


Isolation and Characterization of a Bacterial Strain *Enterobacter cloacae* (Accession No. KX438060.1) Capable of Degrading DDTs Under Aerobic Conditions and Its Use in Bioremediation of Contaminated Soil

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Sonal Suman¹ and Tanuja²

¹Research Scholar Department of Biotechnology, Magadh University, Bodh Gaya, BR, India. ²TPS College, Patna, BR, India.

ABSTRACT: DDT is one of the most persistent pesticides among all the different types of organo-chlorine pesticides used. Among all the degradation methods, bacterial degradation of DDT is most effective. The present study was conducted to isolate different bacteria present in waste samples which have the ability to degrade DDT present in the soil in the minimum possible period of time and to observe the effect of different physical and chemical properties of the soil samples. Many pesticide degrading bacteria were isolated and identified through cultural, biochemical tests and further identified by 16S RNA sequencing method. The most potent strain DDT 1 growth in mineral salt medium supplemented with DDT as the only source of carbon (5-100PPM) and was monitored at an optical density of 600nm. The growth parameters at different physiochemical conditions were further optimized. The result showed that *Enterobacter cloacae* had maximum growth in 15 days. FTIR analysis of the residual DDT after 15 days incubation showed that *Enterobacter cloacae* was able to degrade pesticide into its further metabolites of DDD, DDE, DDNU and other components can be used for biodegradation of DDT present in contaminated soil and water ecosystems.

KEYWORDS: Bacteria, biodegradation, DDT, FTIR

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CORRESPONDING AUTHOR: Sonal Suman, Research Scholar Dept of Biotechnology, Magadh University, Bodh Gaya, BR 824234, India. Email: sonal.micro89@gmail.com

Introduction

Pesticides are natural or synthetic compounds that are used to kill unwanted plant or animal pests. The extensive use of such pesticide has several harmful effects leading to different health and environmental problems like especially to the farm workers. DDT is known to cause cardiopulmonary, neurological, and skin disorders, fetal deformities, miscarriages, lowering the sperm count of applicators, among others.⁴ DDT is one of the most persistent pesticides among all the different type of organo-chlorine pesticides used.¹⁴ It is an organo-chlorine insecticide with the properties like, colourlessness, highly hydrophobic, crystalline solid having a weak chemical odour. Due to its hydrophobic nature it is nearly insoluble in water but reported to have good solubility in many organic solvents, fats and oils. DDT is a synthetic compound produced by the combination of the chloral (CCl₃CHO) and chlorobenzene (C₆H₅Cl) in the presence of sulphuric acid, as a catalyst. The DDT in the environment is persistence to degradation and has caused serious environmental problems worldwide. The study reported that microorganisms can be used in the bio-degradation of DDT and DDE. The conversion of DDT to DDE by microorganisms and the study of the bacterial degradation system of DDE is useful for the development of effective bioremediation technologies for DDE. The bacterial strains reported for DDE degradation are *Pseudomonas acidovorans* M3GY, *Terrabacter* sp. DDE-1, and *Janibacter* sp. TYM3221 are the predominant predator present in the soils.¹⁹

Most of the DDT residues are lipophilic in nature and it can accumulate in the fatty tissues of the organism consuming

the contaminated food by entering the food chain. The biodegradation of DDT and its residues naturally occurring in soil is very slow. To speed-up the process of degradation several in situ strategies are proposed. Among all the most effective method of degradation is DDT-metabolising microbes present in the contaminated soils and the degradation process can be enhanced by manipulating the different environmental factors. Bacteria are capable of degrading DDT in both aerobic and anaerobic conditions, some reported bacterial strains with degradation potential are *Escherichia coli*, *Enterobacter aerogens*, *Enterobacter cloacae*, *Klebsiella pneumonia*, *Pseudomonas aeruginosa*, *Pseudomonas putida*, *Bacillus species*, *Hydrogenomonas*, etc., some fungi are also reported to have degradation potential like *Saccharomyces cerevisiae*, *Phanerochaete chrysosporium*, *Trichoderma viridae* among others.

The present study was conducted to isolate different bacteria present in waste sample with the high potential to degrade the DDT present in soil in minimum possible period of time and to observe the effect on the physiochemical properties of the soil, and to determine the pathways for biodegradation of DDT, DDD, DDE, DDNU and other components.

Materials and Methods

Chemicals

A total of 99% pure DDT of commercial grade of Sigma was obtained, and other chemicals were also purchased from standard manufacturers which were of analytical grade. All used glass wares were kept in the pre-heated hot air oven at 550°C



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before the use and all the other equipment's were washed with analytical grade hexane. DDT stock solution was prepared by dissolving DDT in acetone.

Sampling

Four different soil samples from local agricultural field (Chitkhora, Patna) were collected randomly, with previous history of DDT exposure for the isolation of bacteria with the potential of DDT biodegradation. Hospital waste dumping area soil where DDT is used as disinfectant spray was also used for the isolation. The soil samples were collected by scooping upto 10 cm³ of the topsoil by the help of sterile spatula. The collected soil samples are stored in sterilized packet in cold and humid place for further study (Muchena et al., 1998).¹³

Isolation, maintenance and acclimation and of isolated bacterial strain

Bacterial strain used in this study were isolated and maintained in the laboratory on nutrient agar (NA) media containing peptone 5.0; beef extract 3.0; NaCl 5.0 and agar 20.0 (g/l of distilled water). The bacterial colonies obtained were then streaked on fresh NA-slant and incubated at different temperature that is, 28, 37 and 50°C for 24 to 48 hours to obtain pure cultures at optimum temperature. For screening, enrichment culture technique was used for identification of bacterial isolated with degradation ability. The enrichment was done by incorporating the DDT as the only source of carbon at low concentration of 5 ppm in medium, and was further maintain at optimum physical condition in shake flask as described by Zhu et al²¹

Colony morphology was determined and for further identification, series of biochemical tests were performed like, amylase production test, casein hydrolysis test, H₂S production test, sugar utilization test, litmus milk test, urease test, IMViC test and catalase test (Cappuccino and Sherman, 2001; Bergey and Holt, 1994).^{5,7} The best selected strain with maximum degradation ability was further confirmed on the basis of 16S rRNA sequencing at Yaazh Xenomics (Madurai, Tamilnadu, India). The phylogenetic neighbour-joining tree was made using complete 16S rRNA gene sequence analysis.

Optimization of growth potential of selected isolates in different concentration of DDT as a sole source of carbon

All sets of experiments were carried out in the triplets for determining the degradation percentage of DDT, and the data provided are an average of these 3 values. Using modified Mineral Salt Media (MSM) (Composition: sodium chloride 1 g, CaCl₂ 1 g, MgSO₄ .7H₂O 0.5 g, KH₂PO₄ 1 g, Na₂HPO₄ 1 g, yeast extract 4 g, sterilized distilled water 1 l, pH 7.0 to 7.2, containing DDT at different concentration (5-100 ppm). The culture flasks were incubated in shaker flask at

120rpm by maintaining the ambient physical condition. Samples were taken at regular time intervals of 24 hours for the determination the growth, by spectrophotometer at 600 nm.³

Effect of different physiological and chemicals condition

Isolated strains with potential of DDT degradation were subjected at different physiochemical conditions like pH, temperature, salinity, nitrogen and glucose concentration. The selected strain (DDT-1) was further optimised at different physiological conditions to enhance the DDT pesticide degradation like pH (3.5, 5.5, 7.0, 8.5 and 10.5), temperature (28, 37 and 50°C), salinity (5%, 10% and 15%), nitrogen (yeast extract, casein, beef extract) and glucose concentration (Mannose, Lactose, Sucrose and Dextrose). The control flask was also maintained without inoculating the bacterial culture. All cultures were incubated in a shaker (120 r/min) at 37°C. Growth was observed, taking the optical density (OD₆₀₀) by the help of spectrophotometer after every alternate day and turbidity was measured until the growth becomes constant.¹

Analysis of degraded DDT biodegradation

The initial degradation study was conducted by using the UV-Vis spectrophotometer by using the below formula at different wavelength.²

$$\%D = (1 - A_t / A_0) \times 100$$

Where,

A_0 is the initial absorbance

A_t is the absorbance after time t at the wavelength corresponding to the peak maximum.

In order to estimate the degradation of DDT by isolates to further metabolites like DDT, DDE, DDNU and was analysed by FTIR. It was performed in the CRL, Patna Women's college, Patna.

Result

Physio-chemical analysis of soil sample collected from hospital dumping area and agricultural soil

The sample used for the isolation of the microorganism were collected from a agricultural soil having the prior history of DDT exposure (soil sample designated as R3 & R4) along with a sample from a hospital waste dumping area (waste soil collected from hospital designated as R1 & R2). Analysis of physiochemical properties involved the test for temperature, pH, electrical conductivity (Table 1), and organic carbon, P₂O₅, K₂O, Zn, Cu, Mn and Fe, were also measured as mentioned in Table 2.

The sample R3 is more alkaline with pH 7.2 as compared to other samples, and also the electrical conductivity is high as well. Similarly organic carbon, P₂O₅, K₂O, Zn and Fe is also

high in sample number R3 where as sample number R1 is richer in Mn and Cu.

Isolation, identification and characterization of potent bacterial strains

The growth obtained on NA media was first observed and the number of colony was counted for which quebec colony counter was used. Total 6 selected bacterial colonies were obtained from 4 the samples gram reaction result was noted (Table 3). The selected pure colonies are used for further investigation for DDT degradation potential. The colony characteristics of the selected bacterial strains.

For further identification biochemical tests were performed for all the 6 Isolates. The biochemical tests were, amylase production test, casein hydrolysis test, H₂S production test, sugar utilization test, litmus milk test, urease test, IMViC test and catalase test. Thus, on the basis of the cultural, morphological and biochemical characterization DDT 1 may be *Enterobacter* sp., DDT 2 may be *Bacillus* sp. DDT 3 and DDT 4 may be

Staphylococcus sp. and DDT 5 may be *Streptococcus* sp. and DDT 6 may be *Bacillus* sp. Similar type of result is also reported by Fogh et al¹⁰ the isolated microorganism (both bacteria and fungi) from the different soil samples have the degradation potential of DDT to DDD. As reported by Semple et al¹⁷ that bioremediation process is slow and remediation of DDT contaminated soil takes longer period of time. Among 6 selected isolate, DDT-1 was further identified with maximum potential for degradation and was selected for further study

DDT-1 was further identified as *Enterobacter cloacae* (Accession No. KX438060.1) by 16S rRNA sequencing at Yaazh Xenomics (Madurai, Tamilnadu, India). The phylogenetic neighbour-joining tree was constructed for DDT-1 (Figure 1), using complete 16S rRNA gene sequence analysis. Further in the study of Dileep⁹ several bacterial isolated like species of the genus *Bacillus* and *Staphylococcus* and *Stenotrophomonas* were characterized with the potential of DDT degradation in aerobic condition.

Degradation potential of isolates

All the six isolates were further subjected on the enrich culture media in which DDT is used as a single carbon source as mentioned in Table 5 and were selected on its capability to utilise the DDT. Among 6 selected isolate, DDT-1 with maximum potential for degradation was selected for further study. Degradation at different concentrations of DDT (50-100 ppm) in MSM (DDT as sole source of carbon), in which maximum growth was found at the concentration of 50 ppm on 14th day of inoculation as shown in Figure 2.

The plate count method was initially used for the selection of isolates further the DDT degradation was determined using UV-Vis spectrophotometry. DDT degradation was directly

Table 1. Physical properties of soil samples collected for the isolation of bacterial culture.

S.N.	SAMPLES	PH	TEMPERATURE (°C)	ELECTRICAL CONDUCTIVITY (MS/M)
1.	R1	4.064	31.3	102
2.	R2	3.9	32	106
3.	R3	7.264	46.2	333
4.	R4	7.1	43	290

Table 2. Chemical properties of soil samples collected for the isolation of bacterial culture.

S.N.	SAMPLES	ORGANIC CARBON (%)	P ₂ O ₅ (PPM)	K ₂ O (KG/ HECTARE)	ZN (PPM)	CU (PPM)	MN (PPM)	FE (PPM)
1.	R1	0.62	14	4.09	0.842	0.230	41.60	5.102
2.	R2	0.53	12	3.9	0.73	0.199	34.80	4.09
3.	R3	1.26	43	17.92	1.206	0.138	38.20	7.564
4.	R4	1.02	41	16.02	0.98	0.12	36	6.02

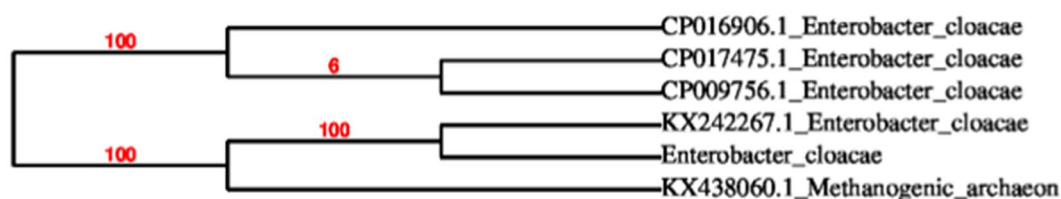


Figure 1. Phylogenetic analysis of strain DDT-1 based on 16S rRNA analysis. The sequence bar equals 0.02 changes per nucleotide position.

Table 3. Results of bacterial isolates from soil samples and colony characteristics.

SAMPLE	COLONY CHARACTERISTICS				GRAM'S REACTION	ORGANISM
	COLOR	TEXTURE	MARGIN	SHAPE		
DDT1	Off white	Slimy	Smooth	Regular	Negative	<i>Enterobacter</i>
DDT2	Off white	Slimy	Smooth	Regular	Positive	<i>Bacillus</i>
DDT3	Pink	Slimy	Smooth	Regular	Positive	<i>Staphylococcus</i>
DDT4	Yellow	Slimy		Regular	Positive	<i>Staphylococcus</i>
DDT5	Cream	Slimy	Smooth	Regular	Positive	<i>Streptococcus</i>
DDT6	Cream	Slimy		Regular	Negative	<i>Coccobacillus</i>

Table 4. Results of biochemical tests of different bacterial isolates.

S. NO.	BIOCHEMICAL TESTS	SELECTED BACTERIAL ISOLATES					
		DDT1	DDT2	DDT3	DDT4	DDT5	DDT6
1	Amylase	+	+	–	–	+	+
2	Casein hydrolysis	–	–	–	–	+	+
3	Catalase	+	–	+	+	+	+
4	Gelatin hydrolysis	+	–	+	+	+	+
5	Nitrate reduction	–	–	+	–	+	–
6	Citrate utilization	–	–	–	+	–	+
7	Indole production	+	–	–	–	–	–
8	MR	–	+	+	+	+	+
9	VP	+	–	+	–	–	–
10	Urease	–	–	–	+	–	–
11	Dextrose	+	+	+	–	+	+
12	Sucrose	+	+	+	–	+	–
13	H ₂ S	+	–	–	–	–	–
14	Motility	+	–	–	–	–	–

‘+’ is for Positive.

‘–’ is for Negative.

Table 5. Growth pattern of isolates on DDT-1 colony on agar media + DDT (different concentration) as source carbon source.

COLONY NO.	GROWTH PATTERN OF BACTERIAL ISOLATE DDT-1 COLONY ON AGAR MEDIA + DDT (DIFFERENT CONC.) AS SOURCE CARBON SOURCE		
	50 PPM	100	250 PPM
1.	Luxuriant growth	Luxuriant growth	Luxuriant growth
2.	Luxuriant growth	Luxuriant growth	Poor growth
3.	poor growth	Luxuriant growth	Poor growth
4.	No growth	Poor growth	Poor growth
5.	Poor growth	Poor growth	No growth
6.	Luxuriant growth	Poor growth	Poor growth

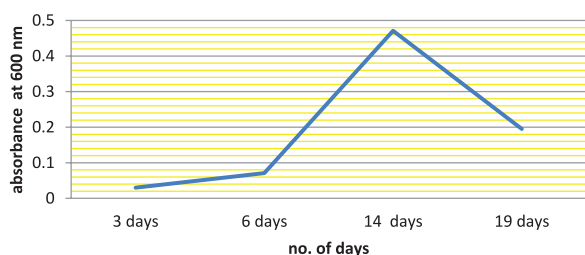


Figure 2. Growth of DDT1 at 50ppm DDT in minimal broth at different interval of time.

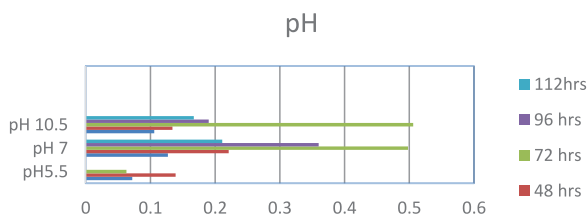


Figure 3. Showing the growth absorbance (mentioned on x-axis) at different pH on growth of bacterial strain DDT-1.

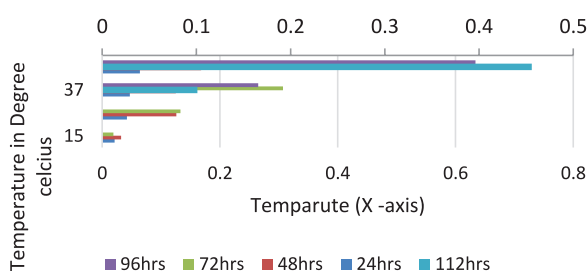


Figure 4. Showing the growth absorbance (mentioned on x-axis) at different temperature on growth of bacterial strain DDT-1.

proportional to growth of the cultures. It was observed that an increase in turbidity corresponded to an increase in DDT degradation.

The uninoculated medium with DDT and inoculated medium without DDT were used as controls the change in the turbidity, and concentrations of DDT was measured at regular time interval. Isolates that grew in the medium and reduced the concentration of DDT as sole carbon source in the medium were judged to be capable of biodegrading DDT. The maximum growths of enrichment cultures observed at DDT concentrations that is, 0, 20, 50 and 100 ppm, after a period of 14 days were different from each other and it was maximum at 50 ppm.

Effect of physio-chemical factor on the growth pattern of DDT-1

Effect of temperature and pH. The bacterial strain DDT-1 was subjected to different temperature and pH, as shown in Figures 3 and 4, strain DDT-1 showed different growth pattern at different temperatures (15, 26, 37, 50°C) and pH (ranging from 5.5 to 10.5), showing maximum optical density at pH 10.5 and at eighth day of incubation at 50°C.

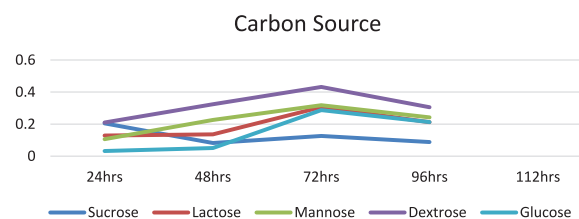


Figure 5. Showing the growth absorbance (mentioned on y-axis) in presence of different carbon source of bacterial strain DDT-1 at different time interval (on x-axis).

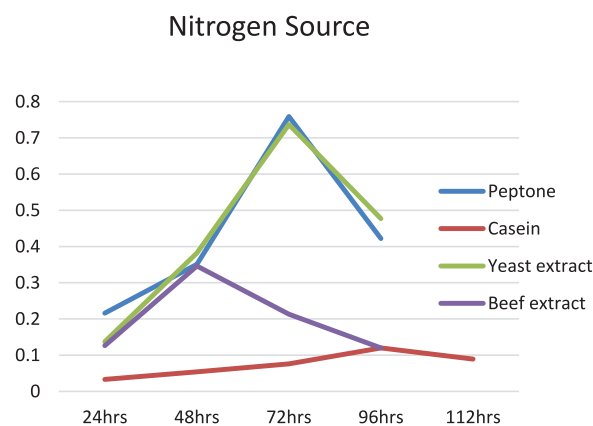


Figure 6. Showing the growth absorbance (mentioned on y-axis) in presence of different nitrogen source of bacterial strain DDT-1 at different time interval (on x-axis).

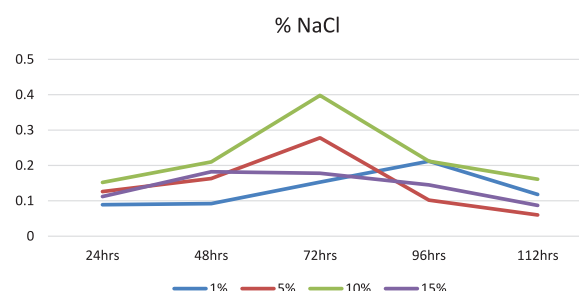


Figure 7. Showing the growth absorbance (mentioned on y-axis, at 600 nm) at different salt percentage of bacterial strain DDT-1 at different time interval (on x-axis).

Effect of chemicals factors (carbon, nitrogen source and NaCl) on growth of DDT-1. To further optimise the growth condition of the selected isolates DDT-1 is subjected to varying carbon (lactose, glucose, mannose and dextrose); Nitrogen (peptone, yeast extract, beef extract and casein); and NaCl (1%, 5% 10% and 15%) condition and optical density was monitored on regular interval of 24 hours (Figures 5–7).

The strain DDT-1 shows the best growth for the carbon source Dextrose on fifth day of inoculation, nitrogen source peptone and yeast extract both shows best growth also on fifth day, 10% NaCl was most optimum for the growth of DDT-1.

Analysis of degradation pathway of DDT by FTIR

The biodegradation of DDT was determined by comparing the FTIR peak profiles of degraded DDT samples and the

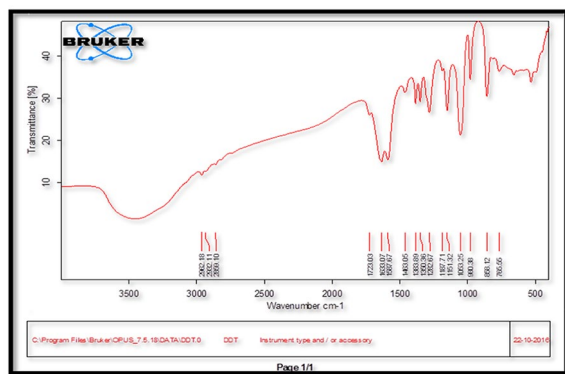


Figure 8. Graph showing the presence of functional group present in DDT that is, C-Cl (765.55 cm^{-1}), C=C (1633.07 cm^{-1}) and C-H (2962.18 cm^{-1}) (control: minimal broth containing 50 ppm of DDT inoculated with bacterial-stain (2%) at 0 days of inoculation).

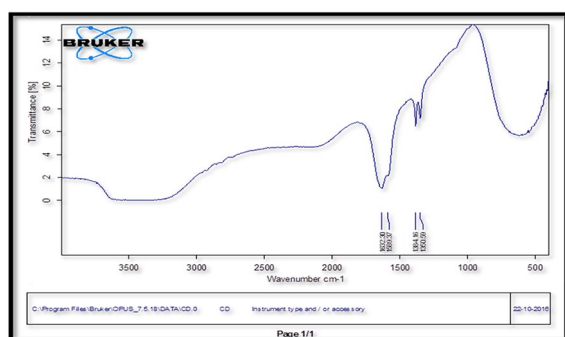


Figure 9. This graph is obtained from the filtrate of minimal broth containing 50 ppm of DDT inoculated with bacterial-stain (2%) after 15 days of inoculation, showing the peak for the presence of functional group C=C and C-H bond present in the degraded compound DDNU from the DDT.

control sample. The selected strain DDT-1 that is, *Enterogens cloacae* (Accession No. KX438060.1) was analysed for simpler degrade compound at above determined optimum condition by FTIR.

The FTIR spectra of pure DDT as shown in Figure 8. The absorption band of the pure DDT (uninoculated sample at 0 day incubation) shows the presence of functional group present in DDT that is, C-Cl (765.55 cm^{-1}), C=C (1633.07 cm^{-1}) & C-H (2962.18 cm^{-1}). Whereas the FTIR spectrum of inoculated sample containing 50 ppm DDT on 14 day of inoculation shows the presence of C=C and C-H bond (the spectrum of C-Cl bond is lacking) showing the present in the degraded compound DDNU from the DDT.

Figure 9 shows the graph obtained from the isolates of DDT 1 showing the maximum degradation of DDT pesticide in minimal broth containing 50 ppm of DDT incubated for 14 days, showing the maximum potential to degrade DDT to DDNU, which is one of the intermediate in microbial DDT degradation pathway. Graph shows the presence of functional group C=C and C-H bond. The above work found in the correlation of the work of Boul.⁶

Discussion

Among 6 different isolated strains our strain DDT-1 was found more optimum to degrade the DDT pesticide, in further study it was identified as *Enterobacter cloacae* (Accession No. KX438060.1). The selected strain DDT-1 can utilise variety of carbon source and shows high tolerance towards alkaline and acidic pH with salinity tolerance up to 10% NaCl. According to the study conducted by the Diamond and Owen,⁸ a selective media and optimum physiochemical conditions was used for determining the microbial capability to transformation and degrade DDT through the major pathway. The factors like pH, temperature can affect the growth of microorganisms and their degradative abilities. However, report by Sreedharan et al¹⁸ pesticides degradation also show the degradation of HCH isomers in soils which was having low or acidic pH by a bacterial strain of *S. paucimobilis*. The strain DDT-1 that is, *Enterobacter cloacae* (Accession No. KX438060.1) is different from those microorganisms previously reported in the literature that could degrade DDT. The degradation mechanism of DDT is generally co-metabolism, and different strains require different substrates, such as glucose and biphenyl, and the substrate of the selected strain in our study is peptone and yeast extract after 3 day of incubation and dextrose as best carbon source. Moreover, not only DDT but also its other metabolite like DDE could be degraded by this strain. The optimal growing conditions were optimized, and the optimal initial conditions included a pH of 10, and temperature of 50°C eighth day of incubation showing that strain is highly acidophilic and thermophilic in nature. From this research, it was determined that the dominant reaction was dehydrochlorination of DDT to DDE, which is predominant under aerobic conditions, whereas there is a reductive dechlorination to DDD under anaerobic conditions.

It was also reported that the growth pattern for different pesticide degrading bacteria at different carbon influences the degradation rates. He also reports that the degradation of pesticide like endosulfan is inhibited by the chemicals like sodium acetate and sodium succinate. The above study was found in correlation with the work of Lai and Saxena,¹² they also report the DDT metabolising microbes isolated from areas with the history of intensive DDT use and were previously exposed to the pollutant. According to the study conducted the main mechanism in microbial degradation is under the reducing conditions, for reductive dechlorination is of both compounds o,p'-DDT and p,p'-DDT further by the isolates resulted from the reductive dechlorination of the aliphatic part of the DDT molecule (Sari, 2012).¹⁵ The substitution reaction happens and substitute the aliphatic chlorine for a hydrogen atom. The bacterial strain *B. subtilis* is reported for producing the metabolite DDD along with other like DDMS, DDMU, DDMS, DDNU, DDOH and DBP. In our study as per graph obtained by FTIR after 15 days of degradation DDT was breakdown to its further metabolites DDNU as the graph shows the presence of functional group C=C and C-H bond, (the presence of functional

group. C-Cl (765.55 cm^{-1}), C=C (1633.07 cm^{-1}) & C-H (2962.18 cm^{-1}) present in DDT). As per the similar study of eco-friendly method of DDT degradation FTIR analysis was used to detect the bioactive compounds, whereas the peak at 1968.41 cm^{-1} is related to the aromatic ring. Similar results have been observed in other studies.¹¹ The absorption band of the aromatic C-C in-ring present in DDT disappeared completely in the liquid sample containing the bacterial isolates, and a new absorption band at 1633.07 cm^{-1} was observed ascribed to the degradation products.¹⁶ From this research, it was determined that the dominant reaction was dehydrochlorination of DDT to DDD that is, a reductive dechlorination, which is predominant under aerobic conditions. As per some of the reports DDE could be degraded by the dechlorination enzymes, dioxygenase and hydrolase in the appropriate conditions. Sari reported that dioxygenase and lignin peroxidase levels were higher with the addition of DDT, and these 2 enzymes play important roles in the degradation of DDT. The further metabolism of DDE could mostly continue under aerobic conditions because there is a double bond structure of the relative instability in the molecule of DDE, and DDE is able to undergo oxidation.²⁰

Conclusion

The present study shows that the bacterial consortia exposed to DDT have the potential to metabolise and bio-degrade the DDT pesticide. The present study identified 6 different bacterial strains with potential of degradation of DDT pesticide, and isolate DDT-1 has the maximum potential to degrade the DDT to its further metabolites like DDNU which is identified as *Enterobacter cloacae* (Accession No. KX438060.1) after mRNA sequence analysis. This suggests that this isolate could be a potent isolate for degradation of DDT present in different environmental condition. This study shows, 50 ppm of added DDT was degraded at 37°C at neutral pH and isolates DDT -1 bacteria culture also show more growth pattern at 50°C , alkaline pH of 8.5 capability of utilising dextrose as best carbon source, peptone and yeast extract as nitrogen, and 10% NaCl concentration. The ability of such potent strain to completely degrade the pesticide DDT to CO_2 or to other non-toxic chlorinated compounds through co-metabolism should be further assessed.

ORCID iD

Sonal Suman  <https://orcid.org/0000-0002-8252-649X>

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