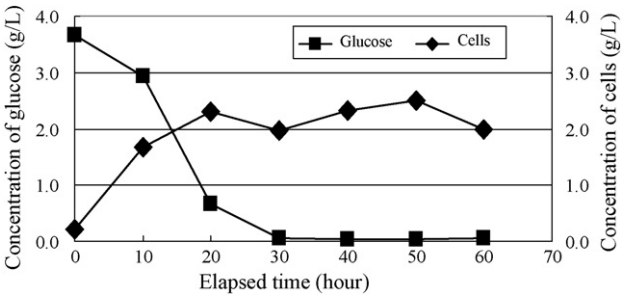

Fig. 1. Growth curve of *P. boydii* with glucose and without 2,3,7,8-TCDD.

2.5.2. Concentration of glucose

The concentration of glucose was determined by the phenol–sulfuric acid method. A 200 μm volume of phenol solution (5% (w/w)) was added to 200 μm of sample. Concentrated sulfuric acid (1 mL) was added to the mixture and the mixture was shaken vigorously. The concentration of glucose was determined by absorption (492 nm) after resting the mixture at 30 °C for 30 min.

2.5.3. Dioxins

To analyze dioxins, 13C-labeled 2,3,7,8-TCDD was added to the sample followed by the addition of 3 mL sulfuric acid and 3 mL nitric acid to break down the *P. boydii* cells. The dioxins were extracted three times using 5 mL toluene.

The extracted dioxins present in the toluene phase were dissolved into hexane and were then applied to a multilayer silica gel column. This was filled, in sequence from the bottom to the top, with 0.5 g silica gel, 3.0 g potassium hydroxide-impregnated silica gel (2%), 0.5 g silica gel, 4.5 g sulfuric acid-impregnated silica gel (44%), 6.0 g of sulfuric acid-impregnated silica gel (22%), 0.5 g silica gel, 3.0 g silver nitrate-impregnated silica gel (10%), 0.5 g silica gel and 3.0 g sodium sulfate. The dioxins in the column were eluted with 150 mL of hexane, and dissolved in 0.1 mL of toluene.

The dioxins were analyzed using a GC/MS/MS (Thermo Quest GCQ plus ion-trap mass spectrometer and TRACE GC 2000 gas chromatograph) according to the method of Kemmochi and Tsutsumi [8].

2.6. Chemicals

The 2,3,7,8-TCDD and 13C-labeled 2,3,7,8-TCDD were purchased from Cambridge Isotope Laboratories (Cat. ED-901 and ED-900, respectively). All the other chemicals used were laboratory grade.

3. Results and discussion

3.1. Growth rate of P. boydii without 2,3,7,8-TCDD

The growth of *P. boydii* coincided with a decrease in the concentration of glucose, as shown in Fig. 1. A logarithmic growth phase was observed over a period of 20 h, and thereafter, the growth rate became more stable. The concentration of cells was measured using the drying method.

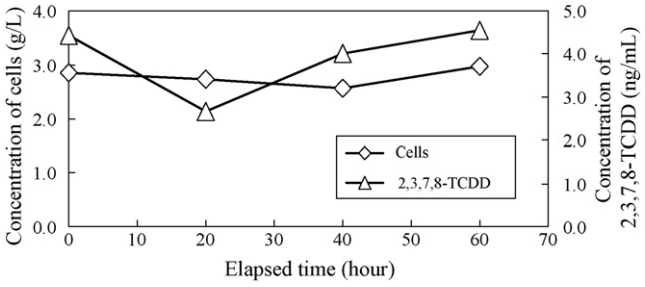

Fig. 2. Time course of 2,3,7,8-TCDD with resting cells and without glucose.

3.2. Degradation of 2,3,7,8-TCDD

3.2.1. Degradation of 2,3,7,8-TCDD by the resting cells

No decrease in the concentration of 2,3,7,8-TCDD was observed in the presence of resting cells of *P. boydii*, as shown in Fig. 2. In addition, the resting cells did not grow in the presence of 2,3,7,8-TCDD as the only carbon source, either because 2,3,7,8-TCDD is not a carbon source for *P. boydii*, or because the concentration of 2,3,7,8-TCDD was too low to support its growth. The concentration of cells was determined by absorption at 620 nm.

3.2.2. Degradation of 2,3,7,8-TCDD using glucose as a carbon source

When *P. boydii* was cultivated in the presence of both glucose and 2,3,7,8-TCDD, the concentration of 2,3,7,8-TCDD decreased at the same rate as the concentration of glucose, as shown in Fig. 3. This observation indicates that the decrease in 2,3,7,8-TCDD concentration coincided with the growth of *P. boydii*, as *P. boydii* grew using glucose, as shown in Fig. 1. The concentration of 2,3,7,8-TCDD decreased during the logarithmic growth phase of *P. boydii*. The concentrations of cells were not measured in this experiment.

3.3. Effect of 2,3,7,8-TCDD on the growth rate of P. boydii

Fig. 4 shows the concentrations of cells when *P. boydii* was cultivated with and without 2,3,7,8-TCDD. The concentration of cells in the medium with 2,3,7,8-TCDD was determined by absorption at 620 nm, as mentioned above. Fig. 4 suggests that there was no effect of 2,3,7,8-TCDD (up to 10 ng/mL) on the growth of *P. boydii* during the logarithmic growth phase. Furthermore, there was no difference in the glucose consumption rate between cultures containing 2,3,7,8-TCDD and those that did not contain 2,3,7,8-TCDD, as shown in Fig. 5.

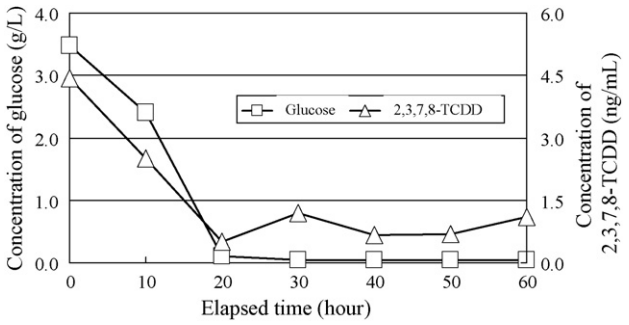

Fig. 3. Time course of 2,3,7,8-TCDD during cultivation of *P. boydii* with glucose.

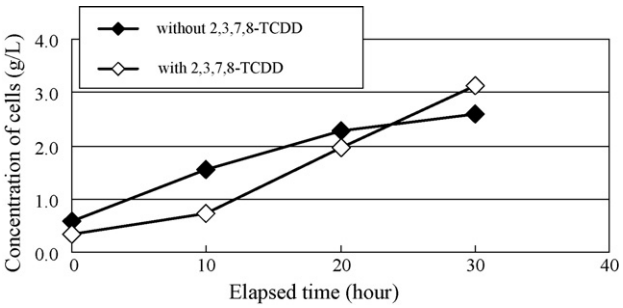

Fig. 4. Effect of 2,3,7,8-TCDD on the growth of *P. boydii*.

3.4. Mechanism of 2,3,7,8-TCDD degradation by P. boydii

Our experiments showed the following results. (1) *P. boydii* grew using glucose as a carbon source. (2) *P. boydii* degraded 2,3,7,8-TCDD during its logarithmic growth phase. (3) Neither the growth of *P. boydii* nor the glucose consumption rate were affected by the presence of 2,3,7,8-TCDD at concentrations up to 10 ng/mL, which is a high concentration compared with those usually found at contaminated sites.

According to our past study [1], *P. boydii* degraded highly chlorinated dioxins without showing any specificity for particular congeners. That is, the concentration of each congener decreased at almost the same rate. Takada et al. [9] also showed that different 2,3,7,8-congeners were degraded by the white rot fungus *Phanerochaete sordida* YK-624 at almost the same rate. The mechanism of 2,3,7,8-TCDD degradation by *P. boydii* will be discussed below.

Many studies on the degradation of dioxins by fungi have not used 2,3,7,8-TCDD, due to its high toxicity. For example, Valli et al. [10] attempted to degrade 2,7-dichlorodibenzo-*p*-dioxin using the lignin-degrading basidiomycete, *Phanerochaete chrysosporium*. Nakamiya et al. [11] found that the cyclic-ether-degrading fungus, *Cordyceps sinensis*, degraded not only DD but also 2,3,7-trichlorodibenzo-*p*-dioxin and OCDD. There are other studies showing that fungi degraded DD, DF, 2,7-dichlorodibenzo-*p*-dioxin or 2,8-dichlorodibenzo-*p*-dioxin [12–15].

Regarding studies on 2,3,7,8-TCDD degradation, Mason and Safe [6] demonstrated, using microsomes isolated from immature male Wistar rats, that the two major mammalian metabolites of 2,3,7,8-TCDD were 2-hydroxy-3,7,8-trichlorodibenzo-*p*-dioxin and 2-hydroxy-1,3,7,8-tetrachlorodibenzo-*p*-dioxin. Regarding enzymes related to the degradation of dioxins, Sakaki et al. [16] added DD, mono-, di-, and trichlorodibenzo-*p*-dioxins to cell cultures containing recombinant yeast that expressed either rat CYP1A1 or rat CYP1A2 (kinds cytochrome P450). They found that the degradation of the dioxins involved multiple reactions, including hydroxylation of dioxin at an unsubstituted position, hydroxylation involving

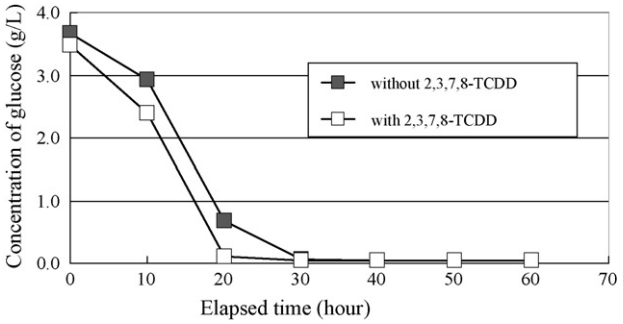

Fig. 5. Effect of 2,3,7,8-TCDD on glucose consumption rate.

migration of a chloride substituent, hydroxylation involving elimination of a chloride substituent and the opening of the dioxin ring. Hu and Bunce [5] showed that 2,3,7,8-TCDD was oxidized by CYP1A2. Therefore, cytochrome P450 enzymes appear to be involved in 2,3,7,8-TCDD degradation, so the multiple reactions reported by Sakaki et al. [16] can occur.

van den Brink et al. [17] said in their review paper that biochemical data suggest that many fungi posses numerous P450 genes. These past studies do not provide any evidence that P450 cannot be involved in the degradation of highly chlorinated dibenzo-*p*-dioxins by fungi. Although metabolites of 2,3,7,8-TCDD were not detected in this study when 2,3,7,8-TCDD was degraded by *P. boydii*, it is possible that *P. boydii* contains a P450 gene that is capable of degrading 2,3,7,8-TCDD.

4. Estimation of the degradation rate of 2,3,7,8-TCDD by *P. boydii*

4.1. Growth rate of *P. boydii*

The Monod equation was applied to the growth rate of *P. boydii* and the rate of glucose consumption, as shown in Eqs. (1) and (2).

$$\frac{dX}{dt} = \frac{\mu_{max} S}{S + K_S} X \quad (1)$$

$$\frac{dS}{dt} = -\frac{1}{Y_{X/S}} \frac{dX}{dt} \quad (2)$$

where X is the cell concentration [g/L], t is the time [h], μ_{max} is the maximum rate of microbial growth [1/h], K_S is the glucose saturation constant [g/L], S is the concentration of glucose [g/L], and $Y_{X/S}$ is the yield coefficient [g cell/g glucose].

4.2. Degradation rate of 2,3,7,8-TCDD

The above experimental results indicated that the concentration of 2,3,7,8-TCDD decreased with the growth of *P. boydii*. Therefore, we assumed that the rate of 2,3,7,8-TCDD degradation was proportional to the growth rate of *P. boydii*, as shown in Eq. (3).

$$\frac{dC}{dt} = -T_C \frac{dX}{dt} \quad (3)$$

where T_C is the transformation capacity [ng TCDD/mg cell]. Since the growth rate of *P. boydii* was not affected by the presence of 2,3,7,8-TCDD in this study, dX/dt in Eq. (3) can be estimated by Eqs. (1) and (2).

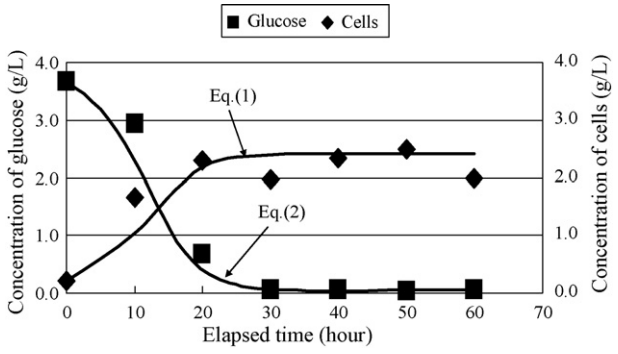

Fig. 6. Curve fitting of Eqs. (1) and (2) with observed data on the growth of *P. boydii* and the glucose consumption.

Table 1
Estimated parameters on the growth of *P. boydii* and the glucose consumption

Parameters	Values
μ_{max} the maximum microbial growth [1/h]	0.56
K_S the glucose saturation constant [g/L]	7.53
$Y_{X/S}$ the yield coefficient [g cell/g glucose]	0.61

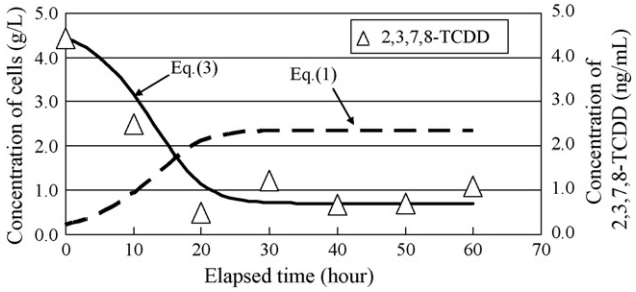

Fig. 7. Curve fitting of Eq. (3) with observed data on the 2,3,7,8-TCDD degradation.

4.3. Determination of parameters in the model

Parameters such as μ_{max}, K_S and $Y_{X/S}$ related to the growth rate of *P. boydii* and the consumption rate of glucose, as shown in Fig. 1, were initially determined using Eqs. (1) and (2). The fitted curves are shown in Fig. 6, and the estimated parameters are shown in Table 1.

Next, we estimated the growth rate of *P. boydii* cultivated with glucose and 2,3,7,8-TCDD, based on the glucose consumption curve as shown in Fig. 3. The broken line in Fig. 7 shows the estimated growth curve of *P. boydii*, calculated from the glucose consumption curve. We also determined the 2,3,7,8-TCDD degradation using this growth curve and Eq. (3), as shown in Fig. 7. The value of T_C was estimated to be 0.57 ng TCDD/mg cell, when the initial concentration of 2,3,7,8-TCDD was 4.4 ng/mL. Other our experiments showed the same behavior, and T_C was estimated to be 0.62 ng TCDD/mg cell. The initial concentration of TCDD was 6.2 ng/mL. Consequently, the degradation rate of 2,3,7,8-TCDD by *P. boydii* was estimated for the first time in this study as part of the process of designing a bioreactor system.

5. Conclusion

The following points were clarified by this study.

(1) The tripartite relationship among *P. boydii*, glucose and dioxins was clarified with respect to the growth of *P. boydii* and the degradation of dioxins.
   (i) *P. boydii* grew using glucose as a carbon source.
   (ii) *P. boydii* degraded 2,3,7,8-TCDD in its logarithmic growth phase.
   (iii) The growth of *P. boydii* and the glucose consumption rate were not affected by the presence of 2,3,7,8-TCDD concentrations usually found at contaminated sites.

(2) An existing mathematical model was successfully applied to the degradation of 2,3,7,8-TCDD by *P. boydii*, based on the above relationship. The degradation rate of 2,3,7,8-TCDD was estimated for the first time in this study, which will help the design of the bioreactor system.

Acknowledgments

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