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Process optimization of γ- Hexachlorocyclohexane degradation using three novel *Bacillus sp*. strains

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ABSTRACT

In the present investigation three lindane degrading bacterial isolates, HP-9, H-10 and HP-13 were isolated from agricultural soil by enrichment culture technique. These isolates were characterized- HP-9 as *Bacillus sp. Cal6F*, HP-10 as *Bacillus sp. Lad2A* and HP-13 as *Bacillus sp. Ym7A* by 16S rRNA sequencing and their 16S rRNA sequence were submitted to National Centre of Biotechnology Information (NCBI) Gene Bank with accession numbers, KJ937291, KJ944283 and KJ944284, respectively. They efficiently degraded up to 100 mg/l of lindane within 10 days under the optimized physiological conditions of: pH 7, temperature 30 °C, shaking speed 120 rpm, NaCl (1–3%) and initial lindane concentration 100 mg/ml. The maximum rate of inorganic chloride ion release was 0.49 mg/ml, 0.40 mg/ml, 0.40 mg/ml and the rate of gamma isomer of lindane involved in degradation observed was 75.65%, 64.77% and 62% for HP-9, HP-10 and HP-13, respectively. Lindane degradation was further confirmed by gas chromatography and *Sphingomonas japonicum* MTCC 6362 was used as a reference strain. Degradation kinetics was studied by using first order kinetic equation. Half-life periods during degradation were found to be 1.35, 1.48 and 1.75 days for the three isolates. The addition of galactose enhanced the degradation rate up to 10% whereas maltose, lactose and xylose had adverse effect and leads to decrease in degradation up to 40%. It was observed that addition of beef extract greatly enhanced lindane degradation rate followed by malt extract and peptone. The addition of glucose as a co-substrate and H_2O_2 as an electron donor greatly enhanced the lindane degradation rate.

1. Introduction

Lindane is the gamma isomer of 1,2,3,4,5,6-hexachlorocyclohexane (HCH), is a cyclic, saturated and highly chlorinated pesticide. It is a broad spectrum insecticide, which has been used worldwide for many decades in the last century to control a variety of pests and insects affecting various crops (Phillips et al., 2005). It has also been applied in human health as scabicide and pediculocide in the form of lotions, creams and shampoos as well as for the treatment of malaria (Bidlan et al., 2004; Lal et al., 2006). It is produced by photochemical chlorination of benzene with UV light; however, during lindane production, a mixture of several stereoisomers is obtained, (Manickam et al., 2007). It was first applied in the form of technical HCH-a mixture of α-HCH (53–70%), β-HCH (3–14%), γ-HCH (10–18%), δ-HCH (6–10%), ϵ-HCH (1–5%), and also traces of other isomers (Geueke et al., 2013). Later, it was also used in a purified form under the commercial name of lindane (> 99% purity), because the γ- isomer is the only one which exhibits insecticidal activity (Nagata et al., 2007). It is estimated that about 600,000 t of lindane was used between 1950 and 2000, and around 1.7 and 4.8 million tons of HCH residues are still present worldwide (Vijgen, 2006).

It has been shown that lindane and its isomers may cause serious damage to health in the short and long term. In mammals, acute lindane intoxication may cause respiratory dysfunction, generalized trembling, hyper-salivation, and convulsions, which can also lead to death in extreme cases (Pesce et al., 2008). For these reasons, currently more than 52 countries have banned or severely restricted the use of this xenobiotic compound (Vijgen et al., 2011). However some countries, mainly developing ones, still use it for economic reasons. As the result, new polluted sites are being aroused, possessing serious environmental threat at global level. Because of its persistence and recalcitrance, lindane and other HCHs residues remain in the environment for a long time and have been recently found in water, soil, sediments, plants and animals all over the world. It was also found in human fluids and tissues, such as blood, amniotic fluid, breast milk and adipose tissue (Herrero-Mercado et al., 2010). In this context, scientists are working all over the world for developing effective lindane remediation technologies. Researchers have isolated a variety of lindane degrading

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microorganisms from different habitats and under diversified conditions. In our previous study, we also have isolated two novel and potent lindane degrading bacterial species as *Kocuria* sp. DAB-IY and *Staphylococcus* sp. DAB-1W (D. Kumar et al., 2016). In the present study, selection of source fields for collecting soil samples was based on consistent pesticide usage history. Since, lindane has been banned for commercial use; it was unlikely to find sites with current application to crop fields. As these sites had a long history of lindane use, there was a possibility of finding higher traces of the pesticide in the selected fields. Hence, it was more probable to isolate potent microorganisms with lindane degrading ability from the current locations. Considering the various environmental impacts and persistence of lindane in the soil for long time and its toxicity, we attempted to isolate and characterize potent bacterial strains involved in degradation of lindane from contaminated soils. Further, these strains may be utilized as the microbial resource for the degradative genes in future and also these genes can be transferred to non-degradative micro-organisms by genetic manipulations.

2. Materials and methods

2.1. Chemicals, strain and collection of soil samples

Lindane (γ-HCH, 97.2% purity) technical grade was procured from Sigma Aldrich (USA). A stock solution of lindane, prepared at a concentration of 4×10^3 mg/l in acetone (Fisher scientific India Pvt. Ltd.) was added to Basal mineral medium with a few modifications at a pH of 7.2 ± 0.5 (Sahu et al., 1990; Pannu and Kumar, 2014). All other chemicals were of analytical grade, procured from Hi-media, Merck and Qualikam India. *Sphingomonas japonicum* (MTCC No. 6362) procured from Institute of Microbial Technology (IMTECH), Chandigarh, has been used as a reference culture for all the biodegradation studies.

Soil samples were collected from three different agricultural fields with crops viz. sweetpoatato, ginger and maize of Hamirpur, (HP) India, which had a long history of pesticide application. Surface soil from 0 to 15 cm depth were collected, placed in plastic bags, transported to the laboratory and stored at 4 °C until analysis. Soil samples were air- dried and sieved through a 10 mm mesh prior to bacterial screening. Physico-chemical characterization of soil samples was carried out using soil quality test kit (K052, Hi-media, Mumbai, India).

2.2. Isolation, screening and substrate tolerance of lindane degrading bacteria

Bacterial isolation was carried out by enrichment culture technique using mineral salt medium (MSM). Two gram of collected soil sample was added to 100 ml of sterile medium supplemented with 10 mg/l of lindane. The flask was mixed thoroughly and incubated at 30 °C for 7 days on rotary shaker at 120 rpm. Subsequently 10 ml aliquots from the flasks were transferred to sterile medium containing same lindane concentration. After that the concentration was increased in a stepwise manner, starting from 10 mg/l to 100 mg/l and the inocula were transferred to fresh media each time. After acclimatization, isolated bacterial colonies were cultured on mineral agar plates by spread plate technique and incubated at 30 °C for 24 h. The plates were preserved at 4 °C inside refrigerator for further research work. The bacterial isolates were further screened for lindane utilization and degradation activity using plate assay and Dechlorinase enzyme assay.

2.2.1. γ-HCH plate assay

The spray plates were prepared with 1.5% agar in mineral medium and cultured by streaking method. The surface of each plate was sprayed with 0.5% of lindane solution and plates were incubated at 28 ± 2 °C for 7days. The appearance of clearance zone surrounding bacterial colonies indicated utilization of lindane by culture organisms. Further, pure isolates were grown into mineral broth supplemented with 100 mg/l of lindane, for 48 h at 30 °C and 120 rpm (Phillips et al., 2005)

2.2.2. Dechlorinase enzyme assay

Colorimetric assay for detecting haloalkane dehalogenase activity was performed in a 96 well microtiter plate according to the method of Holloway et al. (1998) with slight modifications. The assay buffer contains 0.5 mM HEPES (pH 8.2), 10 mM sodium sulphate and 0.5 mM EDTA. Phenol red (dissolved in acetone) was added to the buffer before addition to 96 well plate to give a final concentration 20 μg/ml. Each well was filled with 194 μl of buffer and 3 μl of lindane stock solution (12.5 mg/ml of acetone) prior to the inoculation of 6 μl of cell free extract. During screening process, the microtitre plates were kept covered to prevent volatilization of lindane as well as the reaction mixture. A change in colour from red to yellow was indicative of lindane dechlorination which arises due to decrease in pH. Wells with blank samples were having 4-(2-hydroxyethyl) − 1-piperazineethanesulfonic acid (HEPES) buffer without cell free extract.

2.2.3. Substrate tolerance

Lindane utilizing cultures were further inoculated in half strength mineral salt broth containing various concentrations of lindane as a sole carbon source (i.e. 20, 40, 60, 80, 100, 120 ppm) and incubated at 30 °C at 120 rpm for 15 days to check their tolerance limits.

2.3. Gas chromatography analysis

Residual lindane in the culture was determined qualitatively by GC-ECD method using Gas chromatograph (Shimadzu −2010 Plus, Singapore) equipped with ECD detector and DB-1701 (30 μm × 0.25 μm × 0.25 μm) column. For GC-ECD analysis the residual lindane and the degradation product formed were extracted twice in 1 ml of hexane (HPLC Grade, Qualikam Chemicals, India). Elution were as follows: Helium as carrier gas, detector temperature; 350 °C, oven temperature conditions; 90 °C for 2 min, increase to 250 °C at 5 °C/min, increase to 250 °C at 30 °C/min and held for 5 min. Preliminary test with known standards showed the method to be capable of detecting about 1 μg/ml of lindane in the injected sample (2 μl). All the potent isolates along with *S. japonicum* MTCC 6362 as reference and pure lindane as standard were subjected to the same protocol for GC analysis.

2.4. Biodegradation studies

The potent isolates were inoculated into mineral broth supplemented with lindane (100 mg/l) and kept on rotatory shaker at 120 rpm at 30 °C up to one month to determine their degradation potential quantitatively and qualitatively. Quantitative dechlorination rate was determined by estimation of chloride ions released into the medium by inoculating strains using indirect Argintometric method given by Greenberg et al. (1992). The individual samples were withdrawn at 0, 2, 4, 6, 8, 10, 13 and 15 days of incubation and were analysed for Cl^- estimation. The biodegradation potential of the selected strains was evaluated by analyzing the residual lindane in the medium which was calculated using the following formula:

$$\text{Residual lindane}(\%) = (C_t/C_0) \times 100$$

where, C_0 = initial concentration of lindane in the medium

C_t = lindane concentration at time t.

2.5. Kinetics study of gamma-HCH degradation analysis

In study the degradation kinetics all the degradation experiments in shake flask study were carried out in triplicates. The bacterial isolates HP-9, HP-10 and HP-13 were inoculated into sterile Mineral basal broth