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Isolation, Enrichment and Metagenomic Characterization of Simultaneous DDT and Lindane Degrading Microbial Consortium

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Organochlorine pesticides (OCPs) such as Lindane and DDT (dichlorodiphenyltrichloroethane) have been extensively used for agricultural purposes primarily for pest management and DDT is still the "sought after" for public health care programs to control vector-borne diseases like malaria in developing nations. OCPs, due to recalcitrant nature slowly degrade and pose adverse health effects to the environment and community. Residues of OCPs were detected in soil, water and air leading to potential bioaccumulation in food chains and were considered persistent organic pollutants. Microorganisms were found to be potential bio-degraders of organochlorine pesticides. In this study, the microbial population from aquatic systems, rivers from Yamuna (North India) and Godavari (South India) was isolated and enriched until a Lindane and DDT tolerant population was established. Screening of the population for understanding bioremediation thresholds was done using 5ppm of DDT and Lindane. The populated microbial cells formed the consortium that was subjected to metagenomic analysis to identify the organisms till species level. The 16S amplicon sequencing identified 871 species in the consortium and established the biodiversity of the consortium. The defined consortium was able to degrade DDT and Lindane up to 30 ppm simultaneously in varying order of pesticide concentrations.

Keywords: River Yamuna, River Godavari, Microbial Consortium, Biodegradation, Dichlorodiphenyltrichloroethane (DDT), Hexachlorocyclohexane (HCH), Lindane, Metagenomics, Riverine Metagenome.

Organochlorine Pesticides (OCPs) have been indiscriminately used for agricultural purposes primarily for pest management and OCPs such as DDT (dichlorodiphenyltrichloroethane) is used for public health programs to control vector-borne diseases like malaria. Even though OCPs were proscribed or banned in many countries; being persistent organic pollutants and recalcitrant in nature, they pose a plethora of environmental and health concerns[1, 2]. It is crucial and imperative to develop methods and strategies for removing them from the environment[3].

DDT and Lindane (γ-hexachlorocyclohexane) were the major OCPs that have been ubiquitously used in developing nations[4]. A major sink for persistent organic pollutants discharged into the environment is the water ecosystem i.e. rivers and lake beds[5]. Endrin aldehyde, Endosulfan sulfate and DDT were detected in highest percentage in River Yamuna which demonstrates the pollution of the river with pesticide residues[6]. DDT, Trans-chlordane and Endo- sulfansulfate were the dominant OCPs in soil sediments from River Godavari [7]. Microorganisms are found to be potential degraders of organochlorine compounds,

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notably soil habitants belonging to genera *Bacillus, Pseudomonas, Arthrobacter and Micrococcus* were found to be effective bio-degraders[8]. Several persistent organochlorine pesticides were detected in other rivers where higher concentrations of Endosulfan sulfate and DDT were detected and even found their presence in drinking and bottled water[9]. Hence, it becomes imperative to remove these pollutants from the environment, from the sinks primarily water ecosystems to eliminate their residues.

In the current study, a novel microbial consortium sampled from the River Yamuna (North India) and River Godavari (South India) was enriched until a Lindane and DDT tolerant population was established. This consortium was characterized using metagenomics, 16S amplicon sequencing in Illumina Next Generation Sequencing (NGS) platform[10, 11]. The biodiversity of riverine metagenome was established using QIIME (Quantitative Insight into Microbial Ecology) [12]. The defined microbial consortium was able to degrade DDT and Lindane simultaneously.

MATERIALS AND METHODS

Chemicals

Lindane (γ-HCH) was of 97% purity and obtained from Sigma- Aldrich, USA. DDT, 99.4% pure, was donated by Hindustan Insecticides Ltd, India. All other chemicals and reagents used in the study were of analytical grade and were purchased from standard manufacturers.

Isolation and Enrichment of Microbial Consortium

Water samples from the rivers Yamuna (North India) and Godavari (South India) were collected in clean bottles and brought to the lab in sealed condition. These water samples were mixed and incubated with 1% (w/v) peptone in a rotary shaker maintained at 150 rpm and run in ambient conditions. Once the microbial growth was sufficient to make the broth highly turbid, the culture was starved for a week followed by addition of 0.5% (w/v) peptone, 2 ppm Lindane and 2 ppm DDT. The growing culture was left shaking for a month followed by addition of 0.1% peptone, 5 ppm Lindane and 5 ppm DDT. After shaking for another month, the culture was continuously shaken only in presence of gradually increasing concentrations of Lindane and DDT for many months till a stable Lindane and DDT tolerant population was established in the flask[13]. These populated microbial cells formed the consortium that was used in this study.

Screening of DDT and Lindane Tolerant Microbial Consortium

The established consortium was inoculated to 5ppm Lindane and 5ppm DDT mixture in 25 mL sterile RO water taken in 250 mL Erlenmeyer flasks. The flasks were kept in a rotary shaker set at 150 rpm and maintained under ambient condition. Whole flask samples were drawn at 0 h, 24 h, 48 h and 72 h, acidified by adding 3-5 drops of fuming nitric acid and extracted twice with equal volume of dichloromethane (DCM). Both the organic layers were pooled after passing through anhydrous sodium sulfate and activated fluorisil. The organic solvent was evaporated under ambient conditions and the residual pesticides were re-suspended in HPLC grade acetone before transferring into a microfuge tube followed by complete evaporation of acetone at room temperature (RT) [14]. The residual pesticides were dissolved in a known volume of HPLC grade acetone for further analysis by thin layer chromatography (TLC) and GCMS/MS.

Estimation of Residual Pesticide Concentration Thin Layer Chromatography

TLC was performed on 0.25 mm thick silica gel G plate with cyclohexane mobile phase. The thin layers were air- dried before detecting the pesticide residual spots using *o*-tolidine (2% as acetone solution) spray in bright sunlight. The spots appeared as peacock green. The area under the spot was used for quantifying the residual Lindane and DDT using the relationship that the square root of the area is directly proportional to the log of concentration[15]. The results were further confirmed using GC-MS/MS.

Gas Chromatography-MS/MS

The residual pesticide was quantified by Gas Chromatography using instrument GCMS/MS Triple quad; Model 7000D (Agilent Technologies Ltd) [16]. The column HP-5ms, Agilent 19091S EPC was used for analysis of residual pesticides. These columns have low bleed characteristics, excellent inertness for active compounds, and improved signal-to-noise ratio for better sensitivity. The sample was dissolved in 1 ml MS grade ethylacetate and appropriate dilutions were used for analysis.

The injector was maintained at 70°C initial set point to post run temperature of 280°C, while the column was programmed with pressure 30.797 psi, flow of 3.1793 mL/min, Average Velocity of 54.506 cm/sec and initial temp of 70°C. The ion source was electron ionized (EI) with source temperature of 300°C for triple quadrupole acquisition method.

Metagenomic Analysis of the Consortium

Microbial consortium enriched using organochlorine pesticides was subjected to 16S Metagenomic Study for species-level identification using Illumina Platform. DNA was isolated using Xcelgen Bacterial gDNA kit and quality of gDNA was checked on 0.8 % agarose gel (loaded 5 µl) for the single intact band. The gel was run at 110 V for 30 min. 1 µl of each sample was loaded in Nanodrop 8000 for determining $A_{260/280}$ ratio. The DNA was quantified using Qubit dsDNA HS Assay kit (Life Tech). 1 µl of each sample was used for determining concentration using Qubit® 2.0 Fluorometer. Amplicon library was prepared using Nextera XT Index Kit (Illumina Inc) as per the 16S Metagenomic Sequencing Library and amplicon sequencing was performed using Hi Seq 2500 using Illumina platform.

Fig. 1. Enriched Microbial Consortium (pooled) in Flask and Agar Plate of Mixed populations after enriched by OCPs Mixture

RESULTS AND DISCUSSION

A turbid microbial growth that was continuously shaken only in presence of gradually increasing concentrations of Lindane and DDT for many months till a stable Lindane and DDT tolerant population was established in the flask (Figure-1). These populated microbial cells formed the consortium that was used in this study. As the objective of the study was to genotypically characterize the consortia till species level, no growth based techniques were used for screening individual organism, since such techniques will not able to identify any unculturable microorganisms[17, 18].

The established consortium was incubated with 5ppm Lindane and 5ppm DDT mixture in 25 mL sterile RO water and whole flask samples were drawn at 0 h, 24 h, 48 h and 72 h. The residual pesticides dissolved in a known volume of HPLC grade acetone for analysis by thin layer chromatography (TLC) and GCMS/MS. The consortium was able to degrade effectively DDT and Lindane in varying order of pesticide dissipation simultaneously (Figure-2). A 72 h sample showed around 69% of DDT and 75% of Lindane being effectively degraded through thin

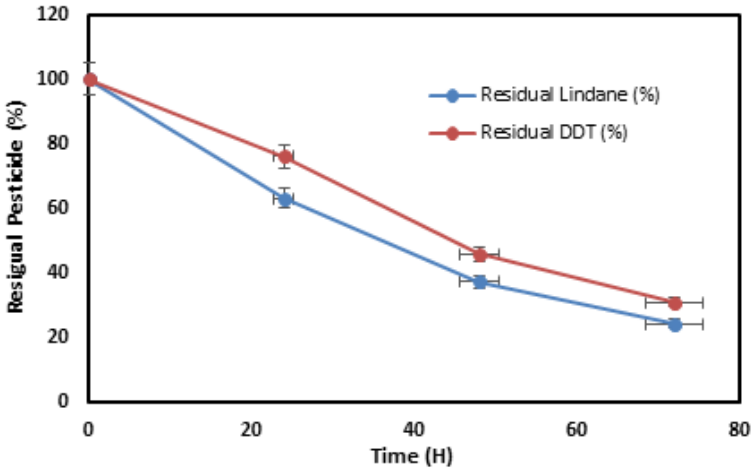

Fig. 2. Simultaneous Degradation of 5 ppm each of DDT and Lindane over a period of 72 h

layer chromatography (TLC) and these profiles were confirmed by GCMS/MS.

The defined microbial consortium was subjected to metagenomic analysis to identify the organisms till species level. The 16S amplicon sequencing identified 871 species in the consortium and established the biodiversity of the consortium[19].The taxonomic distribution of phylum was determined using QIIME (Quantitative Insight Into Microbial Ecology) after analyzing 16S metagenome data from Next Generation Sequencing (NGS) platform in python language (Figure-3).

NCBI Sequence Accession Number

Complete DNA sequences obtained have been deposited at National Center for Biotechnology Information (NCBI) Sequence Read Archive under the bioproject ID PRJNA420925.

The metagenomic analysis identified the 871 species in the Microbial Consortium. The major species in the defined microbial consortia with highest abundance ratio are found to be *Brevundimonas diminuta, Alcaligenes faecalis, Stenotrophomonas acidaminiphila, Bacillus cereus* and *Desulfosporosinus meridiei* (Table 1).

A vast research has been carried out using single organism and a single compound of OCPs in the realm of bioremediation[20, 21]. However, the present study identified a mixture of the microbial population from the river Yamuna and the river Godavari that were characterized till species level using novel metagenomics Illumina platform which could degrade DDT and Lindane up to 30 ppm concentration simultaneously (data not shown).

The biodiversity of microbial population and pesticide removal provides information about

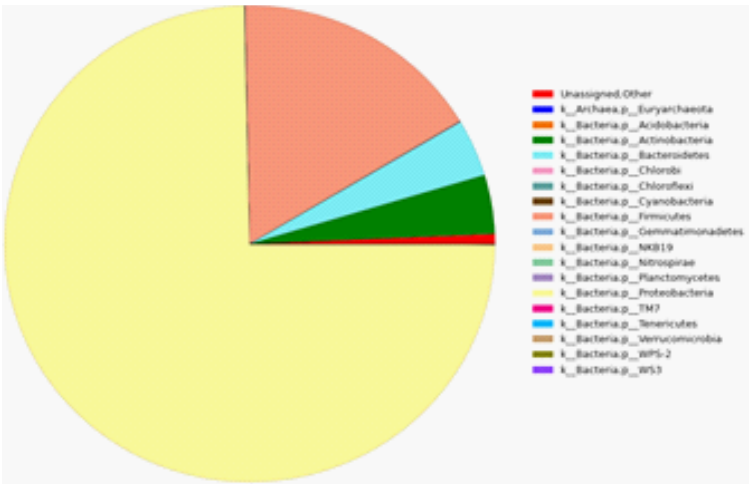

Fig. 3. Pie chart showing the relative abundance of each phylum within the microbial consortium

CCTACGGGCCGCAGCAGTGGGGAATTTTGGACAATGGGGGAAACCCTGATCCAGCCATCCCGCGTGTATGATGAA
GGCCTTCGGGTTGTAAAGTACTTTTGGCAGAGAAGAAAAGGTATCTCCTAATACGAGATACTGCTGACGGTATCT
GCAGAATAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGCAGGGTGCAAGCGTTAATCGGAATTAC
GAGGCGGAAAGCTTGTGTAGGCGGTCTGGAAAGAAAGATGTGAAATCCCAGGGCTCAACCTTGGAACTGCATTT
CTAACTGCCGGGCTAGAGGATGTCAGAGGGGGGTAGAATTCCACGTGTAGCAGTGAAATGCGTAGATATGTGGA
GGAATACCGATGGCGAAGGCAGCCCCCTGGGATAATACTGACGCTCAGACACGAAAGCGTGGGGAGCAAACAG

Fig. 4. Partial 16S Nucleotide Sequence of Riverine Metagenome using Hi Seq 2500

Table 1. Species with Higher Abundance Ratio in the Microbial Consortium

Taxonomy	Abundance Ratio
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Caulobacterales; f__Caulobacteraceae;g__Brevundimonas;s__diminuta	17.57%
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Burkholderiales;f__Alcaligenaceae; g__Alcaligenes;s__faecalis	1.35%
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Xanthomonadales; f__Xanthomonadaceae;g__Stenotrophomonas;s__acidaminiphila	1.09%
k__Bacteria;p__Firmicutes;c__Bacilli;o__Bacillales;f__Bacillaceae;g__Bacillus;s__cereus	0.26%
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales; f__Peptococcaceae;g__Desulfosporosinus;s__meridiei	0.22%

using this defined consortium as a viable strategy for remediation of a mixture of organochlorine pesticides. Further, the consortia were found capable of degrading mixture of organochlorine pesticides simultaneously, a crucial phenomenon which can be a promising solution for removal of DDT and Lindane pesticide mixture in aquatic ecosystems by eliminating pesticide residues thereby enhancing environmental conditions.

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