RESEARCH PAPER

# From Field to Lab: Sequential Screening and Molecular Characterization of High-Efficiency Chlorpyrifos-Degrading Bacteria

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**ABSTRACT**

Chlorpyrifos (CP) remains a global environmental concern due to its persistence and potent acetylcholinesterase inhibition, worsened by accumulation of its metabolite, 3,5,6-trichloro-2-pyridinol (TCP). This study presents a comprehensive bioremediation framework, commenced with a thorough physicochemical analysis of contaminated soil to evaluate the environmental parameters affecting pesticide sequestration. A dual phase microbial screening approach was used, comprising cumulative primary qualitative screening via enrichment culture technique and secondary quantitative study to isolate highly efficient CP degrading bacteria from agricultural soil. Preliminary qualitative assessment screened 18 isolates capable of utilizing CP as sole carbon source, while subsequent quantitative screening involving degradation assay was performed using U.V spectrophotometer which narrowed the selection of the most kinetically proficient bacterial strain; CP 15. A detailed biochemical characterization illustrates the metabolic profile of the isolates, while molecular characterization via 16S rDNA and phylogenetic assessment confirmed their taxonomic position (Bacillus cereus CP15). By correlating soil physicochemical properties with microbial adaptability and efficiency, this research offers a validated, high-efficiency bioaugmentation framework for systematic detoxification of organophosphate contaminated agricultural matrices.

**Keywords:** Biodegradation, Chlorpyrifos, Physico-chemical properties.

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## 1. Introduction

Pesticides are vital part of today's agricultural setup as it is widely used for pest control. Annually, 5.6 billion pounds of pesticide is used worldwide[1]. Low feasibility of close system application of pesticide has been a major concern as this xenobiotic transmitted from soil ecosystem to other ecosystems via environmental route[2]. Only 0.1% of applied dose of pesticide act upon target pest while the residual enters the environmental settings causing eco-toxicity and major threat to biodiversity[3]. CP chemically designated as O, O-diethyl-O- (3,5,6-trichloro-2-pyridinyl)-phosphorothioate, is a potent broad-spectrum organophosphate pesticide (OPP) that constitutes of 36% of total pesticide use on global scale[4]. The broad-spectrum applicability makes it xenobiotic of interest as it they present threat to non-target organisms. Despite recent stringent regulations and bans in several developed countries such as European Union and Northern America, CP remains one of the most widely used pesticides in developing nations to administer soil and foliar pest. Unprecedently and unrestricted used of CP by farmers can threaten environmental integrity by contaminating air, water and soil and risks to human health inclusively neurotoxicological impacts[5]. The chemical characteristics of CP are largely determined by low water solubility (approx. 1.4-2 mg/L) and high lipophilicity (log P≈4.7-5.0), which permits strong adsorption to soil organic matter (OM)[6]. Moderate environmental persistence in

soil(60-120days) can be reasoned with the adsorption potential of this xenobiotic. Though it's persistence might extend upto a year depending on soil type and climatic conditions[7].

Various methods have been explored for remediation of CP such as *ultrasonic treatment*[8], *biochar adsorption*[9], *titanium dioxide photocatalysis*[10] and *synthetic nanocomposites*[11]. All these Chemical and physical methods for eliminating pesticide residue are prone to introducing secondary water soluble and toxic intermediates to environment[12]. Therefore, necessitates utilization of modern yet innovative techniques over traditional approach of removing CP residues from environment. Bioremediation has been considered as most reliable, feasible, sustainable and cost-effective method for pesticide detoxification. Soil microflora, particularly bacteria possess metabolic plasticity to adapt and utilize pesticide as source of carbon, nitrogen and phosphorus. Previous researches are evident that native microbial isolates are more effective than exogenous culture owing to their bio-acclimatization to the environmental stress particularly pesticide.

While numerous microbial isolates have been documented in literature, there remain a need to isolate novel indigenous strains with superior degradation kinetics and ability to tolerate high concentration of pesticide. Furthermore, a polyphasic approach of combining morphological, biochemical and molecular characterization is essential to fully understand the metabolic capabilities and taxonomic diversity of isolates. Therefore, this study aims to isolate potent CP-degrading bacteria from soil having history of pesticide application. The specific objectives are to screen these isolates for primary and secondary degradation efficacy and characterize them through biochemical profiling and confirming their identity using 16S rRNA sequencing. This research contributes to development of microbial consortia for restoration of pesticide contaminates agro-ecosystems.

## 2. Material and Methods

### 2.1 Chemicals and glassware

The chemical used understudy were of analytical grade and sourced from Hi-media to ensure high experimental precision. Experiment was conducted in pre-washed and pre-sterilized borosilicate glassware.

### 2.2 CP Stock solution

For scope of study commercial grade CP (20%EC) was used. To prepare 1000ml of 1000ppm CP stock solution 5ml of 20%EC formulation was dissolved in 95ml acetone. The volume was raised upto 1000ml by adding distilled water. The final stock was filtered through 0.22µm syringe filter to ensure sterility and preventing thermal degradation of pesticide. The master stock was then stored in dark and amber colored borosilicates glass bottle at 4°C to prevent photodegradation.

### 2.3 Soil sampling and site description

Soil samples were collected from top layer (0-15cm) of agricultural field from Ambala, Haryana having a long-term history of CP application. Samples were collected in sterile plastic bags, transported to laboratory under cold conditions (4°C) and sieved through a 2mm mess to remove debris before immediate processing[13].

### 2.4 Soil physicochemical properties

The soil physiochemical properties were characterized using Indian standard (IS) methodologies and laboratory standard procedures (SOPs). Total carbon and organic matter were assessed using the chromic acid wet oxidation method[14], pH (electrometric)[15] and electric conductivity (conductimetric)[16] at 25 °C were determined. The hydrometer method[17] was used to quantify the soil textural classes (sand, clay, and silt). Essential macronutrients—nitrogen, phosphorus, and potassium—while normal Bureau of Indian Standards (BIS)[17] protocols were employed to analyse broader ionic profiles, inclusive of alkanity, water holding capacity, sulphate, nitrate, phosphate, sodium and nitrite. Atomic Absorption Spectroscopy (AAS) was used for quantification analysis of metals (chromium, copper, nickel, and zinc) as it offers high susceptibility for heavy metal load[18]. Pesticide residual analysis (PRA) was done using GC-MS following QuEChERS extraction, with chemical characterization and quantification by mass spectral libraries and matrix-match standards.

### 2.5 Enrichment culture technique for bacterial isolation

Selective enrichment was performed using minimal salt media (MSM). The MSM was composed of (g/L): $K_2HPO_4$ (1.5), $KH_2PO_4$ (0.68), $NH_4NO_3$ (0.2), $MgSO_4.7H_2O$ (0.2), NaCl (4.0), $FeSO_4$ (0.02) and $CaCl_2$ (0.1) with pH adjusted to 7. 1gm of soil was inoculated into 99ml MSM supplemented with 100ppm of CP as sole carbon source. Flask without CP was considered as control.

Flasks were incubated on rotary shaker (150rpm) at 30-37℃ for 7 days. Bacterial growth was measured considering medium turbidity in terms of optical density ($O.D_{600}$) using U.V. spectrophotometer. Enriched cultures were serially diluted upto $10^{-8}$. From each dilution 0.1ml alot of culture was spread on MSM agar plate containing 100ppm of CP as sole carbon source. MSM agar deprived of CP was considered as control. Inoculates plates were incubated at 30-37℃ for 7 days. Distinct colonies were considered as potential degraders and further streaked to obtain pure culture.

**2.6 Multi-step screening process**

**2.6.1 Primary screening: Qualitative degradation on MSM agar**

The maximum tolerance concentration (MTC) is determined by primary streaking of adapted isolates onto MSM agar plates supplemented with increasing concentration of CP (100-500ppm). MSM agar plate without CP served as control. All the plates were incubated at 30-37℃ for 7 days. Plates were observed for growth to identify strains capable of tolerating/ surviving high pesticide concentration.

**2.6.2 Standard curve of CP**

For quantitative determination of CP, a standard calibration curve was establish using a UV-Vis-spectrophotometer by measuring the absorbance of known of known concentration at maximum absorption wavelength ($\lambda_{max}$) at 290nm. Working standards ranging from 20-100 mg/L was made using master stock of 1000 mg/L using dilution approach. The absorbance values were plotted against their respective concentration to generate a linear regression equation equation, $y = mx + c$.

**2.6.3 Secondary screening: Quantitative degradation efficiency in Minimal Salt Media**

All potent bacterial strains were cultured using MSM broth and incubated at 30-37℃ until they reach exponential phase. The pellet was harvested using centrifuge at 6000rpm for 10 min followed by successive washing using 1 M phosphate buffer and standardized for OD=0.5 at 600nm to ensure uniform initial cell density. Standardized inocula (1%v/v) was added to 250 ml Erlenmeyer flask containing 100ml MSM broth supplemented with 100ppm of CP followed by standard incubation. Pesticide degradation was observed by measuring absorbance at λ=290nm using U.V. spectrophotometer. CP degradation was calculated using standard curve of CP. The equation used was:

$$\text{Degradation (\%)} = \frac{\text{Initial Concentration} - \text{Final Concentration}}{\text{Initial Concentration}} \times 100$$

**2.7 Morphological and biochemical characterization**

Bergey's manual of systematic bacteriology was used to characterize the isolates. Morphological characteristics including colony character such as elevation and margins and cell morphology via Gram's staining[19] were studied. Standard biochemical tests including catalase, oxidase, indole, methyl red, Voges-Proskauer (IMViC) and citrate utilization were carried out for most efficient bacterial strains[20].

**2.8 Molecular identification and phylogenetic analysis**

Genomic DNA was extracted from freshly prepared cultures using DNA extraction kit. The gene was amplified via PCR using universal primers 27F(5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5′-GGTTACCTTGTTACGACTT-3′). Purified PCR product was sequenced sing Sanger's method. Sequences were analysed using the NCBI BLAST tool to determine similarity with known species and MEGA X software was employed to construct phylogenetic tree[21].

**3. Result and Discussion**

**3.1 Soil Physico-chemical profile**

Soil physicochemical properties are vital drivers of CP degradation kinetics, with pH and organic matter (OM) acts as substantial regulatory factors. The analysed soil samples exhibit a near neutral range of pH (7.02-7.11). While it is a well-documented fact that alkaline pH (7.5-8.4) typically accelerates abiotic chemical hydrolysis and enhanced microbial catabolism whereas acidic conditions are associated with extended persistence, often leads to half-life exceeding 256 days[22]. Our findings were indicative of low OM content (0.25-0.34% mass/mass) which is a known to affect the bio-availability of xenobiotic as higher OM tends to sequester CP owing to hydrophobic adsorption (Koc values upto 10,000 L/kg)[23]. A study[24] was suggestive of stimulated effect of organic amendments on CP degradation by enriching microbial biomass and diversity. NPK content of soil stimulates the enrichment of biomass. However, the presence of CP residues can lead to a transit decline in availability of nutrients to plant which can be reasoned with *microbial immobilization* and *enzyme inhibitory*

effect. Our samples exhibit high sulphate levels (32-35 mg/Kg) which is likely to play a catalytical role. A study by[25] suggested a negative correlation between soil sulphate content and pesticide persistence and their finding were evident of elevated sulphate content may promoted rapid *mineralization*.

GC-MS analysis to assess residual pesticide reveal history of multi-pesticide application in experimental soil (Table 1). The diverse residue profile confirms the environmental persistence of varied organophosphates and organochlorines, consistent with recent global monitoring efforts. The detection of these compounds validates the transmission of xenobiotics via eco-routes[26], where leaching mechanism facilitates sub-surface bioaccumulation[27], inadvertently posing a severe risk to both pedospheric health and groundwater reservoirs.

**Table: 1** Pedological and chemical profile of control and experimental soil matrices.

| S. No. | Parameter | Test method | Control soil | Experimental soil | Unit |
|---|---|---|---|---|---|
| 1 | **pH@25°C** | IS:2720-26 | 7.11 | 7.03 | - |
| 2 | **Electrical conductivity @25 °C** | IS:2720-21 | 344 | 566 | µS/cm |
| 3 | **Alkalinity** | ITS/LAB/SOP/SOIL | 92 | 116 | Mg/Kg |
| 4 | **Organic matter** | IS:2720-22 | 0.25 | 0.34 | % by mass |
| 5 | **Total carbon** | IS:2720-22 | 0.19 | 0.25 | % by mass |
| 6 | **Water holding capacity** | ITS/LAB/SOP/SOIL | 36 | 26.7 | % by mass |
| 7 | **Sand** | IS:2720-4 | 23 | 43.4 | % by mass |
| 8 | **Clay** | IS:2720-4 | 47 | 25.0 | % by mass |
| 9 | **Silt** | IS:2720-4 | 28 | 21.8 | % by mass |
| 10 | **Texture** | IS:2720-4 | Loamy | Sandy loamy | |
| 11 | **Sulphate** | ITS/LAB/SOP/SOIL | 35 | 32 | Mg/Kg |
| 12 | **Phosphate** | ITS/LAB/SOP/SOIL | 16 | 22 | Mg/Kg |
| 13 | **Sodium (as Na)** | ITS/LAB/SOP/SOIL | 44 | 65 | Mg/Kg |
| 14 | **Nitrite** | ITS/LAB/SOP/SOIL | 0.9 | 1.2 | Mg/Kg |
| 15 | **Nitrate** | ITS/LAB/SOP/SOIL | 43 | 32 | Mg/Kg |
| 16 | **Available Nitrogen** | IS:10158 | 344 | 520 | Kg/hac |
| 17 | **Phosphorus (as P)** | IS:10158 | 13 | 19 | Kg/hac |
| 18 | **Potassium (as K)** | IS:10158 | 144 | 160 | Kg/hac |
| 19 | **Chromium** | AAS | 15 | 22 | Mg/Kg |
| 20 | **Copper** | AAS | 57 | 115 | Mg/Kg |
| 21 | **Nickel** | AAS | 24 | 23 | Mg/Kg |
| 22 | **Zinc** | AAS | 6 | 3 | Mg/Kg |

| | | | | | |
|---|---|---|---|---|---|
| 23 | **DDT** | GC-MS | 0.018 | 0.02 | Mg/Kg |
| 24 | **Chlorpyrifos** | GC-MS | ND | 1.244 | Mg/Kg |
| 25 | **Alderin** | GC-MS | ND | 0.938 | Mg/Kg |
| 26 | **Furadan** | GC-MS | ND | ND | Mg/Kg |
| 27 | **Parathion** | GC-MS | ND | 0.124 | Mg/Kg |

### 3.2 Enrichment and isolation of CP-degrading bacteria

The metabolic capability of indigenous microflora to utilize CP as sole carbon and energy source was evidenced by progressive increase in turbidity ($O.D_{600}$) within the MSM broth after 7day incubation period. This sustained logarithmic phase across all experimental replicates confirm that the chemical structure of CP – despite its chlorinated pyridinyl ring- serves as a viable substrate for microbial catabolism. Following serial dilution and susbsequent inoculation on MSM agar 18 morphologically distinct isolates were recovered. To ensure genetic and phenotypic homogeneity, these isolates underwent rigorous repetitive sub-culturing on nutrient agar yielding axenic cultures.

### 3.3 Primary Screening: Tolerance assay

The catabolic resilience of the isolated microbial strains was systematically evaluated through an agar dilution assay across an escalating gradient of CP concentration. At 100ppm, all 19 isolates exhibit exceptional growth, assessed by rapid colony formation. These results were suggestive of the fact that the indigenous microbial community in pesticide contaminated soil has a basal metabolic readiness to use CP as a carbon source. However, a progressive increase in xenobiotic concentration reveals distinct metabolic threshold limits. As concentration reaches 500ppm, a significant drop in number of viable isolates was observed (Fig.1). This decline in survival frequency is indicative of inherent cytotoxicity of CP, which exhibits antimicrobial property at higher concentration. The qualitative growth patterns and isolate-specific tolerance levels are detailed in Table 2, highlights a selective sub-population of 12 isolates that retains metabolic activity at 500ppm threshold. These high tolerant strains represent the most promising candidate for bioaugmentation, as they can withstand the localized hotspots of high pesticide concentration often found in agricultural spill sites. The sharp decline in viable isolates at 500ppm delineates a critical physiological boundary separating opportunistic tolerators from specialized degraders. The dose dependent attrition is consistent with findings of previous findings[28].

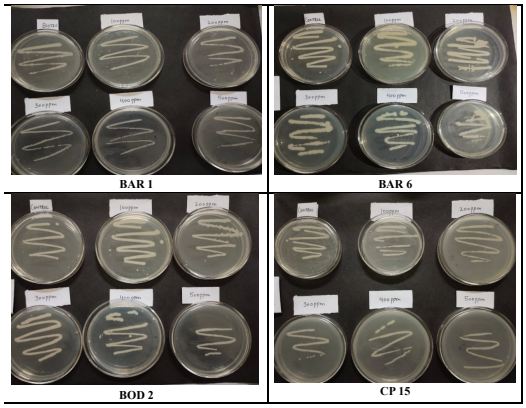


**Figure 1: Growth profile of bacterial isolates in contrast with control (no CP)**

**Table 1** Tolerance assay

| S. No. | Bacterial isolates | 100ppm | 200ppm | 300ppm | 400ppm | 500ppm |
|---|---|---|---|---|---|---|
| 1 | BAR 1 | Yes | Yes | Yes | Yes | Yes |
| 2 | BAR 2 | Yes | Yes | Yes | Yes | Yes |
| 3 | BAR 3 | Yes | Yes | Yes | Yes | Yes |
| 4 | BAR 4 | Yes | Yes | Yes | Yes | No |
| 5 | BAR 5 | Yes | Yes | Yes | Yes | No |
| 6 | BAR 6 | Yes | Yes | Yes | Yes | Yes |
| 7 | BAR7 | Yes | Yes | Yes | No | No |
| 8 | BAR 8 | Yes | Yes | Yes | Yes | Yes |
| 9 | BOD 1 | Yes | Yes | Yes | Yes | Yes |

| | | | | | | |
|---|---|---|---|---|---|---|
| 10 | BOD 2 | Yes | Yes | Yes | Yes | Yes |
| 11 | BOD 3 | Yes | Yes | Yes | Yes | Yes |
| 12 | PB-14 | Yes | Yes | Yes | No | No |
| 13 | Ni-16 | Yes | Yes | Yes | Yes | Yes |
| 14 | SCh11 | Yes | Yes | Yes | No | No |
| 15 | CP 14 | Yes | Yes | No | No | No |
| 16 | CP 15 | Yes | Yes | Yes | Yes | Yes |
| 17 | CP 16 | Yes | Yes | No | No | No |
| 18 | CP 17 | Yes | Yes | Yes | Yes | Yes |
| 19 | CP 18 | Yes | Yes | Yes | Yes | Yes |
| 20 | CP 19 | Yes | Yes | Yes | Yes | No |

### 3.4 Standardization and calibration curve of CP

The spectrophotometric analysis identified the maximum absorption wavelength ($\lambda_{max}$) at 290 nm which was subsequently utilized for all quantitative measurements. The generated calibrated curve exhibits a robust linear relationship across the test concentration of 20-100mg/L. The regression analysis yields the equation y= 0.0314x+0.0578 where y is absorbance and x is CP concentration (mg/L). A high coefficient of determination ($R^2$> 0.9991) (Fig. 2) was achieved, confirming the precision of the method and its suitability for monitoring the degradation kinetics of pesticide throughout the study.

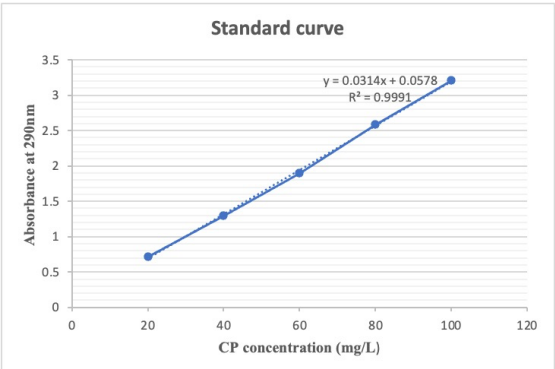


**Figure 2**: Standard calibration curve of CP at $\lambda_{max}$= 290

### 3.5 Confirmation of CP degradation using spectrophotometer

Secondary screening was performed to evaluate the biodegradation potential of the initially screened most tolerant bacterial strains. While the majority of isolate exhibits restricted proliferation and metabolic lag in liquid MSM, CP 15 demonstrate excellent metabolic resilience. Spectrophotometric analysis at $\lambda_{max}$ 290nm revealed a significant time dependent reduction in CP concentration, concomitant with a robust increase in microbial turbidity ($OD_{600}$), confirming that CP serves as the primary carbon source for cellular biosynthesis.

Among the screened candidates, isolate CP 15 emerged as most proficient degrader, achieved a peak degradation efficiency of 83.12± 0.94 within 7-day temporal window. This was followed by isolate BAR 2, which exhibited a substantial degradation rate of 82± 0.62 (Table 2) measure using the spectrophotometric results at 290 nm and standard curve equation.

**Table 2:** Degradation profile of isolates

| S.No. | Bacterial Isolates | % Degradation |
|---|---|---|
| 1. | **BAR 1** | 80± 0.57 |
| 2. | **BAR 2** | 82± 0.62 |
| 3. | **BAR 3** | 67.72± 0.72 |
| 4. | **BAR 4** | 60.06± 0.94 |
| 5. | **BAR 5** | 75.75± 0.60 |
| 6. | **BAR7** | 77.76± 0.63 |
| 7. | **BAR 8** | 61.24± 0.52 |
| 8. | **BOD 1** | 79.13± 0.75 |
| 9. | **BOD 3** | 76.89± 0.82 |
| 10. | **PB-14** | 63.72± 0.84 |
| 11. | **Ni-16** | 60.28± 0.79 |
| 12. | **CP 15** | 83.12± 0.94 |
| 13. | **CP 16** | 46.89± 0.92 |
| 14. | **CP 17** | 72.68± 0.40 |

| | | |
|---|---|---|
| 15. | **CP 18** | 64.88± 0.64 |
| 16. | **CP 19** | 75.79± 0.62 |

### 3.6 Morphological and biochemical profile of potent strains

The most efficient degrader was subjected to detailed phenotypic characterization. CP 15 produces creamish colonies. Table 3 shows the detailed biochemical profile of CP15. Catalase test was found positive for this isolate. It is a well-established fact that catalase is a key biomarker for aerobic microbes for neutralizing reactive oxygen species (*ROS*). Avinash et al. 2025 has identified catalase, decarboxylase and oxidase as key enzymatic drivers in initial stage of CP biodegradation, as they facilitate the oxidative breakdown of phosphorothioate bond.

**Table 3:** Biochemical characterization

| **Characteristics** | **CP15** |
|---|---|
| **Colony Morphology** | Creamish |
| **Gram Staining** | + |
| **Morphology** | Bacilli |
| **Catalase** | + |
| **Urease** | - |
| **Oxidase** | - |
| **Indole** | + |
| **Nitrate reduction** | - |
| **MR** | - |
| **VP** | - |

### 3.7 Molecular characterization and phylogenetic analysis

The 16S rRNA gene sequencing used to identify the bacterial isolate CP 15 demonstrates its tight evolutionary relationship and high resemblance to the *Bacillus cereus* group (Fig. 3). With 100% query coverage and 99.61% sequence identity to several strains, including *Bacillus cereus*, *B. paramycoides*, and *B. tropicus*, the Nucleotide BLAST analysis demonstrated an extraordinarily good match. Further supporting these findings is the phylogenetic tree, which shows that the isolate (particularly identified as *Bacillus cereus* CP15 forms a unique clade that distinguishes it from more distant *Bacillus* species by clustering most closely with *B. paramycoides* (MT256206.1). Although the species-level BLAST findings show strong sequence similarity, the phylogenetic analysis confirms its taxonomic placement within this intricate and tightly linked group of bacteria by offering a more thorough visual depiction of its evolutionary history.

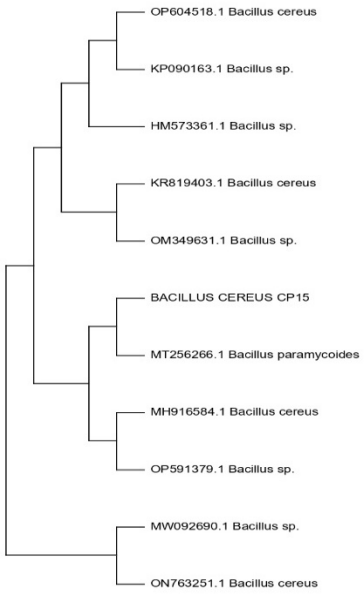


**Figure 3:** Phylogenetic tree

### 3.5 Conclusion

This study provides a comprehensive assessment of bioremediation potential of indigenous soil microflora in chlorpyrifos contaminated environment. Our findings demonstrate the soil physicochemical particularly pH, organic content and NPK content, significantly influence the bioavailability and subsequent degradation kinetics of CP. Through a systemic primary qualitative screening we identified 20 isolates exhibiting high pesticide tolerance, with subsequent quantitative secondary screening revealed a maximum degradation efficiency of CP 15 (83.12± 0.94) at initial concentration of 100mg/L within 7 days. The biochemical characterization of these superior strains indicates the production of catalase which help in initial steps of CP biodegradation. Furthermore, the biochemical characterization via 16S rDNA technology identify CP 15 as *Bacillus cereus* CP 15 confirming their phylogeny and special niche adaptation. It also suggests a specialized evolutionary adaptation to pesticide-stress niches.

Collectively, these results suggests that the integration of site-specific microbial isolates with optimized soil conditions offers a scalable and eco-strategy for *in-situ* detoxification of organophosphate-polluted agricultural soils. This

research provides a robust framework from laboratory scale *proof-of-concept* to viable field application. Future research should be focused on the expression of functional degradation genes under varying field stress to ensure the long-term efficacy of bio-augmentation strategies.

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