Enhanced Biodegradation of 2,3,4,5,6-pentachlorocyclohexan-1-ol -- a β-HCH Metabolite -- in the Presence of α- and γ-HCH Isomers by *Pseudomonas aeruginosa ITRC-5*

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ABSTRACT

Chlorinated insecticide hexachlorocyclohexane (HCH), which is still used in many countries in agriculture and forestry, leads to environmental problems. HCH consists of a mixture of four isomers: α-, β-, γ-, and δ. A bacterium *Pseudomonas aeruginosa ITRC-5* degrades not only α-, γ-, and δ- HCH isomers but also β-HCH which is the most recalcitrant due to its chemical stability. The degradation of β-isomer is accompanied with the formation of a metabolite 2,3,4,5,6-pentachlorocyclohexan-1-ol (PCCOL).

In the present study, effectiveness of ITRC-5 strain in further degradation of PCCOL was evaluated. γ-HCH grown ITRC-5 cells show higher degradation of PCCOL than the t-HCH grown cells do. Furthermore, degradation of PCCOL by γ-HCH grown cells is enhanced in the presence of α- or γ-HCH isomers. Cloned genes of ITRC-5 bacterium showed 50% degradation of PCCOL in 16 hrs. The ITRC-5, therefore, demonstrates potential for the bioremediation of β-HCH and PCCOL

Keywords: *Pseudomonas aeruginosa ITRC-5*, β-Hexachlorocyclohexane, 2,3,4,5,6-pentachlorocyclohexan-1-ol, Biodegradation.

1 INTRODUCTION

A technical mixture of hexachlorocyclohexane (t-HCH) consisting of four major isomers α- (60-70%), β- (5-12%), δ-HCH (6-10%) and 10-12% of γ-HCH (also known as Lindane) remained a popular insecticide formulation and was used extensively worldwide prior to the 1990s [1]. Although HCH has been banned or restricted in many developed countries because of its toxic and persistent nature, it is still in production and in use in many parts of the world [2]. Agricultural soils and groundwater around the world has been contaminated with HCH compounds to various degrees [3]. Efforts have been made for the remediation of soils and groundwater contaminated with the toxic and persistent HCH isomers through biodegradation processes [4-6].

In particular, β-HCH isomer has higher degree of persistence compared to other HCH isomers and, therefore, β-isomer residues have been reported predominantly from soil, water, and food commodities [7-9]. It is the most problematic of the HCH isomers as it is quite resistant to biodegradation and is also suspected to cause endocrinal disruption as well as breast cancer [10,11]. It is also reported to have estrogenic effects in mammalian cells and fish [2]. From various places around the world, β-HCH has been reported to be found in human breast milk [3,12]. It poses a serious threat as it has potential to be transferred from one generation to the next via bioaccumulation in various food chains.

Only a few bacterial strains have been found responsible for the degradation of β-HCH isomer [13,14]. Nagata et. al. (2005) reported that haloalkane dehalogenase linB in the UT26 bacterial strain converted β-HCH to PCCOL, a metabolite which could not be further degraded [15]. In a recent study linB2 gene in *Sphingomonas sp.* BHC-A bacterial strains converted not only β-HCH to PCCOL but also PCCOL to tetrachlorocyclohexanediol [16].

The isolated bacterium *Pseudomonas aeruginosa ITRC-5* was reported to degrade all four major isomers (i.e. α, β, γ, and δ) of HCH [17]. However, the degradation of β–HCH isomer resulted in a dead-end product of PCCOL. In the present study, we found that the ITRC-5 bacterial strain grown in media containing γ-HCH and t-HCH was able to further degrade PCCOL in aerobic condition. The degradation of PCCOL was enhanced in the presence of additional α- or γ-HCH. Furthermore, the cloned genes of ITRC-5 expressed in *E. coli* DH5-α could successfully degrade PCCOL.

2 MATERIAL AND METHODS

2.1 Chemicals

Technical HCH was obtained from India Pesticides Limited, Lucknow, India. β-HCH and γ-HCH were purchased from Riedel-de Haën, Germany. Silica gel TLC

plate, and all other chemicals of AR grade used in the experiments were purchased from Merck India Ltd.

2.2 Microorganisms and culture conditions

The bacterium *ITRC-5,* isolated earlier in the ITRC lab [17], was used in aerobic condition. It was grown in flasks that were precoated with 200 ppm t-HCH and contained 50 ml of W^+ medium [18]. The bacterium was incubated in the flasks at 28°C with shaking at 180 rpm for 1 week. In order to maintain fresh culture of the bacterium cells throughout the experiments, 2 ml of the grown culture was transferred to the fresh flasks and grown as above. *E. coli DH5-α* was used in gene cloning experiments which was cultured in Luria broth medium containing an appropriate concentration of ampicillin antibiotic and incubated overnight at 37°C.

2.3 Biodegradation of PCCOL by γ-HCH and t-HCH grown cells

Three sets of 25 ml flasks were coated with 1 ppm of PCCOL dissolved in acetone. Eight flasks in each set were used. The flasks were kept open in a laminar hood at room temperature. After evaporation of the acetone, 10 ml of sterile W^+ medium was added into each flask. While the first set of flasks was kept un-inoculated, the second and the third set of flasks were inoculated with 1 ml of the *ITRC-5* cells previously grown in W^+ medium with γ-HCH and t-HCH, respectively, as the carbon source. The flasks were inoculated in duplicate and were incubated for 6 days on a rotary shaker (150 rpm) at 28°C. Two flasks from each set were removed from the reaction setup at the interval of 2 days and the contents were extracted and analyzed by GC.

2.4 Biodegradation of PCCOL by "γ-HCH grown cells" in the presence of α- and γ-HCH

In this experiment four sets of 25 ml flasks were coated with 1 ppm of PCCOL dissolved in acetone. The first set was used as a reference and therefore, kept un-inoculated. The second set was inoculated with 0.2 ml of the "γ-HCH grown cells" of *ITRC-5* in the absence of α- or γ-HCH. The flasks of sets 3 and 4 were coated additionally with 50 ppm of α-HCH and γ-HCH, respectively. The flasks were kept open in a laminar hood at room temperature. As acetone evaporated from the flasks, 10 ml of sterile W^+ medium was added into each flask. The flasks were inoculated in duplicate and were incubated for 6 days on a rotary shaker (150 rpm) at 28°C. Two flasks from each set were removed from the reaction setup at the interval of 2 days and the contents were extracted and analyzed by GC.

2.5 Cloning of gene responsible for degradation of PCCOL

ITRC-5 and *DH5-α* bacterial cells were used for cloning procedure. *ITRC-5* bacteria were incubated at 28°C in 1 liter W^+ medium containing 500 ppm of γ-HCH, whereas *DH5-α* bacteria were incubated at 37°C in 50 ml of Luria broth medium. pUC18 plasmid vector and ampicillin (50 mg/ml) antibiotic were used in the gene screening process. The alkaline lysis method was used to isolate plasmid DNA of *DH5-α* and the DNA was further dephosphorelated with calf enzyme [19]. Marmur's method was used to isolate genomic DNA from *ITRC-5* strain [20]. Genomic library was constructed for *ITRC-5* bacterial DNA which was partially digested with Sau3AI restriction enzyme. Isolated DNA fragments of 5 kb in size were ligated with the dephosphorelated plasmid vector pUC 18 digested with Bam H1 restriction enzyme. The ligation product was transfected into *E. coli DH5-α* and screened for ampicillin resistance on agar plates. The library in *DH5-α* was stored at 4°C.

2.6 In vivo assay for PCCOL degrading gene

ITRC-5 gene library was assayed for PCCOL degrading gene. A small quantity of a colony was suspended in 1ml of assay solution (1 ppm PCCOL, 100 ppm of each α, β, γ and δ-HCH in 1/3rd LB medium). The suspension was incubated at 37°C over night. Then 1 ml of hexane and acetone (9:1) was added, and the mixture was vortexed for 3 min. After 5 min. the hexane and acetone layer was recovered. 1 μl of this extract was analyzed by GC for PCCOL degradation.

2.7 GC Analysis

A gas chromatograph (GC, Netel Chromatograph; Michro 9100) fitted with 63Ni-ECD was used for this study. The column, injector and detector temperatures were kept at 190°C, 250°C and 250°C, respectively. Nitrogen was used as the carrier gas and maintained at a pressure of 15 psi. The GC was calibrated with standard HCH isomers and PCCOL.

3 RESULTS AND DISCUSSION

3.1 Biodegradation of PCCOL by γ-HCH and t-HCH Grown Cells

The purified PCCOL was degraded by γ-HCH grown as well as t-HCH grown *ITRC-5* cells. The concentration of PCCOL in the media decreased with increased time period of incubation. TLC analysis of PCCOL degradation by γ-HCH is shown in Fig.1. After 6 days of incubation, approx 61% and 58% degradation of PCCOL was achieved by γ-HCH grown cells and t-HCH grown cells, respectively [Fig.2].

In un-inoculated condition, a small degradation of PCCOL was observed after 2nd day of incubation, as shown

NSTI-Nanotech 2009, www.nsti.org, ISBN 978-1-4398-1783-4 Vol. 2, 2009

in Fig. 2, which could be due to hydrolysis, photodecomposition, volatilization, or other abiotic transformations of PCCOL. Furthermore, under inoculated condition, higher degradation of PCCOL was observed with γ-HCH grown cells in comparison with t-HCH grown cells. It is suspected that the genes responsible for PCCOL degradation might be more active in the presence of γ-HCH.

3.2 Biodegradation of PCCOL in the Presence of α- and γ-HCH by γ-HCH Grown Cells

After 6 days of incubation of γ-HCH grown *ITRC-5* bacterial cells, 75.5% degradation of PCCOL was observed in the presence of additional 50 ppm γ- HCH whereas 71% degradation was observed in the presence of α-HCH [Fig. 3]. The results show an increase in the degradation of PCCOL by ~14.5% and ~10% in the presence of γ-HCH and α-HCH, respectively. Presence of γ–HCH shows higher degradation of PCCOL than the presence of α-HCH isomer.

Increased degradation of PCCOL in the presence of α- or γ-HCH could be due to additional carbon source provided by α- or γ-HCH for the proliferation of ITRC-5 bacterial strain. Furthermore, ITRC-5 utilizes relatively easily γ–HCH isomer than α-HCH isomer as a carbon source owing to the difference in spatial arrangements of chlorine atoms. This possibly explains the observed higher degradation of PCCOL in the presence of γ-HCH than α-HCH. No further degradation of PCCOL was observed after 6 days of incubation, possibly because of the depletion of the growth supporting α- or γ- isomers by this time.

3.3 Screening of Clones with PCCOL Degradation Activity

The gene library of *ITRC-5* was constructed in *DH5-α*. The clones responsible for PCCOL degradation activity were screened by GC analysis. Out of 200 clones that were tested for insert DNA using plasmid isolation method, 100 clones showed positive results. Fig.4 shows 4.5kb, 4.5kb, 5kb, 5kb and 4.5kb insert DNA with plasmid DNA band in lane 1, 2, 5, 6 and 7, respectively. Only three clones out of 100 positive clones showed PCCOL degradation activity. These three clones showed approx. 50% degradation of 1 ppm PCCOL in 16 hrs. The degradation activity was not detected in other clones having insert DNA possibly due to lack of PCCOL degrading gene. The gene responsible for the degradation of PCCOL has not been characterized in the present paper.

pUC18 plasmid vector in this research has not been used for functional assay. Although pUC18 is not equipped with promoter, regulator sequences, large genomic DNA fragment of ITRC-5 up to 5 kb size used in this study may contain gene as well as all things needed for expression of protein. Therefore, the gene involved in the degradation pathway of PCCOL could be expressed by three clones.

The gene of ITRC-5 responsible for the degradation of PCCOL might be very similar to LinB2 gene. ITRC-5 has full copy of LinB gene designated as LinB1 and LinB2 gene [21]. Jun et al. (2007) reported that LinB2 gene in *Sphingomonas sp.* BHC-A was involved in PCCOL degradation pathway [16].

4 CONCLUSIONS

In conclusion, the present study suggests that an effective remediation of persistent β-HCH isomer and its metabolite PCCOL can be achieved by the addition of ITRC-5 bacterium. Furthermore, degradation activity of ITRC-5 bacterium is induced by the additional α-HCH and γ-HCH isomers in the medium. Further experiments are necessary to characterize the PCCOL degrading gene which seems to have very strong degrading activity. These findings are very important as it is one step further in the elucidation of probable β-HCH biodegradation pathway.

5 ACKNOWLEDGEMENTS

This work was supported through the research assistantship by the University Grant Commission (UGC), New Delhi in form of a Senior Research Fellowship to Anita Singh. We would also like to thank Industrial Toxicology Research Centre (ITRC) for providing logistical support for this research.

6 FIGURES

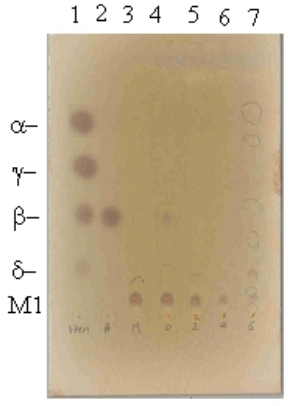

Figure 1: TLC shows biodegradation of PCCOL metabolite (M1) after 0 time, 2, 4 and 6 days of incubation. Heptane and 5% acetone were used as solvents for run. Rf value: α-HCH 0.63, γ-HCH 0.47, β-HCH 0.32, δ-HCH 0.15, M1 0.05.

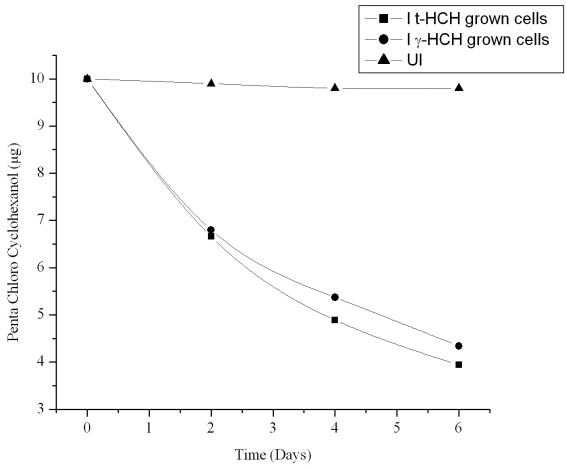

Figure 2: Degradation of PCCOL metabolite (M1) under un-inoculated (UI) and inoculated (I) conditions**.**

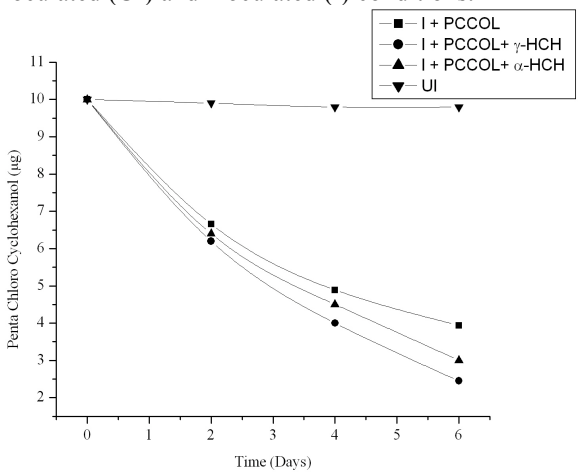

Figure 3: Biodegradation of PCCOL under un-inoculated (UI) and inoculated (I) conditions. Biodegradation of PCCOL under inoculated conditions in the presence of additional γ-HCH (I+γ), α-HCH (I+α) by γ-HCH grown cells, is also shown.

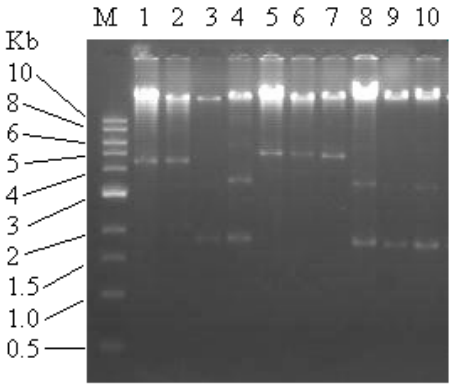

Figure 4: Lane 1, 2, 5, 6 and 7 shows pUC18 plasmid vector with insert DNA from *ITRC-5*.

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