DIOXIN DIOXYGENASE & DIHYDROXYBIPHENYL DIOXYGENASE GENE SEGMENTS IN BACILLUS NOVEL THERMOPHILE THAT DEGRADES TCDD

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Introduction

Isolation of strains of *Bacillus midousuji*, a species of thermophilic bacteria, which degrades various toxic and non-toxic waste materials, includes industrial wastes. *Bacillus midousuji* HB1030 is a unique thermophilic microorganism that has optimum growth temperature at 62C[1]. When 2,3,7,8 dioxin is 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

Bacterial suspension was prepared so that the concentration of *B. midousuji* HB1002 and HB1030 (both variant strains) would be 10^6 cell/ml by adding Tripticasse Soy Broth (BBL). Four hundreds ml of the medium was placed into the round bottom longneck flasks, and 2 samples, one containing 30 ml (equivalent to 10^8 /ml cells) of bacteria, the other without (blank), were prepared. For both conditions, 300pg of 2,3,7,8-TCDD substrate in DMSO were added to the media. Both test tubes were shaken for 24 hours at 65C temperature, and then TCDD levels were measured by established method.

Pretreatment Method[2]

After incubation, mixtures were transported into glass bottles with screw caps. Following treatment by hydrochloric acid, distilled water was added to increase the total amount. As clean-up spike, 10 ng of $^{37}Cl_4$-2, 3,7,8-TCDD was added to this solution.

After adding toluene, the reaction tube was sealed, then shaken for 10 minutes. The toluene layer was then separated by pipette, and was transferred to 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 was 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.

Characterization of products[2]
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.

DNA search for dioxin-dioxygenase or dihydroxybiphenyl dioxygenase enzymes in *B. midousuji*
DNA homology search for dioxin dioxygenase (*dxn*A1) and dihydroxybiphenyl dioxygenase (*bph*A1) gene sequence was performed under the collection of PCR products using primers conserved region in its enzyme gene from *Sphingomonas* sp. Strain RW1[1], *Pseudomonas stutzeri*, *Pseudomonas mendocina*, *Alcaligenes xylosoxydans*, *Rhodococcus rhodochrous*[4]. Consensus amino acid sequences CNGRHHT and RHTNDHM from these strains 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'. These sequences positioned 964-1002 and 104-132 of *dxn*A1 gene from *Sphingomonas* sp. Strain RW1.
DNA homology search was done with DNASIS Multiple Edit 7.

Results and Discussion
(1) 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 of reactant was used to examine the products and degraded substance of non-Cl-DD, but catechol, a degradation product, could not be detected. On the other hand, for samples tested under reacting conditions (65C), 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.

(2) When 2,3,7,8-chlorinated dioxin is incubated at growth condition of *B. midousuji* at 62C, dioxin level decreases to 54% of original concentration (0.5ng/ l) within 24 hours[1].

(3) When *B. midousuji* was incubated with non Cl-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.

(4) DNA homology search of dioxin-dioxygenase (*dxn*A1) and dihydroxybiphenyl dioxygenase (*bph*A1) gene sequence was performed using conserved region of its enzyme gene from *Sphingomonas* sp. Strain RW1, *Pseudomonas stutzeri*, *Pseudomonas mendocina*, *Alcaligenes xylosoxydans*, *Rhodococcus rhodochrous*[3,4]. PCR with the consensus oligomer primers was performed with 55C annealing temperature to *B. midousuji* HB1030 and four clear DNA bands were observed in gel electrophoresis.
DNA homology of *B.midousuji* PCR products to *dxn*A1 and *bph*A1 of these species were highly found in this search. This consensus sequences positioned 964-1002 and 104-132 of *dxn*A1 gene from *Sphingomonas* sp. Strain RW1.
By DNA homology search, No.11 contigue was found a 62% identity in *dxn*A1 gene (955-1005). Scachared homologous regions were also found in *dxn*A1 with this search.

(5) Considering to higher mutational rate in thermophile, this result might suggest *dxn*A1/*bph*A1 genes were carried in DNA of *B. midousuji* HB1030. Comparing with two strains of *B. midousuji*, DD metabolites of these strains are different, systems of dioxin degradation enzymes might not be identical situation.

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