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Bioremediation of DDT in soil by genetically improved strains of soil fungus *Fusarium solani*

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Abstract

Bioremediation of DDT in soil by genetically improved recombinants of the soil fungus *Fusarium solani* was studied. The parent strains were isolated from soil enriched with DDD or DDE (immediate anaerobic and aerobic degradation products of DDT), as further degradation of these products are slow processes compared to the parent compound. These naturally occurring strains isolated from soil, however, are poor degraders of DDT and differed in their capability to degrade its metabolites such as DDD, DDE, DDOH and DBP and other organochlorine pesticides viz. kelthane and lindane. Synergistic effect was shown by some of these strains, when grown together in the medium containing DDD and kelthane under mixed culture condition. No synergism in DDE degradation was observed with the strains isolated from enriched soil. DDD-induced proteins extracted from individual culture filtrate (exo-enzyme) when subjected to SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) showed complementary polypeptide bands in these strains i.e., each strain produced distinct DDD degrading polypeptide bands and the recombinant or hybrid strains produced all of the bands of the two parents and degraded DDD better than the parental strains. Recombinant hybrid strains with improved dehalogenase activity were raised by parasexual hybridisation of two such complementary isolates viz. isolate 1(P-1) and 4(P-2) showing highest complementation and are compatible for hyphal fusion inducing heterokaryosis. These strains are genetically characterised as $Kel^{+}Ben^{R}DBP^{-}Lin^{-}$ and $Kel^{-}Ben^{r}DBP^{+}Lin^{+}$ respectively. Recombinants with mixed genotype, i.e., $Kel^{+}Ben^{R}DBP^{+}Lin^{+}$ showing superior degradation quality for DDT were selected for bioremediation study. Recombination was confirmed by polypeptide band analysis of DDD induced exo-proteins from culture filtrate using SDS-Polyacrylamide Gel Electrophoresis (PAGE) and RAPD (Random Amplified Polymorphic DNA) of genomic DNA using PCR (Polymerase Chain Reaction) technique. SDS-PAGE showed combination of DDD induced polypeptide bands characteristic of both the parents in the recombinants or the hybrids. PCR study showed the parent specific bands in the recombinant strains confirming gene transformation.

Introduction

Organochlorine insecticide DDT is highly persistent in the environment and was banned in most of the advanced countries way back in the seventies. Its presence, however, is still felt in soil, air, water, food, etc. in many remote areas. Persistence of DDT in tropical soils is relatively less compared to the temperate regions. This is due to higher volatilisation rate and faster degradation to DDD or DDE under anaerobic and aerobic condition of soil respectively (Castro & Yoshida 1971). However, subsequent degradation is a slow process resulting in accumulation of these products in soil (Mitra & Raghu 1978). National Standards and Guidelines for Pesticides in Water, USA has given these a priority pollutant status and classified as probable human carcinogen based on animal studies (Nowell & Resek 1994). In India, DDT was used intensively and indiscriminately in agriculture and public health purposes till recently, resulting in

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widespread environmental contamination. Factory affluents further contaminate the surrounding soil and water constantly. Adverse effects of DDT residues was earlier reported on plant growth (Mitra & Raghu 1989, 1998c), yield of certain economically important crop plants (Mitra & Raghu 1998b) and on soil fertility (Mitra & Raghu 1998a).

Accumulation of these products in soil, therefore, is undesirable from the environmental point of view and needs quick removal from the terrestrial and aquatic ecosystem. Microbes are natural biological detoxifying agents in soil. Microbial detoxification of hazardous chemicals has opened up a promising new area in the environmental bioremediation research. Naturally occurring organisms present in soil are poor degraders of DDT and several theories were put forward earlier regarding their poor performance in soil (Watanabe 1973; Subba Rao et al. 1982; Bumpus et al. 1982; Goldstein et al. 1985). Enhancement of biological activities by genetically manipulated organisms has attracted the attention for soil bioremediation considerably (Head & Cain 1991). Strain improvement by genetic recombination through inter-specific and intra-specific DNA hybridisation via parasexual cycle in strains compatible for hyphal fusion are routinely practised in asexual industrial (Ball 1984) and pathogenic fungi (Hastie 1981). The importance of this technique for breeding high potential strains with superior enzyme systems in asexual fungi is commendable due to simplicity of this technique and time saving quality compared to other conventional practices like protoplast fusion or genetic transformation. The present study describes development of recombinant strains with improved DDT degrading enzyme systems from naturally occurring low potential DDT degrading strains of soil fungi by parasexual hybridisation and their efficacy in bioremediation processes.

Materials and methods

Substrates

DDT (1,1-bis(*p*-chlorophenyl)-2,2,2-trichloroethane), *DDD* (1,1-bis(*p*-chlorophenyl)-2,2-dichloroethane); *DDE* (1,1-bis(*p*-chlorophenyl)-2,2-dichloroethylene); *Kelthane* (1,1′bis(*p*-chlorophenyl)-2,2,2-trichloroethanol); *DBP* (4,4′-dichlorobenzophenone), *DDOH* (1,1′-bis(*p*-chlorophenyl)-2-hydroxyethane), *Lindane* (gamma isomer of hexachlorocyclohexane) were procured from Aldrich Chemical, USA and *Benomyl* (Benlate 50 DF) was acquired from Du Pont Co., Wilmington, Del.

Culture media

Potato Dextrose Agar (PDA) – for routine maintenance and enumeration of conidia in conidial suspension used as inoculum.
Basal medium (BA) – Carbon free mineral salt agar medium (Leadbetter & Foster 1958)
Limiting broth/Agar medium (LB/LA) – BA + DDT (5 mg ml^{-1})
Screening Medium (SM) – 1. BA + DDD (5 mg ml^{-1}); 2. BA + DDE (5 mg ml^{-1}); 3. BA + Kelthane (5 mg ml^{-1}); 4. BA + DDOH (5 mg ml^{-1}); 5. BA + DBP (5 mg ml^{-1}), 6. BA + Lindane (5 mg ml^{-1}).

Strain isolation and characterisation

DDT degrading organisms were isolated from DDD and DDE amended soil by soil enrichment culture technique since our earlier studies showed much slower degradation rate of these products as compared to the parent compound DDT (Mitra 1978). Soil (20 g) treated with these chemicals (50 ppm) in glass tubes were incubated for several months with 40–60% moisture level at room temperature (25 ± 2 °C) under laboratory condition. Moisture level was maintained by adding water at intervals. Soil samples after incubation were extracted with 50-ml water by shaking them at 150 rpm for 30 minutes on a gyratory shaker. Aliquots of clear supernatant from soil extract were plated out with LA. Colonies formed were transferred to PDA slants for identification. Growth and sporulation behaviours of these isolates were studied using different media viz. Czapek Dox Agar, Sporulation medium of Blom et al. (1952), yeast extract malt extract agar and nutrient agar for classification. Fungal isolates showing better DDT degradability among the strains isolated were selected for further studies as fungi play an important role for DDD degradation in soil. These fungal strains were identified as *Fusarium solani*.

Biochemical characterisation of these strains was done by transferring each of them to plates containing LA with benomyl (20 ppm) in a checker board manner (Mitra & Chaudhuri 1966). Very high level of benomyl was added to the medium to be sure about their tolerance limit. Isolates capable of growing in this medium are benomyl tolerant and characterised as Ben^R. Substrate utilisation of these isolates were studied by screening them in the similar fashion against

a series of screening media viz. SM-1, SM-2, SM-3, SM-4 or SM-5 and SM-6 containing DDD, DDE, Kelthane, DDOH, DBP, and Lindane respectively. To check any growth due to carbon contamination in the medium from chemicals used for media preparation cultures were also grown in BA.

Inoculum

Cultures were grown in PD broth on a gyratory shaker (150-rpm) at room temperature 25 ± 2 °C for 7–10 days and allowed producing conidia. Spores were harvested by filtration and washed with sterile water by centrifugation. Sterile water with Tween-20 (1 : 10,000) was added to the pellet to bring the conidial concentration to approximately 10^{12} ml^{-1} in the suspension.

Selection of complementary strains for heterokaryosis

Complementary strains were selected on the basis of substrate utilisation of the isolates. For screening complementary pairs, conidial suspension of different fungal isolates with diverse degradative characteristics were streaked on LA in the petri plate serially and allowed to dry. Plates were then cross-streaked with the same set of spore suspensions at right angle to the previous ones and allowed growing. Better growths at the junction of two streaks indicated complementary effect of that particular pair for DDT degradation. For confirmation the complementary pairs were grown individually and in combination (keeping the total spore concentration in all samples approximately the same i.e., 10^{12} per flask) in SM-1, SM-2 and SM-3 to study the effect on DDD, DDE and kelthane (immediate degradation products) degradation, since these products are degraded slowly in the soil. Levels of DBP (end product) and other products and the rate of substrate degradation in the culture filtrates were determined from the extracts of acidified culture filtrate with acetone: hexane mixture (1 : 4). Quantitative analysis was done by Reflection Densitometry at 235 nm of TLC (Thin Layer Chromatography) plates using *n*-heptane/acetone/ethanol (98 : 2 : 1) mixture as running solvent. Degradation rates of substrates by the mixed cultures were compared with the individual cultures. Complementary pair showing highest degradation under mixed culture condition was selected for parasexual hybridisation and recombination studies.

Selection of recombinants through parasexual hybridisation

Heterogeneous spores were produced from complementary strains of *F. solani*, by growing them together on PDA to form heterokaryons. Complementary strains, with heterogenic genetic markers, used for parasexual hybridisation are as follows:

P-1 (Isolate 1/Parent 1): capable of utilising DDD (D^+), Kelthane (Kel^+) or DDOH (OH^{++}) as the sole carbon source and resistant to benomyl (Ben^R).
P-2 (Isolate 4/Parent 2): capable of utilising DDD less effectively ($D^{\pm}$) compared to DBP (DBP^+) or Lindane (Lin^+) and sensitive to benomyl (Ben^r).

These isolates were grown independently and in combination on PDA and allowed to form heterokaryons (in mixed culture) and conidiospores. Conidia were harvested and washed with Tween-20 water and plated out with screening medium SM-5 containing DBP and benomyl (20 ppm) to score revertants or hybrid spores, and also with PDA to enumerate the total number of spores present in the conidial suspension. Revertants or recombinant spores with dbp^+ben^R genotype, capable of utilising DBP and benomyl, only could survive in this medium and form colonies while parental type (P-1 and P-2) spores with genotype DBP^-Ben^R and DBP^+Ben^r, were unable to utilise DBP or benomyl and failed to grow in this medium due to the presence of DBP as sole carbon source and benomyl added to this medium. Possibility of reverse mutation in P-1 and P-2 at DBP^- and Ben^r sites to produce dbp^+ben^R mutant spores or revertants, capable of utilising this medium and form colony can not be ruled out. Reverse mutation frequencies in the parent cultures were, therefore, determined by counting the number of dbp^+ben^R colonies from P-1 and P-2 on SM-5 and the total number of colonies in PDA at a given dilution. This was done to compare the frequencies of such spore types in individual parent cultures and in the mixed culture. Higher frequency in the mixed culture will indicate the possibility of heterokaryon formation under mixed culture condition. Such colonies with heterologous nuclei carrying dbp^+ben^R genes, appeared on SM-5 medium in individual (P-1 or P-2) and mixed culture (P-1 + P-2), were scored and transferred to PDA for further characterization. Nuclei with dbp^+ben^R gene in individual parent strains (P-1 or P-2) can only develop by mutation while in mixed

culture (P-1 + P-2), other than mutation these may also develop by (1) heterokaryotic binuclear spores formed after hyphal fusion or (2) heterokaryotic diploid spores or (3) recombinant spores produced by parasexual hybridisation of heterokaryotic diploids. Mutants involving single locus i.e., *DBP* or *Ben*, from mixed cultures were easily identified by screening the $dbp^{+}ben^{R}$ genotypes against a second set of markers viz. kelthane degrading gene (Kel^{+}) of the parent P-1 and lindane degrading gene (Lin^{+}) of P-2. Colonies ($dbp^{+}ben^{R}$), isolated earlier from mixed culture after first screening were, therefore, transferred further to two other screening media, i.e., SM-3 containing kelthane and SM-6 containing lindane as a substrate. Cultures failed to grow in either of these media were mutants and were discarded. Those capable of utilising these substrates could be heterokaryons, double mutants, diploids or recombinants (hybrids). Studying the segregation pattern of the remaining isolates, heterokaryons were identified as these segregated and produced parental type spores which were unable to survive in SM-5 containing DBP and benomyl. As a result, spore suspension from such colonies when plated out with SM-5 and PDA, lesser number of colonies were formed in SM-5 compared to PDA. Such colonies were eliminated from the rest which were subjected to the test for double mutants or diploids or recombinants. Similarly double mutants and the mutation frequencies in parental strains (P-1 and P-2) were also determined by studying the survival of $dbp^{+}ben^{R}$ isolates from two parents in screening media SM-3, SM-6 and comparing their survival rate with that of PDA. Isolates surviving in both the media were double mutants and number of such colonies in screening medium against the total number of colonies appeared on PDA in that dilution showed double mutation frequency in the parent strains. Higher frequency in mixed culture would indicate presence of diploids or recombinants. Double mutants and diploids were distinguished from heterologous haploids by subculturing them repeatedly for several generations(>50) and looking for the segregation of parental type of spores which may form during subculturing by reverse mutation in the mutants and occasional haploidization of diploid nuclei. Genetically stable strains are recombinants.

Enzyme complementation and DNA homology

Polypeptide band of heat denatured protein (Laemmli 1970) on SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and dehydrochlorinases enzyme bands on native gel using monochloro acetic acid as the substrate (Thomas et al. 1992) were carried out in complementary strains to find out enzyme complementation in them. Extracellular proteins from the culture filtrate of the parent strains and the recombinants growing in basal medium (broth) containing DDD as the major carbon source were precipitated by acetone. Extra-cellular protein and DNA profile was studied to identify the homology between the improved DDT degrading heterogenic recombinants (hybrid strains) raised by parasexual hybridisation and the parent strains.

Molecular homology of genomic DNA was studied by RAPD (Random Amplified Polymorphic DNA) using PCR (Polymerase Chain Reaction) technique. For PCR, primers were obtained from Operon Technology, USA, the nucleotides from Boehringer-Mannheim and the enzyme Taq polymerase from Bangalore Genei, India. Reaction mixture for PCR (50 μl) contained 10 mM tris-HCl; pH 8.3; 50 mM KCl; 1.5 mM $MgCl_2$; 200 μM each dATP, dTTP, dCTP and dGTP; 0.2 μM primer; 5 ng DNA and 2 units of Taq polymerase. Amplification reaction was done in Hybaid OmniGene, PCR, UK. Initial denaturation was carried out at 94° for 4 min. 45 cycles of following program were used for amplification: 94 °C for 1 min, 72 °C for 2 min, and 36 °C for 1 min. The product of amplification were analysed by electrophoresis in Tris acetate-EDTA buffer using 1.5% agarose gel followed by staining in ethidium bromide and viewing in UV-transilluminator.

DDT degradation in culture medium and soil

Studies were carried out under laboratory condition in culture tubes using 5 ml LB with DDT at 5 ppm level. Tubes were inoculated with 10^{12} spores of parents and hybrids and were allowed to grow for 10 days on a shaker at room temperature. Acidified culture media were extracted with equal volume of acetone/hexane mixture (1 : 4) following Katayama & Matsumura (1993). For soil study, 10 g autoclaved or unautoclaved (control) soil was contaminated with DDT at 5 ppm level, were flooded in glass tubes in triplicate and allowed to reduce sufficiently to carry out DDT degradation. Tubes were then inoculated with 10^{12} spores of P-1, P-2 and selected recombinants (hybrids) independently under outdoor laboratory conditions. Tubes were subjected to few cycles of unflooding (40–60% moisture) and flooding – a

prerequisite for the cometabolic degradation of DDT (Guenzi & Beard 1968) for 6 months. At intervals, soil samples were extracted with acetone partitioned with Hexane and residues were measured quantitatively by GLC (Gas Liquid Chromatography) following Mitra & Raghu (1978).

Results and discussion

Preliminary screening of DDD and DDE enriched soils yielded several colonies of bacteria, actinomycetes and fungi (Mitra 1999). Fungi are of special interest for this study as they play an important role in bioremediation of soil due to the filamentous nature of mycelium, which can reach the remote crevices in the soil in search of food where other organisms can not survive due of absence of water. Apart from this, fungi are primarily responsible for the degradation of DDD to non-toxic water-soluble products (Malone 1970). Naturally occurring soil fungi have limited capacity for DDT degradation. Improvement of DDT degrading enzymes in native fungi was induced by parasexual hybridisation mediated by heterokaryosis between two strains having complementary enzyme systems and compatibility for hyphal fusion. Heterokaryosis is an important and prevalent phenomenon in the life cycle of these asexual fungi belonging to the group Fungi Imperfecti. It serves as the first step for transmitting genetic materials from one strain to the other during heterozygous nuclear fusion, followed by occasional haploidization that results in the production of hyphae or cultures with haploid recombinant nuclei, carrying genetic material of original components of the heterokaryon. Asexual fungi, thus, achieve the benefits of diploidy and recombinants are produced achieving complementation, heterosis or synergistic effect. In the present study, selections of such strains are made on the basis of substrate utilisation and compatibility for hyphal fusion. Substrate utilising capability of some of the compatible and complementary strains is shown in Table 1. It is evident from the table that isolate-1 is deficient or limiting of enzymes degrading DDT, DDE, DBP and lindane. Isolate-4 is deficient of kelthane and DDOH and relatively poor degrader of DDT, DDD and DDE; isolate-5 of DDOH and DBP and isolate-12 of DDT, DDE and DBP, and relatively poor degrader of DDD and kelthane. Thus, it shows that these strains differ from each other in their spectrum of substrate utilising capability. End product of DDT and Kelthane is DBP, which is followed by ring

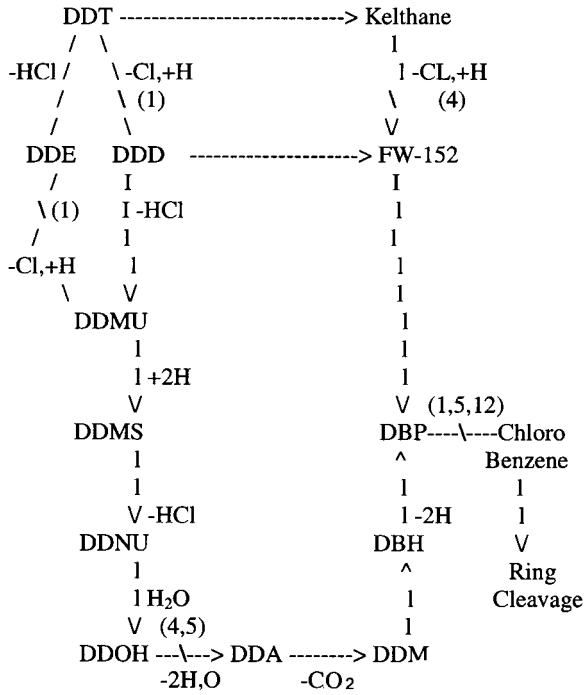

Figure 1. Schematic diagram showing DDT and Kelthane degradation pathways and probable recombination sites in isolates Nos. 1, 4, 5 and 12.

separation and ring cleavage producing chlorobenzene and water-soluble products respectively (Bumpus & Aust 1987). Figure 1 shows probable sites of enzyme blockage in DDT and kelthane degrading pathways in different strains. It shows only isolate-4 had capacity to degrade DBP while it was unable to degrade DDOH and kelthane which other strains could do. These characteristics probably have made the basis for enzyme complementation between isolate-4 and other three strains viz. 1, 5 and 12 giving synergistic effect in DDT degradation when grown together under mixed culture condition (Table 2).

Degradation of DDD, DDE and Kelthane by these strains was studied by growing these complementary strains independently and in combination (mixed culture) in basal medium (broth) containing any one of the above substrates as the sole carbon source. Solvent extracts of the culture filtrates were subjected to TLC. Percent degradation and product formation of these substrates as evaluated by Reflection Densitometry Technique of TLC plates are presented in Table 2. Synergistic effect was observed only with DDD and Kelthane. As expected from substrate utilization capability of these strains, synergistic effect (heterosis) in DDD and Kelthane degradation was shown by isol-

Table 1. Substrate utilisation of different fungal isolates from soil, compatible for hyphal fusion or heterokaryosis.

Isolate No.	Substrates added to LA						
	DDT	DDD	DDE	Kelthane	DDOH	DBP	Lindane
1	*	+	*	+	++	*	*
4	(+)	(+)	(+)	*	*	++	+
5	+	+	+	+	*	*	+
12	*	(+)	*	(+)	++	*	++

*Growth same as in LA without carbon source (C).
+, ++ Degree of growth stimulation compared to C.
(+) Slow growth.

Table 2. DDD and kelthane degradation by different complementary strains of *Fusarium solani* after 12 weeks (% distribution on TLC plates by reflection densitometry at 235 nm).

Isolate No.	Substrate degraded		Products formed		
	DDD	Kethane	DDE	DBP	Polar metabolites
1	42.0	–	9.6	21.0	11.4
4	69.0	–	1.6	–	10.2
12	33.0	–	5.8	41.7	6.1
1 + 4	19.9	–	2.2	67.7	5.3
12 + 4	22.0	–	3.9	65.0	6.7
4	–	50.0	1.6	22.6	12.6
4	–	23.3	6.4	64.2	6.4
4 + 5	–	14.5	1.2	47.4	27.0

Table 3. Screening schedule for isolation of mutants and recombinant from conidial populations of parents grown independently and under mixed culture condition.

Spore type	Genotypes*	Growth in S. media + benomyl		
		1st screening	2nd screening	
		SM-5** (+DBP)	SM-3** (+Kel)	SM-6** (+Lin)
P-1 (Parent-1)	$DBP^{-}Ben^{R}Kel^{+}Lin^{-}$	–		
P-2 (Parent-2)	$DBP^{+}Ben^{r}Kel^{-}Lin^{-}$	–		
Single mutant of P-1	$\boldsymbol{dbp}^{+}Kel^{+}Ben^{R}Lin^{-}$	+	+	–
Single mutant of P-2	$DBP^{+}Kel^{-}\boldsymbol{ben}^{R}Lin^{+}$	+	–	+
Heterokaryons from mixed culture of P-1 and P-2	$DBP^{-}Kel^{+}Ben^{R}Lin^{-}$ + $DBP^{-}Kel^{-}Ben^{r}Lin^{+}$	+	+	+
Double mutant of P-1	$\boldsymbol{dbp}^{+}Kel^{+}Ben^{R}\boldsymbol{lin}^{+}$	+	+	+
Double mutant of P-2	$DBP^{+}\boldsymbol{kel}^{+}\boldsymbol{ben}^{R}Lin^{+}$	+	+	+
Recombinant/hybrid from mixed culture	$DBP^{+}Kel^{+}Ben^{R}Lin^{+}$	+	+	+

*Parental wild type genes – expressed with higher case & italics; mutated alleles – expressed with lower case & italics.
** Screening media.

Table 4. Genotypes and frequencies of isolation of heterogenic spores from parental and mixed culture conidial population.

Isolate	Parental genotypes	Frequency of hybrid genotypes	
		First screening* dbp^+/ben^R	Second screening** dbp^+lin^+/kel^+ben^R
1 (P-1)	$Kel^+Ben^RDBP^-Lin^-$	1.5×10^{-9}	9.0×10^{-11}
4 (P-2)	$Kel^-Ben^rDBP^+Lin^+$	6.9×10^{-9}	7.0×10^{-11}
1 + 4(P-1 + P-2)	$kel^+ben^Rdbp^+lin^+$		
Total reverts	or	1.9×10^{-6}	1.9×10^{-7}
Recombinants	$Kel^+Ben^RDBP^+Lin^+$		7.0×10^{-8}

*With SM-5 medium.
** With SM-3 and SM-6 media.

ate 4 when grown with three other isolates viz. 1, 12 and 5. No synergism was observed for DDE degradation with any of these strains when grown in combination with each other. Complementary pair 1 and 4, showing highest degradation of DDD was selected for parasexual hybridisation to produce improved DDT degrading hybrid recombinants, as it is the major degradation product of DDT under anaerobic condition and highly persistent in the soils. As discussed earlier, faster degradation under mixed culture condition could be due to complementary activity of these strains to remove enzyme blockage at DDT, DBP and DDOH degradation sites in the respective isolates, i.e., isolate1 and isolate 4 enabling them to utilize the substrates more efficiently. Confirmation of enzyme complementation is shown by Polyacrylamide Gel Electrophoresis of heat denatured polypeptide bands on SDS-PAGE and DDD degrading enzymes on native gel electrophoresis (Figure 2). A scan of polypeptide band profiles on SDS-Gel by a gel scanner showed abundance of a low molecular weight (∼14 kd) DDD induced protein (polypeptide) band in isolate-4 which is absent or present in a very low quantity in other three strains viz. isolate 1, 5 and 12 (Figure 2a). This could be responsible for faster degradation of DDD when isolate-4 was grown with any one of the latter two strains under mixed culture condition (Table 2). Similarly presence of a relatively high molecular weight (∼35 kd) protein in isolate-5 which is absent in isolate-4 could be the basis of complementation in kelthane degradation between these two strains. Dehalogenase enzyme bands pattern of DDD induced exoenzymes of different complementary strains on native gel is shown in Figure 2b. This shows predominance of a low molecular weight protein band in isolate-4, which is absent, or present in a very low quantity in other three strains. A relatively high molecular weight protein band is shown in 5, which is absent in 4. Another protein of intermediate size, which is present in a very low concentration, is observed in the isolates 1 and 12 only and not present in isolate-4. Presence of these different DDD induced enzymes in different isolates may indicate the basis of enzyme complementation between isolate-4 with other strains.

Table 3 shows the screening schedule for isolation of mutants and recombinant/heterologous haploid genotypes from the total population of conidia of individual parents and mixed culture of the two parents. Genotypes pertaining to the parents and different spore types derived from the spore suspension of different cultures are presented under the head 'Genotypes'. First screening shows genotypes formed involving two genes viz. Ben^R and DBP^+ and second screening involves Kel^+ and Lin+ of the parents P-1 and P-2 respectively. In the first screening the parental spore types, i.e., dbp^- Ben^R and DBP^+ ben^r genotypes were unable to survive in the screening medium SM-5 containing DBP as sole carbon source and benomyl added to the medium, due to the absence of DBP degrading gene in P-1 and benomyl resistant gene in P-2. Mutant spores of P-1 and P-2 carrying dbp^+ or ben^R genes, however, survive in this medium forming colonies. However, some of these spores failed to grow in the media used for second screening, i.e., SM-6 containing lindane or SM3 containing kelthane due to the absence of lin^+ gene in P-1 and kel^+ gene in P-2 and were discarded. Spores growing in all these media were double mutants, heterokaryons, diploids or recombinants.

Frequencies of hybrid genotypes in the total population of parental and mixed culture conidia from first and second screening are shown in Table 4. Frequencies of dbp^+ben^R genotype in mixed culture were

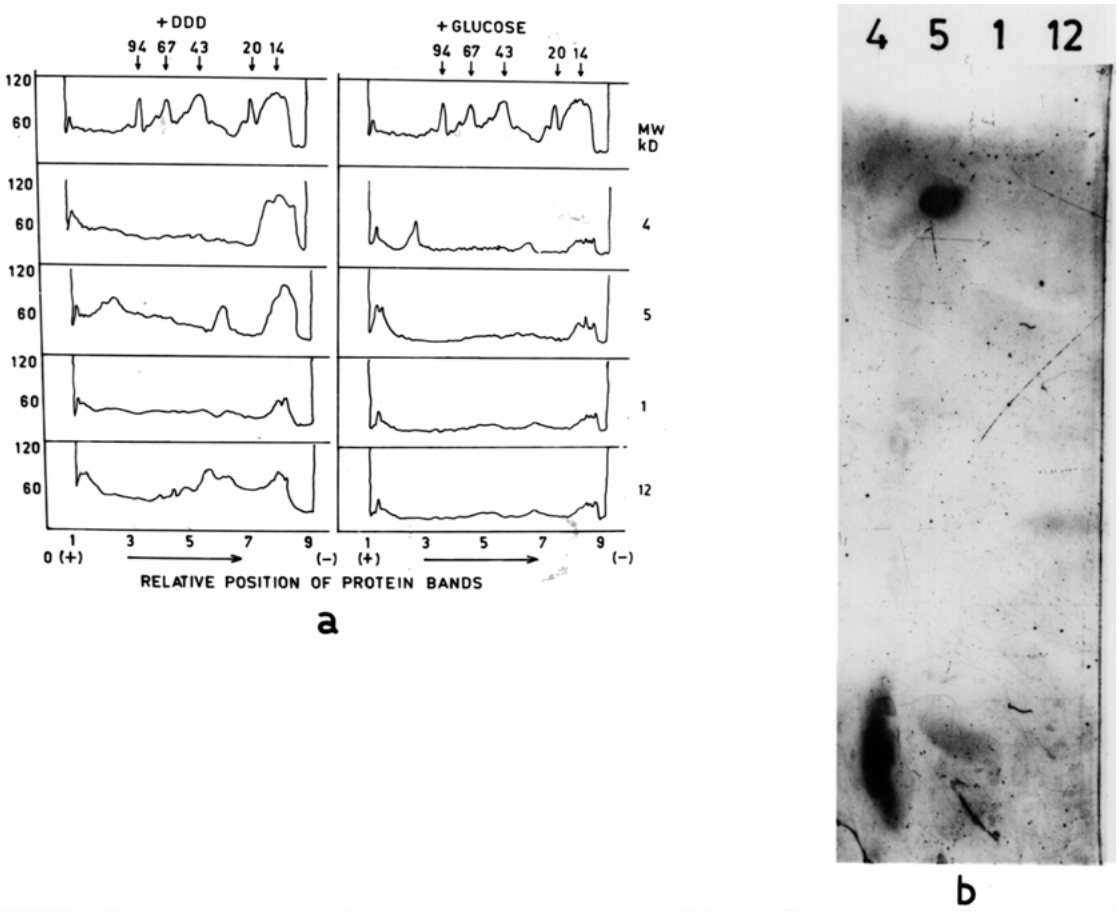

Figure 2. (a) Protein profile of DDD induced enzymes different complementary solates on SDS and (b) native poly acrylamide gel electrophoresis.

found to be much higher (1.9×10^{-6}) compared to P-1 (1.5×10^{-9}) and P-2 (6.9×10^{-9}). This is because in mixed culture, in addition to single mutation, heterokaryotic bi-nuclear spores, and recombinant spores were also formed by haploidization of heterokaryotic diploid spores. Single mutations in parents were eliminated by subjecting these mixed genotype spores for second screening. Colonies with genotype $dbp^{+}ben^{R}lin^{+}kel^{+}$ from individual parents and mixed culture were isolated by growing $dbp^{+}ben^{R}$ genotypes in SM-3 containing kelthane and SM-6 containing lindane as a substrates in the respective media. Spores with single reverse mutation could survive on either of these media due to the absence of the lindane degrading gene in P-1 and kelthane degrading gene in P-2. However, a double mutant with reverse mutation at both the marker sites could grow in both the media. Double mutation frequency in parental populations was found to be 9×10^{-11} in P-1 and 7×10^{-11} in P-2. In mixed culture such spores were more frequent and their frequency was 10^{-7}. Hence, possibility of double mutation among this genotype ($dbp^{+}ben^{R}lin^{+}kel^{+}$) in mixed culture population may be ruled out due to their occurrence at such high frequency. However, some of these could be heterokaryotic binuclear spores or heterologous diploids or recombinant genotypes developed during haploidization of the diploid nuclei. Studying the segregation pattern of this biotype eliminated heterokaryotic binuclear spores. Those isolates segregated into parental type conidia, when plated out on SM-5 medium, produced lesser number of colonies in this medium compared to PDA and were discarded as heterokaryons. Remaining $dbp^{+}ben^{R}lin^{+}kel^{+}$ isolates without reverting to the parental genotypes for several generations and showing better degradability of DDT compared to the parents were selected as genetically improved heterologous haploids or recombinants or hybrid strains

Table 5. DDT degradation in soil by hybrid strains (% increase over control, i.e., soil without inoculum).

Sample	% Increase over control
− Inoculum (control)	−
+ P-1	0.3 ± 0.04
+ P-2	12.5 ± 0.8
+ H-2	22.0 ± 1.6
+ H-30	14.2 ± 1.9
+ H-40	25.2 ± 0.0
+ H-46	34.0 ± 3.8

with improved enzyme system . Their genotype is designated as $DBP^{+}Ben^{R}Lin^{+}Kel^{+}$. These biotypes were used for bioremediation experiments.

Biodegradation potential of different genetically improved strains as expressed by percent increase in DDT degradation in soil over native population (uninoculated control) in soils is shown in Table 5. Highest increase in percent degradation was observed with the hybrid H-46 showing 34.0 ± 3.8% over control soil (uninoculated).

Probable attributers for enhanced degradation in hybrids were studied by SDS- PAGE of fungal exoproteins. Figure 3a. shows diagrammatic representation of polypeptide bands of DDT induced proteins on SDS-PAGE in parents and few recombinants showing faster degradation of DDT. Presence of the DDD induced polypeptide bands characteristic of two parents is seen in the recombinant strains. Presence of a <14 kd polypeptide of P-2 and two little larger sized polypeptides of P-1 in the hybrid recombinants indicated complementation of different DDT degrading proteins in genetically improved strains of *Fusarium solani*.

The characterisation of recombination in H-46 is based on RAPD analysis of genomic DNA. Characteristic bands of the two parents P-1 and P-2 was studied by PCR using the primers OPA-01 to 10 from Operon Technology Inc. USA. Distinct differences in band pattern were shown in two parents with primers 01 (CAGGCCCTTC), 06 (GGTCCCTGAC), 07 (GAAACGGGTG), 09 (GGGTAACGCC), and 10 (GTGATCGCAG). To establish homology of the recombinant H-46 with two parents PCR was done with these primers. H-46 showed absolute homology for P-2 with primer 01, 07 and 09; with primer 10 homology and differences from both the parents at certain regions were observed. Homology and differences in

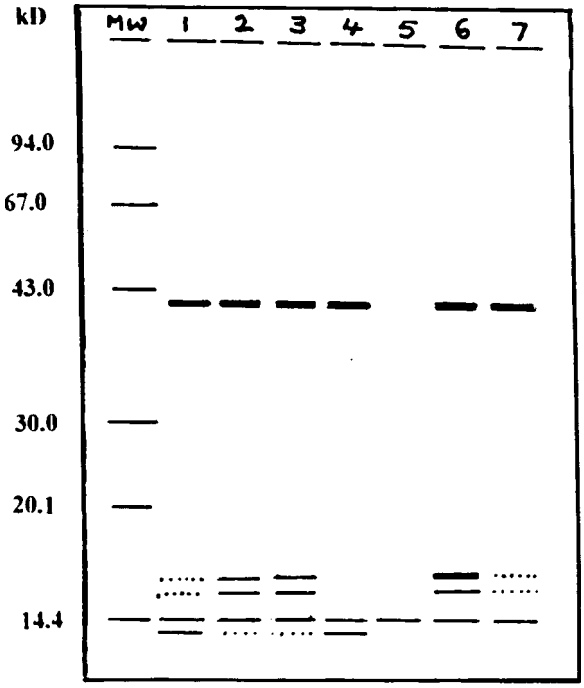

Figure 3a. Diagrammatic representation of polypeptide band pattern of DDD induced enzymes in hybrids and parents.

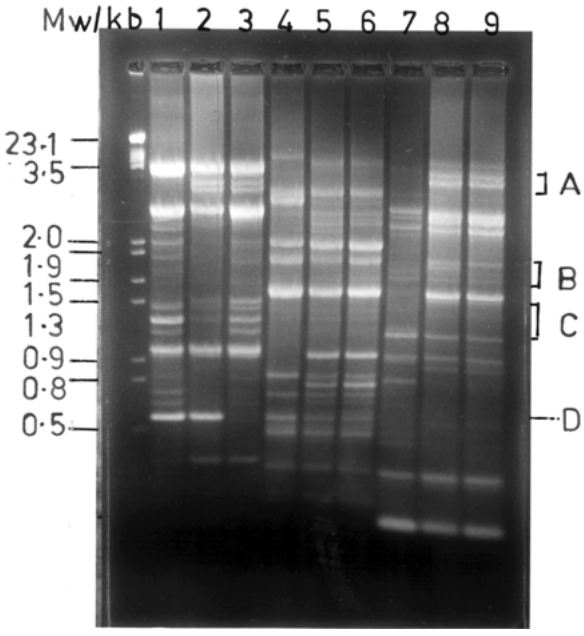

Figure 3b. RAPD of genomic DNA of P-1 (Lanes 1, 4, 7), P-2 (Lanes 2, 5, 8) and H-46 (Lanes 3, 6, 9) using primer OPA-6 (Lanes 1–3), OPA-9 (Lanes 4–6), and OPA-10 (Lanes 7–9).

Table 6. DDT degradation by parents and hybrid (H-46) in liquid medium under laboratory condition.

Strain	μg DDT		μg DDD		μg DDE	
	Live culture	Heat killed	Live culture	Heat killed	Live culture	Heat killed
Blank*	21.8		0.286		1.069	
P-1	12.3	17.0	0.057	0.165	0.055	0.205
P-2	10.6	14.9	0.065	0.206	0.036	0.165
H-46	6.1	18.8	0.063	0.070	0.040	0.057

* Without inoculum.

H-46 from the parents were found to be more obvious with primer 06. Figure 3b shows RAPD of H-46 and two parents with primers 06, 09 and 10. It shows absolute homology between H-46 and P-2 with primer 09 (lane # 4–6). With primer 10 (lane # 7–9), H-46 shows homology toward both the parents. One band, which is present in P-2 around 1.9 kb (B) region is missing while another band present close to this region in P-1, is added to its band profile. Homology with both the parents is more apparent with primer 06 (lane # 1–3). H-46 with this primer showed homology with P-2 around 3-kb (A) region and with P-1 between 0.9–1.3 kb (C) region. Also with the same primer one major band at 0.5–0.8 kb (D) region of both the parents is missing in H-46 totally. The parent specific bands in the recombinant strain H-46 indicates origin of this strain from P-1 and P-2 by recombination during parasexual hybridisation of heterogenic diploid nuclei in the heterokaryons. Missing band could be a result of cross over within the primer region.

Pure culture study with this recombinant strain along with two parents in mineral salt medium (broth) is shown in Table 6. Blank values represent recovery of DDT and the degradation products DDD and DDE from uninoculated medium due to chemical degradation during the experimental period. Differences between blank and heat- killed samples will show adsorption due to mycelium and the differences between heat killed and live culture will give actual degradation due to the organism after 10 days. Mycelial adsorption was found to be 4.8, 6.9 and 3.0 μg in P-1, P-2 and H-46 respectively while actual degradation of DDT was 4.7 μg, 4.3 μg and 12.7 μg after 10 days in the respective cultures. Thus as observed earlier in soil, H-46 in pure culture also shows at least three times more degradation of DDT than the parent strains. Poor recovery of DDD from the culture filtrate is due to further degradation of this compound to other products as indicated by lesser recovery of DDD and DDE in live cultures compared to the heat killed samples.

Thus, genetic manipulation made this way through parasexual recombination technique to produce potentially superior organism from low potential native fungi can be utilised successfully for bioremediation of DDT pollution in soil. To achieve further improvement in bioremediation processes and to make it an ecofriendly agent, multiple copy number of the degradative genes in the recombinant strains for overproduction of DDT degrading enzymes can be induced and the organism or enzymes extracted from culture media may be immobilised for better results.

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