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Isolation, Identification and Characterization of p, p'-DDT Degrading Bacteria from Soil

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ABSTRACT

DDT (dichlorodiphenyltrichloroethane), one of the organochlorine pesticides was widely used in agriculture and healthcare. Though its usage is banned in most of the countries, DDT residues cause varying negative effects through bioaccumulation and bioamagnification. Biodegradation is an potential method to detoxify the recalcitrant compounds and this study is an investigation to isolate and characterize the p, p'-DDT degrading bacteria from DDT contaminated soil. An p, p'-DDT [1,1,1-trichloro-2,2-bis(4-chlorophenyl)ethane] degrading bacterium was isolated and identified by 16S rRNA studies. The role of temperature, pH and DDT concentration in the degrading ability of the isolate has also been investigated. Based on the analysis of the phenotype, biochemical characteristics and 16S rRNA, the strain was identified to belong to the bacterial genera *Bacillus* and was named as *Bacillus* strain GSS. The isolate had an optimum pH of 7.0 at 35°C and was able to degrade DDT at a wide range of concentrations with complete degradation of the DDT (10 mg L^{-1}) and 89.3% of 15 mg L^{-1} in 120 h, whereas 100% degradation of 5 mg L^{-1} concentration was observed within 48 h. Significant degradation was observed at 72 h and 96 h for 15 and 20 mg L^{-1}, respectively. At the end of 120 h, 73% of 20 mg L^{-1} and 34% of 25 mg L^{-1} was recorded by the isolate *Bacillus* strain GSS.

Key words: *Bacillus*, DDT, biodegradation, 16S rRNA

INTRODUCTION

Among the xenobiotic compounds, organochloride pesticides are recalcitrant in nature and highly resistant to biodegradation (Diaz, 2004; Dua *et al*., 2002). Organochloride pesticides are cumulative in the organisms and pose chronic health effects, such as cancer and neurological and teratogenic effects (Vaccari *et al*., 2006). This class of pesticides includes the chlorinated derivatives of diphenyl ethane (dichlorodiphenyltrichloroethane-DDT, its metabolites dichlorodiphenyldichloroethylene-DDE, dichlorodiphenyldichloroethane-DDD and methoxychlor), hexachlorobenzene (HCB), the group of hexachlorocyclohexane (α-HCH, α-HCH, γ-HCH, α-HCH, or lindane), the group of cyclodiene (aldrin, dieldrin, endrin, chlordane, nonachlor, heptachlor and heptachlor-epoxide) and chlorinated hydrocarbons (dodecachlorine, toxaphene and chlordecone) (Menone *et al*., 2001; Patnaik, 2003). Non-target organisms including human beings are affected when recalcitrant chlorinated pesticides undergo bioaccumulation and biomagnifications.

Dichlorodiphenyltrichloroethane [1,1,1-trichloro-2,2-bis-(p-chlorophenyl)ethane] or p, p'-DDT, is the most well known pesticide from the organochlorine group. Its high efficiency and mode of action along with low water solubility has lead to increasing usage by many countries. Though they have contributed considerably to agricultural outcomes, their accumulation is adversely affecting the environment health. DDT is highly persistent in the environment with a reported half life between 4-35 years and is immobile in soils (Corona-Cruz *et al*., 1999; Howard, 1991). Due to its recalcitrance, high concentrations of DDT and its metabolites have been found in soil, water and sediment samples though use of DDT has been banned from agricultural and public health use (Thomas *et al*., 2008; Barragan-Huerta *et al*., 2007). In some regions, organo chlorine pesticides are still found in soils and DDT accounts for approximately 93% of the total organo chlorine pesticide content (Wang *et al*., 2006). DDT residues have been found in many foodstuffs and accumulate in the fatty tissues of ingesting organisms due to their lipophillic nature which leads to serious health effects such as nausea, vomiting, dizziness, headache, loss of muscle control and tremors (Kannan *et al*., 1992; Smith, 1991).

Biodegradation of hazardous compounds and their detoxification is one promising strategy to maintain the environmental health. Microorganisms possess many enzymes to degrade organochlorine pesticides through reductive dechlorination, dehydrochlorination, oxidation and isomerization of the parent molecules (Nawab *et al*., 2003). Microorganisms could degrade DDT and its primary metabolites, DDD and DDE into 4-chlorobenzoic acid or 4,4-dichlorobenzophenone under aerobic and anaerobic conditions (Cutright and Erdem, 2012; Kamanavalli and Ninnekar, 2004; Nadeau *et al*., 1994). Microbial degradation of pesticides involving their biochemical and molecular mechanisms has been well documented earlier (Singh *et al*., 1999; Kumar *et al*., 1996). Use of microorganisms to remove pollutants from contaminated sites is an effective, minimally hazardous, versatile and environment-friendly strategy. The present study described the isolation and genetic characterization of the organochlorine pesticide (p, p'-DDT) degrading bacterial strain which was isolated from the DDT contaminated soil. Further, the role of various factors viz., temperature, pH and concentration of DDT in the degrading ability of the isolate has been investigated under aerobic conditions.

MATERIALS AND METHODS

Chemicals and enzymes: DDT [1,1,1-trichloro-2,2-bis-(p-chlorophenyl)ethane], lysozyme, proteinase K and all other chemicals were obtained from Sigma Aldrich (Bangalore).

Media for bacterial growth: Luria-Bertani medium (LB) was prepared from the following components: (per litre of distilled water) 10 g BactoTryptone; 5 g yeast extract; 10 g NaCl (HiMedia Laboratories, Mumbai) as an enriched medium to rapidly grow the cells of sample isolates. For growth on a solid surface, the media were supplemented with 15.0 g Bacto Agar.

Minimal Salt Medium (MSM) medium: The Minimal Salt Medium (MSM) medium had the following composition: (per litre of distilled water) 0.1 g $CaCl_2.2H_2O$; 0.08 g $Ca(NO_3)_2$ $4H_2O$; 0.5 g $MgCl_2.6H_2O$; 1.0 g Na_2SO_4; 1.0 g KH_2PO_4. Immediately prior to inoculation, MSM was supplemented with DDT (0.1 mg mL^{-1}). The final inoculation into MSM ensures that the only carbon source available to the bacterial isolates comes in the form of the pesticide p, p'-DDT and therefore sustained growth in this media is possible only if the isolate can metabolize p, p'-DDT.

Isolation of DDT-metabolizing microbial species from soil samples: Soil samples were collected from sites of a farmer's field located in Nelamangala taluk, in Karnataka province with history of continued farming activities for more than 30 years. Air-dried soil (1 g) was suspended in 50 mL of the LB medium. The suspension was kept for 2 days at 30°C on a shaker. Any insoluble materials were allowed to settle down and an aliquot (100 μL) from the cleared supernatant was used to inoculate 3 mL of p, p'-DDT-supplemented MSM. The culture was incubated for 1 week at 30°C on a rotary shaker at 200 rpm. After incubation, 100 μL of the bacterial suspension was transferred into 3 mL of fresh MSM containing p, p'-DDT and the incubation step was repeated. After five consecutive sub-cultivations, the isolates were inoculated on to MSM agar plates containing p, p'-DDT (100 μg mL^{-1}) for overnight incubation at 37°C and the isolates formed were frozen at -80°C.

Biochemical characterization: The isolates were subjected to morphological and biochemical studies which included gram staining, motility test, endospore and capsule staining. Standard biochemical tests included indole, methyl red, Voges Proskauer, citrate test, nitrate reductase, urease and 1% sugar solutions of sucrose, glucose, lactose, xylose, maltose and mannitol with methyl red indicator were carried out for identification of the isolate.

Isolation of genomic DNA: The isolates were used to inoculate 3 mL LB medium and incubated overnight at 37°C and 200 rpm for 24 h. The resulting bacterial suspension (OD_{600} = 0.6) was pelleted at 10,000 rpm for 5 min and the genomic DNA was extracted using the method outlined by Schmidt *et al*. (1991). In brief, cell pellets were thawed on ice in 4.5 mL of 40 mM EDTA, 0.75 M sucrose, 50 mM Tris-HCl (pH 8.3). Lysozyme was added to a final concentration of 1 mg mL^{-1} and the suspension was incubated at 37°C for 30 min. After the addition of 800 μg of proteinase K and Sodium Dodecyl Sulfate (SDS) to a final concentration of 0.5% (w/v), the mixture was incubated for an additional 2 h at 37°C. The protein and polysaccharide complexes were removed by extraction with an equal volume of chloroform-isoamyl alcohol (24:1), followed by extraction with phenol-chloroform-isoamyl alcohol (50:49:1). Nucleic acids were recovered by the addition of 0.6 volume of isopropanol and centrifugation for 10 min at 10,000 rpm and the pellet was suspended to a concentration of between 50 and 100 μg mL^{-1} in TE buffer. Genomic DNA was visualized by ethidium bromide fluorescence and 16S rRNA sequencing was done.

16S rRNA sequencing: In order to amplify ~1.4 Kb gene from the isolated genomic DNA, 16S rRNA gene primers [FP: 5'-AGA GTT TGA TCC TGG CTC AG-3' and RP: 5'-ACG GCT ACC TTG TTA CGA CTT-3'] were used (Eppendorf). PCR conditions were set as follows: Initial denaturation at 94°C for 4.5 min, 32 cycles consisting of denaturation at 94°C for 0.5 min, annealing at 52°C for 0.5 min and extension at 72°C for 1 min and final elongation at 72°C for 4 min. PCR product obtained was gel purified and quantitated and sent for sequencing with 16S rDNA forward primer. DNA alignments were made using ClustalX 2.0.12 and the sequences were compared to sequences in the public databases with the BLAST search program on the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/) to identify the isolate.

Degradation of DDT by the isolated strain: The degradation ability of the isolated was determined *in vitro* by pre-culturing in LB medium for 18 h, centrifuging and washing the cell pellets with fresh MSM medium. After adjusting the OD_{600nm} of cell density to 0.6, the cells were

inoculated into MSM media containing varying pH (4.0, 5.0, 6.0, 7.0, 8.0, 9.0 and 10.0) with increasing DDT concentrations (5, 10, 15, 20 and 25 mg L^{-1}) and incubated at different temperatures (25, 30, 35, 40 and 45°C) under shaking conditions (150 rpm) in triplicates along with control. At constant intervals, aliquots of the sample were removed for the determination of cell growth (UV-VIS) and DDT concentration (GC-FID). Data were interpreted by analysis of variance ($p<0.05$), multiple range analysis and correlation analysis. Each experiment was performed in duplicate and the results were considered significant when $p<0.05$.

RESULTS AND DISCUSSION

In this study, a bacterial strain that was able to grow in p, p'-DDT supplemented media was isolated and named it as GSS. To identify the species collected in the study, morphological and biochemical tests were performed (Table 1). Further, 16S rRNA gene of the isolate was probed by PCR using universal bacterial primers as described by Baker *et al.* (2003) and Frank *et al.* (2008). The 16S rRNA gene sequence of GSS showed the greatest similarity to the members of the genus *Bacillus* (Fig. 1).

The role of temperature, pH and concentration of p, p'-DDT were tested during the degradation process. The *Bacillus* strain GSS could efficiently degrade DDT at temperatures in the range of 25-40°C with an optimum temperature of 35°C. To study the effect of pH on the degradation of DDT, the isolated strain was inoculated into MSM medium at a pH ranging from 4.0-10.0. Degradation was observed at pH values between 5.0 and 8.0 with an optimum pH of 7.0. The strain was able to degrade DDT at a wide range of concentrations (Fig. 2) with complete degradation of the DDT (10 mg L^{-1}) and 93% of 15 mg L^{-1} in 120 h, whereas 100% degradation at the concentration of 5 mg L^{-1} was observed within 48 h. The degradation was significant at 72 h (15 mg L^{-1}) and 96 h (20 mg L^{-1}) of incubation time. However, only 31.2% degradation of the highest DDT concentration (25 mg L^{-1}) was observed in the end of 120 h incubation period.

The isolate has degraded 38% of the total DDT, at a concentration of 10 mg L^{-1} in first 48 h and 84% at the end of 96 h. Degradation of 15 mg L^{-1} concentration was significant from 72 h of incubation and 89.3% was degraded after 120 h whereas, 73% of 20 mg L^{-1} and was 34% of

Table 1: Biochemical characteristics of the bacteria isolated from DDT contaminated soil

Biochemical test	Result
Gram's staining	+
Indole	-
Methyl red	+
Voges proskauer	-
Citrate utilization	+
Urease	+
Nitrate reductase	+
Oxidase	-
Catalase	+
Glucose fermentation	+
Lactose fermentation	+
Xylose fermentation	+
Mannitol fermentation	+
Sucrose fermentation	+
Maltose fermentation	-

+: Positive, -: Negative

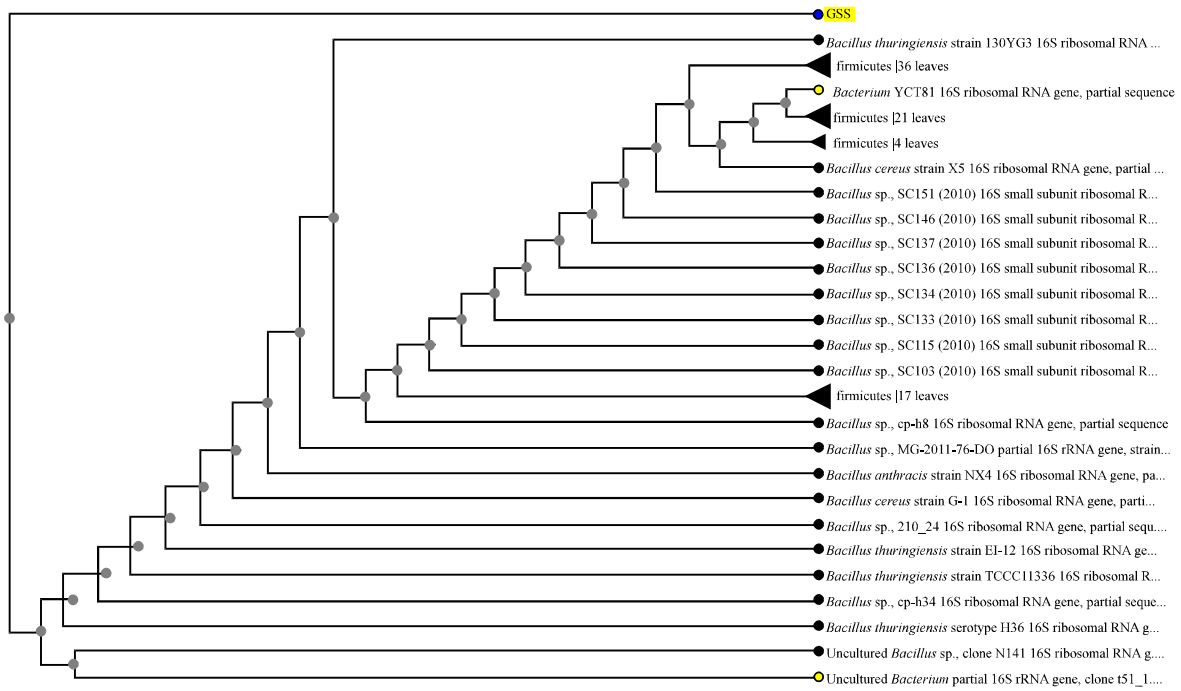

Fig. 1: Phylogenetic tree showing the relationship of 16S rRNA gene sequences within GSS and closely related *Bacillus* sp. The bar indicates a genetic distance of 0.5

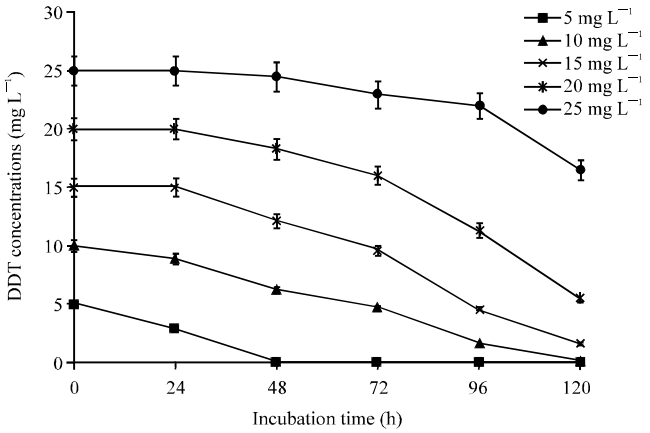

Fig. 2: Effect of incubation period on biodegradation of DDT by *Bacillus* strain GSS

25 mg L^{-1} was degraded at the end of 120 h. There was a delay in the degradation process and cell density at the initial incubation period in medium containing higher DDT concentrations and one possible explanation could be that the isolate needed an adaptation period to produce necessary degrading enzymes.

Microbial degradation of DDT is an effective method to reduce toxicity of the parent compound thereby maintains environmental health and more than 300 bacterial strains have been identified to degrade DDT (Zhang and Bennett, 2005). Gao *et al.* (2011) has isolated an *Alcaligenes* sp. which was effectively degrading DDT. Fang *et al.* (2010) has isolated DDT degrading soil isolate and identified as *Sphingobacterium* sp. Mwangi *et al.* (2010) has isolated *Bacillus, Staphylococcus* and

Stenotrophomonas sp. from soil which degrades DDT. Wang *et al.* (2010) have isolated *Pseudoxanthomonas* sp. from long term DDT contaminated soil sample. Species of the genus *Bacillus* (Kuhad *et al.*, 2004) and *Staphylococcus* (Singh, 2008) have been reported to degrade DDT. Other bacterial genera that have been implicated in DDT degradation are *Aerobacter*, *Alcaligenes*, *Agrobacterium*, *Clostridium*, *Hydrogenomonas*, *Klebsiella*, *Streptomyces*, *Xanthomonas* and *Stenotrophomonas* (Singh and Ward, 2004; Ahuja and Kumar, 2004; Nadeau *et al.*, 1998; Juhasz and Naidu, 2000). In this study, most of the DDT was degraded by the *Bacillus* strain GSS with an optimum pH and temperature of 7.0 and 35°C, respectively at the end of incubation period.

CONCLUSION

Due to the recalcitrant and slow degrading ability of p, p'-DDT *in situ* conditions, microbial degradation and or mineralization of p, p'-DDT is gaining popularity. The main focus of the study was to isolate and characterize the potential p, p'-DDT degrading bacteria and to study its degrading ability at different temperature, pH and DDT concentrations. Growth experiments conducted had shown that *Bacillus* strain GSS was able to grow in the presence of high concentrations of DDT and utilized it as sole carbon source. Utilization of p, p'-DDT by the soil microorganisms as carbon source and its degradation is a crucial phenomenon to remove pollutants from contaminated sites and the isolate *Bacillus* strain GSS is one potential candidate to maintain environmental health.

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