BIODEGRADATION PATHWAY OF DIOXINS BY NOVEL RAPID GROWING THERMOPHILE

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Introduction

Bacillus midousuji HB1030 is a unique thermophilic microorganism that does not show growth under 62 °C. When non-or low-chlorinated (Cl=1-4) dioxins are incubated at growth condition of *B. midousuji*, dioxin levels decrease[1]. For the current study, this degradation mechanism was examined.

Methods and Materials

(1) Preparation of substrate solution, bacterial suspension and medium

The substrate Dibenzofuran (DF) was measured, and was diluted to 1 mg/ml by methanol. Final 20 ug/ml of DF in methanol became the substrate solution-1.

One mg of the Compound Dibenzo-p-dioxin (DD) was measured, and was diluted to 1 mg/mg by adding methanol (substrate solution-2).

Bacterial suspension was prepared so that the concentration of *B. midousuji* HB1002 and HB1030 would be 10^6 cell/ml by adding Tripticasse soy broth.

(2) Dibenzofuran sorption to the bacterial cell

For sorption to the bacterial cell, since *B. midousuji* start to react at temperatures above 62°C, examination took place under room temperature conditions where reaction will not occur, and comparison was made between those with bacteria included and those without (blank).

As with former paper[1], Tripticase soy broth was used as the medium. One ml of the medium was placed into the test tube, and 2 samples, one containing 10 µl (equivalent to 10^8 /ml cells) of bacteria, the other without (blank), were prepared. For both conditions, 50 µl (equivalent to 1µg) of Dibenzofuran (DF) substrate solution-1 was added to the media. Both test tubes were shaken for two days at room temperature, and then DF levels were measured by established method.

(3) Biodegradation reaction

Two hundreds and fifty µl of bacterial suspension (app. 10^8 /ml) was added to 25 ml of medium and after the addition of 25 µl of Dibenzo-p-dioxin (DD) substrate solution-2. This mixture was Incubated for 3 hours at 65 °C.

(4) Pretreatment Method

After incubation, mixtures were transported into test tubes with screw caps.

Following treatment by hydrochloric acid, distilled water was added to increase the total amount to 4ml. As clean-up spike, 10 ng of $^{37}Cl_4$-2, 3,7,8-TCDD was added to this solution.

After adding 500 µl of toluene, the reaction tube was sealed, then shaken for 10 minutes. The toluene layer was then separated by pipette, and was transferred to 3-ml mini-vials. This procedure was repeated 3 times. Each toluene layer from the samples were returned to the same vials and mixed.

Nitrogen flow was gently sprayed to the toluene in the vial, to remove surplus toluene. The final volume of the solution became approximately 100 μl.
Two ul of injection samples were analyzed by gas chromatography mass spectrometry (GC-MS). Analytes were measured by internal standard method with $^{37}Cl_4$-2, 3,7,8-TCDD as the internal standard.
(5) Characterization of products
With the reaction system, extraction took place by using ethyl acetate ester, under neutral condition around pH7. This became the neutral fraction. Then, after the liquid was acidified to pH2 by adding hydrochloric acid, extraction again took place by using ethyl acetate ester, and this became the acidic fraction. Both were evaporated, then concentrated under nitrogen flow. They were analyzed by GC-MS.
At the retention time where emergence of catechol was expected, total ion chromatograph (TIC) was obtained at the retention time around when the substrate emerged. By comparing it with the TIC of blank samples, peaks not observed in the blanks were detected.
Characterization and determination was conducted by GC-MS using gas chromatograph as separation system.
(6) DNA search for dihydroxy-dioxygenase or extradiol meta-cleavage enzymes in *B. midousuji*
DNA homology search for Dihydroxybiphenyl dioxygenase (BphC) gene sequence was performed under the collection of conserved region in its enzyme gene from *Sphingomonas* sp. Strain RW1[2], *Pseudomonas stutzer i*, *Pseudomonas mendocina*, *Alcaligenes xylosoxydans*, *Rhodococcus rhodochrous*[3].

Results and Discussion
When samples with bacterial suspension added were compared with those without under room temperature, no changes in the amount of remaining substrate could be observed in either sample.
Twenty-ml (20 times the norm) of reactant was used to examine the products and degraded substance of DD, but catechol, a degradation product, could not be detected. On the other hand, for samples tested under reacting conditions (65°C), emergence of peaks were observed instead of at blank samples.
The above results show that even under 37C, unlike incubation at 65C, show no dioxin decrease in either samples, with *Bacillus midousuji* and without. This suggests that dioxin decrease can not take place by processes other than growth processes including sorption to the bacteria. It was suggested that the dioxin decrease is a result of a biological reaction stemming from the process of *B. midousuji* 's growth.
When *B. midousuji* was incubated with DD, peaks that were different from that of blank samples emerged. For strain HB1002, peaks were detected more in the neutral fraction than in the acidic fraction. In contrast, for strain HB1030, peaks were detected more in the acidic fraction than in the neutral fraction. Also, peaks with different retention time appeared between HB1002 and HB1030. It was interesting that different two thermophilic strains HB1002 and HB1030 show differences in metabolic process.
If there were metabolites, the fact that catechol could not be detected by TIC after incubation under conditions of growth of *B. midousuji* suggests the possibility of a rapid degradation with 3 hours incubation at 65C or a new different metabolic pathway.
The fate of DD nor catechol product could not be observed in these results. This would mean that there is a high possibility of *B. midousuji* not having a pathway where 2,2', 3-THB ether is the intermediate metabolite. TIC data might be suggesting an existence of a metabolic pathway different than this one. On the other hand, it is possible that it indeed has a 2,2', 3-THB ether metabolizing pathway, but produced that catechol underwent further metabolism.

DNA homology search of Dihydroxybiphenyl dioxygenase (BphC) gene sequence was performed using conserved region of its enzyme gene from *Sphingomonas* sp. Strain RW1, *Pseudomonas stutzeri*, *Pseudomonas mendocina*, *Alcaligenes xylosoxydans*, *Rhodococcus rhodochrous.* Amino acid sequences CNGRHHT and RHTNDHM were reversed to oligomer DNA sequences as the results of 5' tgc aay grh sgm cac cac ac 3' and 5' cgc cac acc aat gac cac atg 3'.
PCR with these oligomer primers was performed with 55°C annealing temperature to *B. midousuji* HB1030 and the clear DNA band was observed in gel electrophoresis.
This result might suggest the BphC gene was carried in the DNA of *B. midousuji* HB1030.

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References

1. Hoshina S., Figurski, D. H. F., Weinstein, I.B., Gohda, H., and Furuichi, T. (1999) Organohalogen Compounds 40, 503-505.
2. Armengaud J., Timmis K. and Wittich R-M. (1999) J. Bacterial. 181, 3452-3461.
3. Takami, H., Kudo T. and Horikoshi K. (1997) Biosci. Biotech. Biochem., 61, 530-532.