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Research Article
Proficient Biodegradation Studies of Chlorpyrifos and its Metabolite 3,5,6-Trichloro-2-pyridinol by *Bacillus subtilis* NJ11 Strain

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Abstract

Background and Objective: Chlorpyrifos (CP) is one the most widely used broad spectrum organophosphorus (OP) pesticide in agricultural fields worldwide particularly in Punjab, India. Scarcity of the knowledge in farmers regarding the usage, handling and safety concerns of pesticides leads to massive and uninhibited use of CP. Upon hydrolysis, CP usually generates diethyl thiophosphoric acid (DETP) and 3,5,6-Trichloro-2-pyridinol (TCP), later one possess antimicrobial attributes. Residues of both CP and TCP have been detected in ecosystems and imparts numerous toxicological effects on the various life forms particularly in humans. Hence, their elimination from the contaminated environmental sites is highly needful and bioremediation is the most convenient option at present as it is cost-effective and ecofriendly. Thus objective of the present study was to isolate a bacterial strain having the capability to degrade CP and TCP both completely in to less toxic forms. **Materials and Methods:** Bacterial strain was isolated from CP and TCP contaminated soils of Malwa region of Punjab where CP has been used continuously in agricultural fields. The CP utilization capabilities of bacterial strain were analyzed by HPLC, HPTLC and other chemical based colorimetric methods. Minimum inhibitory concentration of CP and TCP on the strain was analyzed and also influence of pH and temperature on the growth of isolate was assessed. **Results:** Strain was found to be Gram positive and was able to use CP as well as TCP as sole carbon source. Molecular characterization based on 16S rRNA gene sequence homology confirmed its identity as *Bacillus subtilis*. The HPLC studies revealed almost complete degradation of 150 ppm of CP within 5 days without accumulation of TCP in the system. Isolate was competent to tolerate initial CP concentration as high as 800 ppm and present study report firstly of tolerating such higher concentration by *Bacillus subtilis* strain. Isolate was well adapted to grow within the wide temperature (25-40°C) and pH (6.0-9.0) range. **Conclusion:** The isolate *Bacillus subtilis* NJ11 was found to be efficient in the remediation of CP and TCP in liquid medium by converting them into lesser toxic intermediates. Present study suggested that strain has potential to clean up the CP and TCP contaminated sites.

Key words: Organophosphorous, chlorpyrifos, HPLC, tetrazolium reduction assay, TCP, *Bacillus subtilis*

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Pesticides are the chemical compounds that are used to manage the pest population and control crop-damage and vector-borne human disorders[1,2]. Based on the chemical constituents they are classified as organochlorines, organophosphates (OPs), carbamates, synthetic pyrethroides inorganic pesticides, etc[3]. The OPs are more effective, less toxic and possess relatively short half-life then other types thus use of OPs in agriculture has been increasing considerably[4]. From literature survey, it has been acknowledged that out of total pesticide consumption globally, OPs account for about 38%[5]. Chlorpyrifos (O,O-Diethyl O-3,5,6-Trichloropyridine-2-yl phosphorothioate), CP is a moderately toxic, non systemic OP consumed by all over the globe to control pest populations in agricultural crops like cotton, cereals, vegetables and fruits. It was in the use for last more than 60 years to cut down the agricultural losses as it displayed insecticidal activity against a wide variety of insects. Globally, chlorpyrifos ranks first among all the conventional pesticides used in the agricultural sector in 2007[6].

The CP was a widespread contaminant of environment leading to serious damage to non target organisms. The CP possess a half-life period of 2 weeks to over 1 year and it varies with soil characteristics like organic content, porosity and soil pH and exhibits moderate persistent in the environment[7]. Due to its slow remediation rate, CP can remain in environment for extended time periods which impose life threatening effects on various life forms[8]. Various toxic effects and target of CP in humans are: Immune system[9], AChE activity in developing fetus[10], mammalian cell cultures[11], neurodevelopmental disorders[12] etc. The TCP is one of the main hydrolysis product of CP, possess relatively elevated antimicrobial attributes than CP and have the capability to inhibit the growth and proliferation of CP degrading microbial populations[13]. One of the other intermediate is DETP, which possess the tendency to damage DNA of non-targeted live forms and thus affects their health[14].

According to Centre for Science and Environment India, farmers in Punjab are spraying 6 times more pesticides including CP than recommended values due to lack of the knowledge regarding the application and safety concerns of pesticides. Accumulation of higher concentrations of CP and other pesticide in the living organisms has generated detrimental results in this area[15]. Nearly 17 different pesticides residues have been detected in Malwa region of Punjab. This part was also described as India's "Cancer Capital" due to abnormally high number of cancer cases that has been increased 3 fold in the last 10 years[16]. Also there was sharp increase in many pesticide related diseases in Punjab population[17]. The CP, TCP and DETP impose toxicological effects on human health if even present in trace amounts therefore monitoring and complete removal is essentially required to lower down the perilous effect of these hazardous chemicals[18,19].

As compare to other physical and chemical methods employed to degrade the pesticides, biodegradation is the best option at present. Bioremediation being cost effective, easy to apply, reliable and eco-friendly has received particular attention as an effective approach to degrade synthetic chemicals. To date, few microorganisms capable of degrading both CP and TCP have been reported in the literature including *Alcaligenes faecalis*[4]. *Sphingomonas* sp., Dsp-2[20], *Paracoccus* sp., TRP[21], *Bacillus pumilius* C2A1[22], *Ralstonia* sp., T6[23], *Athrobacter* sp.[24] *Cupravidus* sp., DT-1[25], *Mesorhizobium* sp., HN3[26], *Ochrobactrum* sp., JAS2[19] etc.

To the best of the knowledge CP and TCP bioremediation capabilities of *Bacillus subtilis* has not been used exploited fully till date. Objective of present study was to isolate a bacterial strain exhibiting methyl parathion (a type of OP), CP and TCP degradation capabilities. Growth of bacteria in liquid culture medium under different environmental factors like temperature, pH was investigated to optimize the conditions for chlorpyrifos biodegradation. The HPLC and HPTLC studies were performed to investigate the bioremediation of CP and TCP in liquid medium. Complete mineralization of CP and TCP in to safer metabolites was also checked by chemical based methods. Present study aimed to elucidating a possible application of isolated bacterial strain for remediation of chlorpyrifos and TCP contaminated environments.

MATERIALS AND METHODS

Chemicals and growth media: Chlorpyrifos (CP), 3,5,6-Trichloro-2-pyridinol (TCP) (HPLC grade, 99% purity) were purchased from Sigma- Aldrich, Germany. All other reagents and solvents used for HPLC, HPTLC and various studies were of high purity and of analytical grade. Mineral salt medium (MSM) for the CP and TCP utilization studies (MSM, pH$\pm$7.2) was composed of 1.5 g K_2HPO_4, 0.5 KH_2PO_4, 1.0 g $(NH_4)_2SO_4$, 0.5 g NaCl, 0.2 g $MgSO_4$, 0.02 g $FeSO_4$, 0.05 $CaCl_2$ into 1000 mL of distilled water.

Isolation and characterization of CP and TCP utilizing bacterial isolate: Strain NJ11 having OPH (Organophosphorus hydrolase) activity was isolated from pesticide contaminated soils of Punjab and was initially employed for methyl parathion degradation studies in our

laboratory (data not shown). Study was carried out during March, 2016-November, 2016. Isolate was then checked for its CP and TCP utilization efficacies. Filter sterilized CP and TCP (150 ppm) was added in autoclaved nutrient broth separately and inoculated with the bacterial culture. Freshly grown bacterial culture was streaked on the MSM agar plates supplemented with 150 ppm CP and TCP, respectively and incubated at 30°C for 48 h and observed for growth. Bacterial culture was subjected to standard physiological, biochemical and morphological examination according to the procedures mentioned in Bergey's Manual of Determinative Bacteriology. Isolated microorganism was identified from IMTECH (Institute of Microbial Technology), Chandigarh, India. Growth profile of isolate was studied by taking O.D (optical density) at 600 nm after the interval of every 3 h up to 60 h.

Minimum inhibitory concentration assay for CP and TCP: Bacterial tolerance for CP and TCP was studied by monitoring its growth in MSM containing increasing concentration of CP and TCP as the sole carbon source. The MSM agar plates supplemented with varying CP and TCP concentrations ranges between 100-1000 ppm were streaked with freshly grown bacterial culture. Plates were then incubated at 30°C for 48 h and observed for growth.

Silver nitrate assay to check TCP utilization by strain: For the confirmation of utilization of TCP by the isolate, chloride ions release was determined implying the modified procedure as described by Li *et al.*[23]. Fifty milliliters autoclaved MSM supplemented with 150 ppm TCP as the only carbon source was inoculated with 1 mL (1×10^8 cells mL^{-1}) of freshly grown bacterial culture and incubated for 48 h at 30°C on a rotatory shaker. Bacterial cell number i.e., 1×10^8 cells mL^{-1} was referred as 1 unit throughout the experimental study. Post incubation time period, 10 mL of sample was removed and centrifuged to obtain cell free medium. To the supernatant, 0.1 mL of indicator $K_2Cr_2O_4$ (50 g L^{-1}) was added and then mixture was titrated with silver nitrate solution (0.0141 mol L^{-1}) until a red brown color appeared. Same procedure was followed with control run also.

Test for determining the biodegradability of chlorpyrifos by tetrazolium reduction assay: To evaluate the biochemical oxidation of CP by bacterial isolate, tetrazolium chloride reduction test was performed as described by Bhagobaty and Malik[27]. Upon the oxidation of CPs, yellowish color of tetrazolium dye which functions as an artificial electron acceptor was converted into non soluble purple colored formazon by dehydrogenase activity of bacteria. Autoclaved mineral salt media (MSM) was added with a 150 ppm of CP aseptically. One unit of freshly grown culture was inoculated into media and incubated at 30°C in a rotary shaker for 48 h. Post incubation, 1 mL of sample was removed and 5 mL of freshly prepared 0.02% tetrazolium chloride was added into the test tubes containing test organism. Test tubes were boiled for 5 min and incubated at 30°C for 4 h at room temperature and then observed for color change.

Effect of media pH and temperature on the growth of NJ11: To study the effect of pH and temperature on the growth of *Bacillus subtilis* NJ11, bacterial culture was freshly grown in nutrient broth containing 100 ppm CP as an inducer. From the growth medium microbial cell biomass was harvested at its exponential phase by centrifugation, washed twice and finally mixed with normal saline and cell count was adjusted to one unit. For temperature optimization, 25 mL MSM medium in Erlenmeyer flasks containing 200 ppm CP were inoculated with 1 unit of freshly grown culture and were kept at different temperatures (25, 30, 37 and 40°C) on rotatory shaker for 1 week. After every 24 h of incubation, optical density of medium was recorded at 600 nm. Similarly for the optimization of pH, 1 unit of freshly grown microbial inoculum was added into MSM containing flasks adjusted to different pH values i.e., 5-9. The CP 200 ppm, was added aseptically and flasks were then placed on to the rotatory shaker 130 rpm at 30°C . After every 24 h of incubation period, microbial growth of medium was noticed at 600 nm for 6 days.

Biodegradation studies of chlorpyrifos by HPTLC and HPLC: Shake flask biodegradation studies were carried out to investigate the CP removal capabilities of isolate. One hundred milliliters of autoclaved mineral salt media (MSM) containing 150 ppm of CP was inoculated with 1 unit of NJ11 strain and incubated at 30°C on a rotatory shaker at 130 rpm shaker for 7 days. The CP containing MSM flask without culture was used as a control.

To study the CP degradation by HPTLC after 5 days of degradation 10 mL of culture was withdrawn from the MSM flasks, centrifuged at 7200 rpm for 10 min to obtain cell free medium. Residues of CP and TCP in sample were extracted from supernatant using equal volume of dichloromethane (DCM) twice. Organic layer of DCM was evaporated and finally the residues were dissolve in HPLC grade acetonitrile and spotted on Merck Silica gel 60 TLC plates. Standard of 50 ppm of TCP and CP solutions were also spotted along with sample using HPTLC pump (Camag Scientific Washington, NC) and plates were developed in solvent system with composition of hexane:chloroform:methanol (4:1:0.25, v:v:v (Modified). Compounds were visualized under UV light in TLC Scanner 4 and were analyzed.

For HPLC studies culture flasks were sampled after 2 and 5 days of incubation period to determine the residual concentration of CP and TCP system. Five milliliters of culture was withdrawn from the aqueous medium, centrifuged to obtain a cell free medium and extracted thrice with an equal volume of (DCM). After extraction, the pooled organic phase was dried over anhydrous calcium chloride and evaporated at room temperature 28±2°C. Dried residue were dissolved in HPLC grade methanol and final volume was adjusted to 5 mL for HPLC analysis. The extracted samples were analyzed by using isocratic HPLC, Waters 51, HPLC pump 2489 (Switzerland) equipped with dual absorbance (2489) U.V. detector, using C18 column (Ascentis 3 µm, 4.6 mm×15 cm). The isocratic mobile phase was composed of a mixture of methanol:water (85:15, v:v), which was pumped through the column at a flow rate of 1 mL min^{-1}.

Identification of expected CP metabolites by chemical test analysis: For the identification of metabolites generated upon CP and TCP mineralization, chemical tests were carried out as a preliminary approach following the standard protocols. The CP degradation pathway proposed by Singh and Walker[5] discussed the generation of various intermediate entities with different functional groups. Sample containing residues of CP metabolites was removed from the MSM culture flasks (as discussed in HPLC section), centrifuged and processed with standard procedures for the identification as described by Mann and Saunders[28]. For the detection of carboxylic acid group litmus paper test, sodium bicarbonate test, sodium hydroxide test and ferric chloride test were performed. For alcoholic group identification, ester test and iodoform test were performed. For the identification of compounds with aldehyde group-silver mirror test or tollen's reagent test, fehling's solution test, 2,4-Dinitrophenylhydrazine test were performed.

RESULTS

Isolation and characterization of bacterial isolate: Only a few bacterial species capable of degrading both CP and TCP have been listed in the scientific reports. From the enrichment culture technique, a number of bacterial stains has been isolated from pesticide contaminated soils in the lab capable of methyl parathion, chlorpyrifos etc. Using pure culture technique, colonies of one of the isolate were streaked repeatedly to obtain pure culture and subjected to biochemical and molecular identification. From biochemical depiction of isolate it was found that bacterium was Gram positive, rod shaped, positive for dextrose utilization, citrate utilization, catalase presence and motility whereas, Gram-negative for indole utilization. Comparative analysis of 16s DNA gene sequence of bacterial isolate confirmed its identity as *Bacillus subtilis* (Genbank Acession No. KM401571). Phlylogenetic tree of the isolate is given in Fig. 1.

Utilization and maximum tolerance of CP and TCP: Distinct colonies were visualized on MSM agar plates supplemented with CP and TCP (100 ppm) in 24 h fig 2. This observation evaluated the capability of the isolate to utilize both compounds within short time period. Efficacy of the bacteria to tolerate higher concentrations of CP and TCP was checked further. Bacterium was observed to tolerate CP up to 800 ppm as bacterial colonies were appeared with in 48 h of incubation. Growth of bacterium was inhibited by CP concentrations higher than 800 ppm. Similarly in case of TCP, bacteria with stand up to 200 ppm concentration with in 48 h. Growth profile of the isolate was studied by preparing its growth curve as given in Fig 3. In the nutrient broth where CP was not supplemented additionally, it was found that post 9 h of incubation, bacterium attained a log phase followed it up to 15 h and then and entered in to a stationary phase. In other set where in nutrient broth, 100 ppm CP was added, culture attained log phase after 15 h of incubation managed it up to 20 h and then followed a stationary phase up to 60 h. This reveals that isolate remain in its metabolically active state within 10-20 h. It was found that nutrient broth containing CP as an inducer do not imparts negative effects on the growth of isolate Fig. 3.

Effect of pH and Temperature on the growth of isolate: The pH and temperature are the two main important factors that influence the growth of microorganisms and also the degradation of xenobiotic compounds. Growth of isolate was studied for 5 days in sterilized MSM supplement with 200 ppm chlorpyrifos maintained at different temperature (25-40°C) and pH (5-9) conditions. Growth of isolate was found to be maximum in nearly neutral pH conditions and optimal value was recorded as 8. However, acidic and highly basic pH conditions less usage of CP was observed which corresponds to less growth. It has been also found that growth of microbe was well supported in temperature range between 30-37°C. Growth at other temperature conditions i.e., 25 and 40°C was comparatively low but not supposed to impart extreme inhibitory effects on microbial growth. The effect of temperature and pH on utilization of CP by the isolate is shown in Fig. 4a and b, respectively.

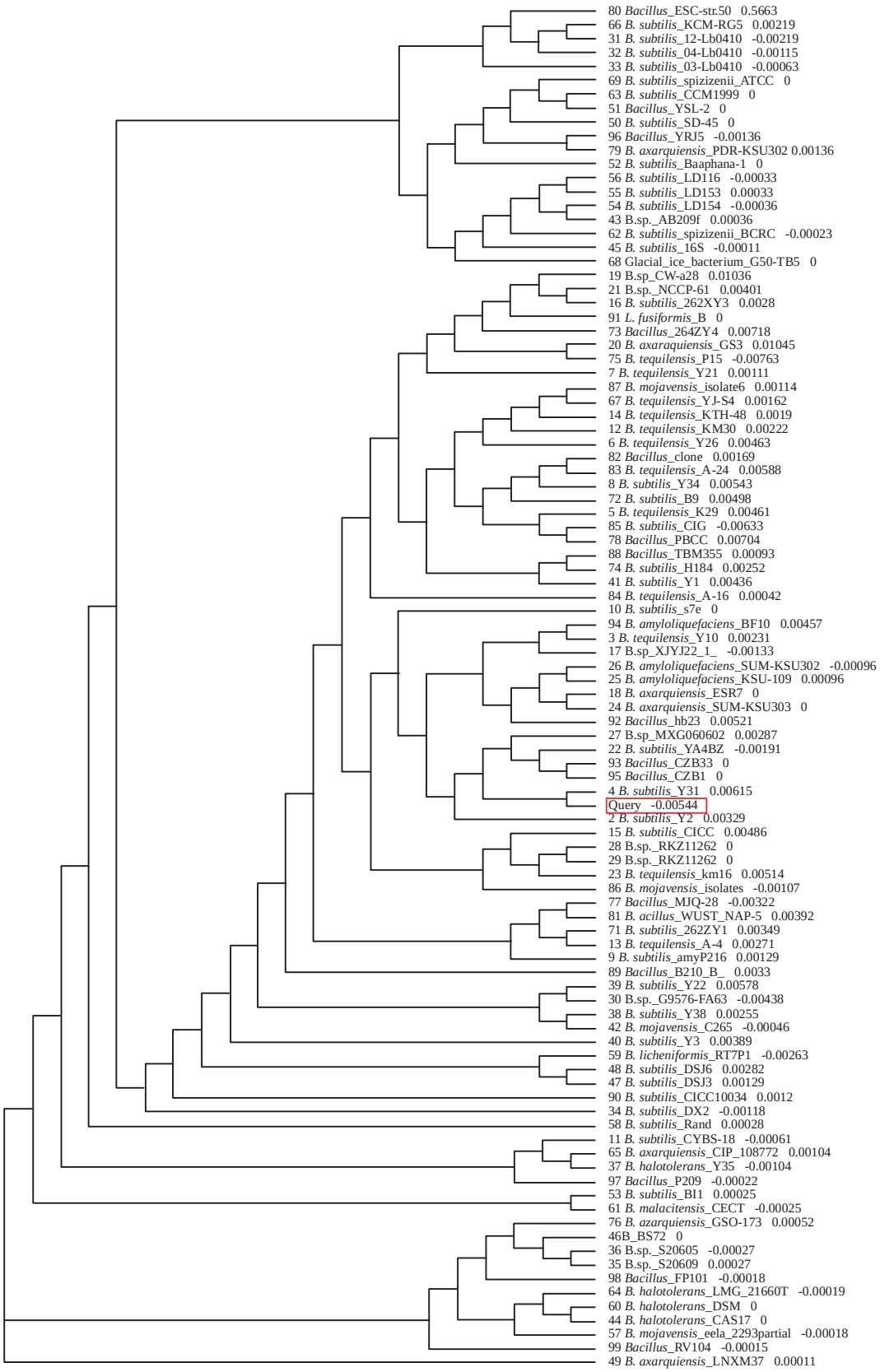

Fig. 1: Phylogenetic analysis of 16s rRNA sequence of *Bacillus subtilis* NJ11

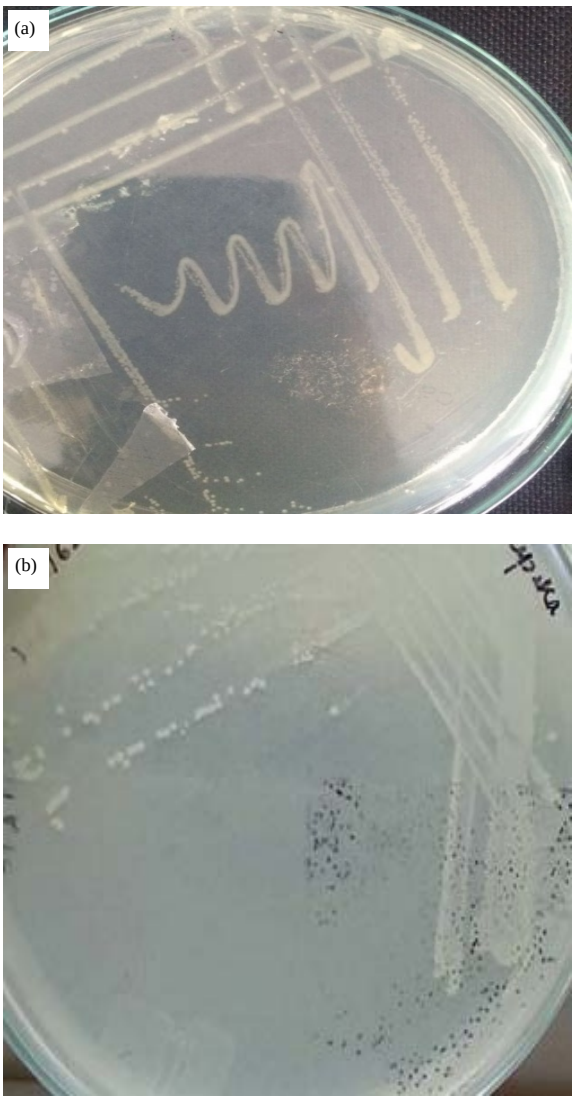

Fig. 2(a-b): *Bacillus subtilis* growth on MSM agar plates supplemented with (a) CP (a) and (b) TCP

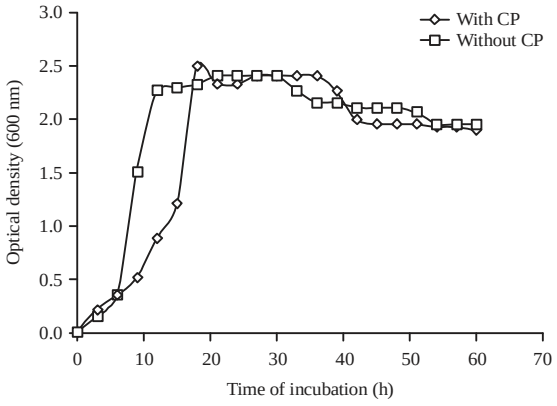

Fig. 3: Growth profile of isolate in nutrient broth

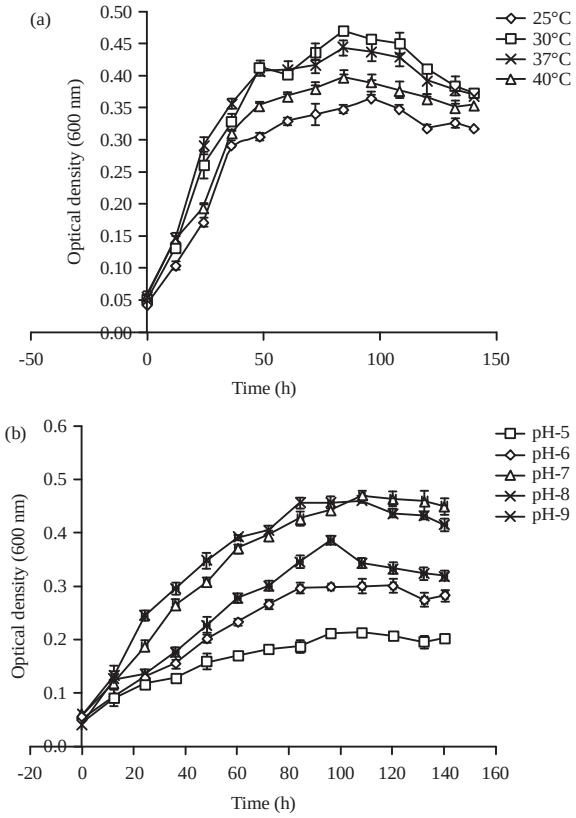

Fig. 4(a-b): Growth pattern of isolate NJ11 in MSM containing CP in different (a) Temperatures and (b) pH values
Bars mentioning Standard deviation

Qualitative analysis of CP and TCP utilization employing tetrazolium reduction assay and silver nitrate assay: The tetrazolium chloride reduction test was carried out to assess the biochemical oxidation of chlorpyrifos by bacterium. It was found that NJ11 strain was able to oxidize CP in the minimal salt medium. As soon as isolate starts degrading CP, tetrazolium chloride act as an artificial electron acceptor reduced by dehydrogenase of bacteria and gets converted in to a highly colored product formazon. Generation of this highly red colored product supports the CP biodegradation potential of isolate qualitatively. No color development was observed in the control run which suggest that CP remain in intact form in un-inoculated medium. Bacterial isolate was also checked for its capability to degrade TCP simultaneously using silver nitrate titration method. Upon utilization of TCP by isolate in MSM, chloride ions were supposed to released. Titration of the sample containing chloride ions with standard silver nitrate solution produce reddish brown color precipitates supporting the TCP degradation capability of the isolate. No color was appeared in control run.

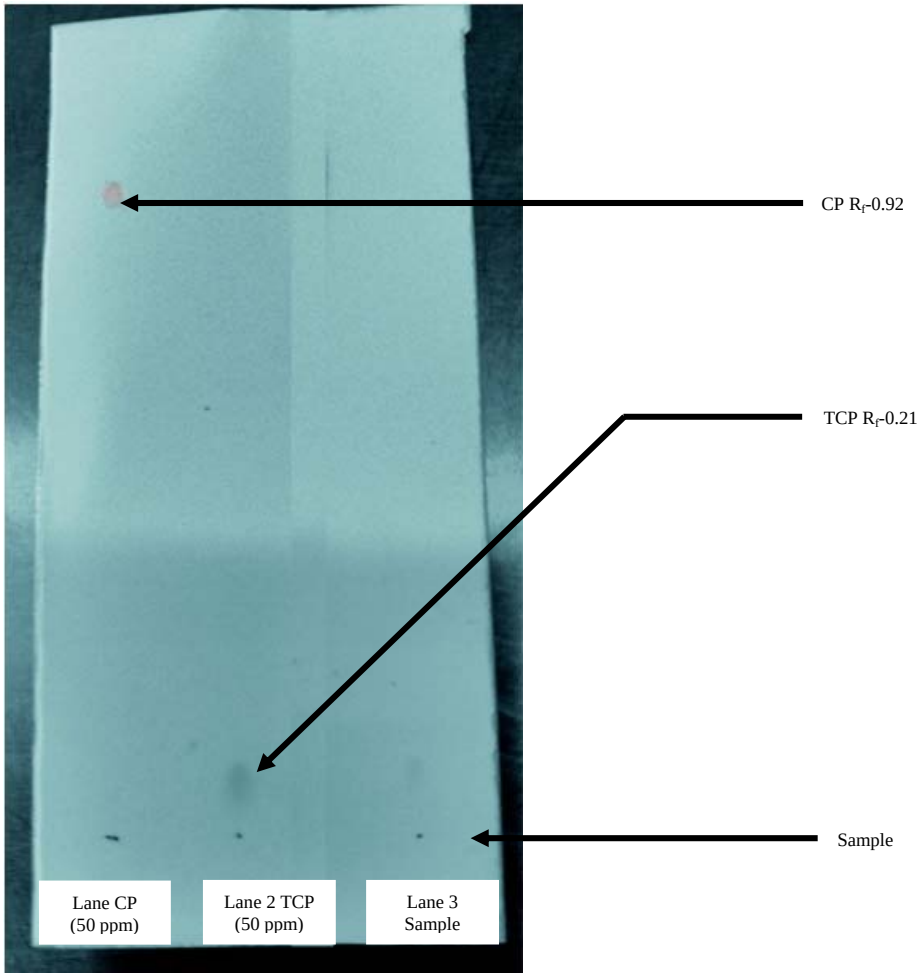

Fig. 5: HPLTC profile of standard CP, TCP and Sample

Degradation studies of chlorpyrifos by the isolate in MSM liquid medium by HPTLC and HPLC: Degradation dynamics of CP and TCP were studied in liquid culture medium inoculated with strain NJ11. Bacterial isolate was capable to mineralize 150 ppm CP completely within five days of degradation progression and negligible amount of TCP accumulation was observed in the system. Biodegradation studies by HPTLC suggest the complete removal of the CP from the liquid medium as no spot of CP or TCP was appeared in the sample when compared to standard CP and TCP HPTLC profiles. The R_f values recorded for standard CP and TCP were 0.92 and 0.21, respectively (Fig. 5). A number of modified solvent systems were developed for HPTLC studies although in the present communication authors are mentioning only one i.e., hexane: chloroform:methanol (4:1:0.25). Being more sensitive then HPTLC, HPLC studies reveals 39% degradation after two days which was elevated up to 95% after the completion of five days. HPLC chromatograms of the standards of both CP and TCP with their retention time, 7.2 and 1.6 min are given in Fig. 6a and b, respectively. A very small peak of CP and negligible amount of TCP was observed in sample after five days of degradation. In the control flasks, CP degradation, as a result of a biotic factors was negligible and insignificant (5%). Percent degradation of CP was calculated from the standard plot of CP and TCP concentration. Growth of bacteria in the medium was also monitored up to a period 7 days of incubation and it was observed that after 6 days of incubation culture attained the stationary phase (Fig. 7). The CP degradation was found to be directly proportional to the increase in bacterial growth with a time dependent loss of CP in MSM medium.

Characterization of functional groups of metabolites produced by chemical methods: Four chemical tests were performed to check the presence of carboxylic group in the sample. Litmus paper test was found to be negative.

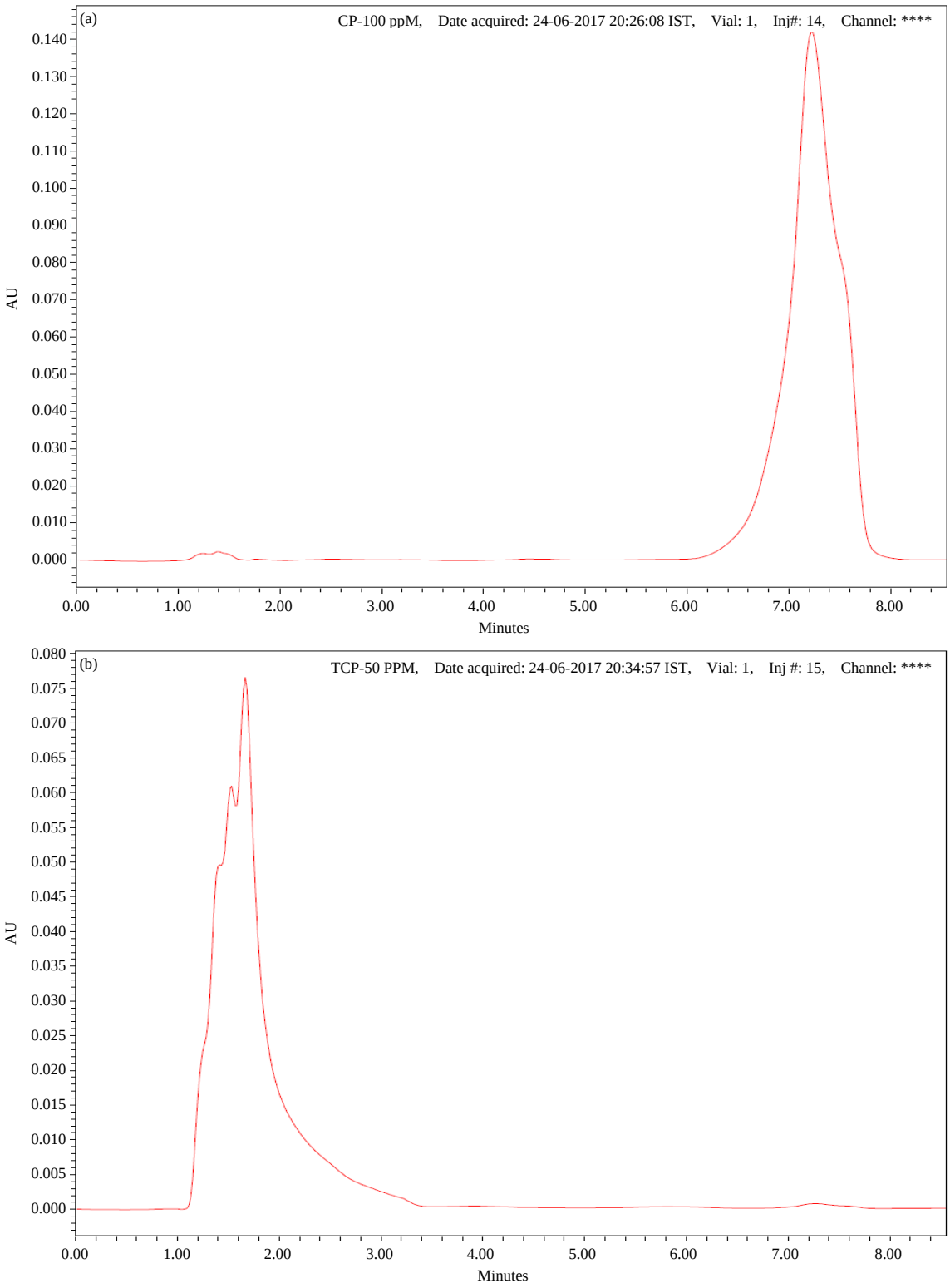

Fig. 6(a-b): Chromatogram of CP (a) Retention time-7.2 min and (b) Retention time-1.6 min

Sodium bicarbonate test was found to be positive as on the addition of 5% solution of $NaHCO_3$ to the sample, brisk effervescence was observed. Sodium hydroxide and ferric chloride test were also found to positive and gave conformance of the presence of carboxylic group.

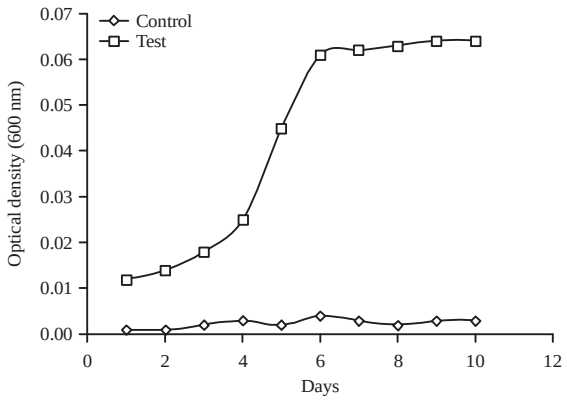

Fig. 7: Growth pattern of bacteria in MSM supplemented with 150 ppm CP

Table 1: Identification of the metabolites generated upon CP degradation

Test	Results	Inference
Silver mirror test	+ve	Aldehyde group present
2,4-Dinitrophenylhydrazine test	+ve	
Fehling's solution test	+ve	
Ester test	+ve	Alcohol group present
Iodoform test	+ve	
Litmus paper	-ve	Carboxylic group present
Sodium hydroxide test	+ve	
Ferric chloride test	+ve	
Sodium hydrogen carbonate	+ve	

For the detection of alcoholic group two tests were performed. Ester test was found to be positive with the appearance of fruity odor and iodoform test also gave positive result. For the detection of aldehyde group three tests were performed. Silver mirror and 2,4-Dinitrophenylhydrazine test gave positive inference. In Fehling's solution test, formation of red colored precipitates also indicate the presence aldehyde group in the sample. Summary of the results of all the tests is given in Table 1.

DISCUSSION

The current study describes the isolation and characterization of a proficient bacterial strain *Bacillus subtilis* NJ11 from pesticide contaminated soils of Malwa region of Punjab capable of degrading methyl parathion, chlorpyrifos and TCP. Isolation site becomes contaminated because of continuous exposure to CP and other pesticides over an long duration of time. This led to the evolution of metabolic pathways in micro-organisms to adapt in to the pesticide contaminated environments and use them. Microorganisms due to repeated encounter of synthetic toxic chemicals become resistant and develop capabilities to degrade these compounds with rapid remediation efficiency of contaminated sites. The CP has been used continuously continuous worldwide for almost past 50 years world widely[5]. The earlier pioneer in CP degradation studies, isolated six chlorpyrifos-degrading bacteria from an Australian soil showing enhanced degradation of chlorpyrifos. The first CP-degrading bacterium, *Enterobacter* B-14, isolated by them hydrolyzed the CP into diethylthiophosphate (DETP) and TCP and utilized DETP for growth. Although till now many bacterial strains has been reported in the literature showing the capability to degrade CP. However, there is scarcity of reports in scientific community, mentioning the CP and TCP removal efficacies of *Bacillus subtilis*. Degradation studies of 50 ppm CP was carried out using *Bacillus subtilis* and after 10 and 30 days of incubation 56 and 85% of degradation was recorded simultaneously[29]. Isolation and characterization of a *Bacillus subtilis* strain Y242 from agricultural wastewater was done in Egypt[30]. Isolate degraded over 95% of CP within 48 h but accumulation of toxic a metabolite i.e., 3,5,6-Trichloro-2-methoxypyridine (TMP) in the liquid medium was observed[31]. In both the studies no observation was made regarding complete utilization of CP and TCP by the strains in to non toxic metabolites. Also there was no efforts were made to study the individual CP and TCP utilization by isolates.

From best of knowledge there is no report available in the literature demonstrating the potential of *Bacillus subtilis* in the degradation of CP, TCP and methyl parathion individually. It was found that isolate has the potential to remediate chlorpyrifos contaminated sites in to the safer levels. Two significant colorimetric methods were also developed to investigate CP and TCP usage by microbial cultures qualitatively. These methods are robust, less time consuming, cost effective and are the good alternative to analyze CP and TCP utilization by microbe on preliminary basis. Test organism offered positive result for the terazolium reduction test by reducing tetrazolium chloride into reddish-brown-purple colored product upon the bio mineralization of CP. Bioremediation of another chemical entity, p-nitrophenol (degradation product of methyl parathion) was also by studied using the same principle[27]. It was found that the isolate, *Pseudomonas aeuroginosa* utilized the compound and appearance of reddish color was reported. Intensity of color was found to be directly related to the amount of chemical substrate degraded and maximum color intensity was recorded when all the carbons in culture medium exhausted. The TCP utilization by strain by silver nitrate assay also give positive results.

Further biodegradation potential of strain was studied by HPTLC. Two solvents system were developed and were used as a mobile phase in the chromatographic studies to investigate the CP removal on qualitative basis. The TLC plates shows no visible spot for both CP and TCP in sample when compared with the TLC profile for standards of CP and TCP which indicates the complete mineralization of CP. The HPTLC system standardized in the lab was found to detect upto 20 and 10 ppm of CP and TCP, respectively. The TLC studies of CP were also investigated by Bhagobaty and Malik[27] employing different mobile phase. Also from the HPLC studies it was found that bacterium was able to degrade 150 ppm CP almost completely in the liquid medium without accumulation of TCP. It was observed and inferred that bacterium possess the capability to degrade both CP and TCP simultaneously in MSM liquid medium and proliferation the strain was unaffected by. There are only few reports accessible in the scientific writings related to the isolation and characterization of *Bacillus* sp. capable of degrading of CP and its antibacterial intermediate TCP. Bacterium was able to tolerate and grow in MSM agar plates supplemented with 800 ppm CP and 200 ppm TCP concentration respectively. Present results were in contrast to some previous studies which suggested that higher concentrations of these compounds impose inhibitory effects on bacterial growth[32]. The available literature reveals that most of the bacterial species could tolerate CP from 15-300 ppm[12]. Due to the repeated exposure of CP, NJ11 strain may have evolved the capability to tolerate such higher concentrations.

Notably, abiotic factors like pH and temperature greatly influence the capability of microorganisms to degrade such xenobiotic compounds and different bacterial strains possess varying optimal values of temperature and pH[25]. However, in present work bacterial isolate was able to grow temperature range 25-40 and at 30°C bacterial growth was most efficient. Thus all degradation experiments during present study were carried out at 30°C. Enhanced growth of isolate was recorded at neutral and slightly basic conditions whereas optimum pH was 8. This is due to the fact that main enzyme (s) responsible for chlorpyrifos degradation have their maximum activity at neutral pH whereas highly alkaline and acidic pH values impose inhibitory effects[12]. Rate of biodegradation of pesticide in alkaline conditions proves to be more efficient as compare to acidic conditions[22,33].

In the present study experiments were performed to detect the metabolites generated upon CP degradation and results were analyzed. Proposed CP degradation pathway also supports outcomes of the present study[5]. Expected metabolites produced upon CP and TCP mineralization must be alcoholic compounds, aldehyde group containing Compounds, acidic groups containing compounds. Taking this in to consideration, sample was withdrawn from the degradation system and processed with standard protocols. Litmus paper test for carboxylic group was found to be negative which is due to less sensitivity of this test then other tests. Results are concluded in Table 1 and confirm the presence of acidic group, alcoholic group and aldehyde group in the sample. These out comes suggested the fact that the bacterium mineralize CP and TCP in to lesser toxic metabolites.

CONCLUSION AND FUTURE RECOMMENDATIONS

A chlorpyrifos (CP) and 3,5,6-Trichloro-2-pyridinol (TCP)-degrading bacterium NJII that observed to be more efficient than other reported *Bacillus subtilis* was isolated, characterized and studied for the mineralization of CP and TCP. Strain NJ11 is capable of tolerate and proliferate at wide range of initial CP concentration, temperature and pH conditions. Isolate was able to tolerate as high as 800 ppm of CP concentration and convert CP in to lesser toxic metabolites. Two new colorimetric methods were also developed to study CP and TCP utilization efficacies of isolate. Bacterial isolate can prevent the accumulation of CP and its toxic metabolites in soil, water and in other various environmental sites thus minimizing supposed adverse toxicological effects on other non-targeted populations can be minimized.

Further research is necessary to explore more soil micro biota having CP and TCP utilization capability and exhaustive investigation of the metabolic pathway of CP degradation. The state and centre governments should come forward to support field trials with such microbial populations to reduce pesticide load in soils, water and other ecosystems.

SIGNIFICANCE STATEMENTS

This study discovered a bacterial strain that has been isolated from the pesticide contaminated soils of Punjab, (India). Bacterial isolate was identified as *Bacillus subtilis* and was found to degrade CP (chlorpyrifos) and its toxic metabolite TCP (3,5,6-Trichloro-2-pyridinol) completely within five days without the accumulation any toxic intermediate that can be beneficial to clean CP contaminated sites. *Bacillus subtilis* NJ11 isolate was found to be more efficient than then other reported *Bacillus subtilis* strains engaged in CP degradation. There is no report available in the scientific community demonstrating the potential of *Bacillus subtilis* in the degradation of methyl parathion, CP, TCP in to non-toxic forms. This study will help the researchers to explore the

potential of such type isolates to clean up the soil, water and various CP contaminated environmental sites thus minimizing the adverse toxicological effects of CP on various life forms specifically in humans.

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