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Screening and molecular identification of monocrotophos degrading bacterial isolates.

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Abstract
The unregulated use of organophosphate group of pesticides leads to severe pollution of the soil, water and food chain due to its long term persistent nature. Monocrotophos is one of the majorly used pesticides of this group. The use of microorganisms is one of the best ways to degrade the monocrotophos. The soil samples were gathered from various agricultural fields located in Haryana for this research. The different bacterial strains were isolated from these soil samples using enrichment method. The degradation of the pesticide was confirmed using HPLC analysis. Among all these strains three bacterial strains namely, *Bacillus weidmannii* (HR-29), *Bacillus subtilis* (HR-100), and *Microbacterium sacchrophilum* (HR-112) were further explored for their degradation capability on the HPLC. The results revealed that HR-29 and HR-100 demonstrated almost similar degradation up to 73.14 % and 73.43% whereas, 69.11% degradation was showed by HR-112.

Introduction

The Indian economy is significantly influenced by the agricultural sector, which ensures food availability for over one billion people. Agriculture not only sustains the population but also supports industrial development. Approximately 60-70% of the Indian population depends on agriculture. To meet the growing demands for food and clothing, a tremendous pressure is anticipated on annual food grain production. Minimizing crop loss and achieving high yields require a balanced use of fertilizers and pesticides, along with the adoption of modern farming techniques.

Out of the various categories of pesticides employed globally, the organophosphorus group stands out as the most extensively utilized, constituting approximately 38% of the total global market (Singh *et al*.,2006). The pesticides of organophosphorus group are responsible for worldwide health hazards, with approximately three million poisonings and 2,00,000 deaths every year (Karalliedde *et al*., 1999, and Sogor *et al*., 2004). These pesticides have been linked to various muscular and nerve disorders in humans (Ragnarsdottir, 2000). Some of the main agricultural pesticides in the organophosphorus group are Monocrotophos (MCP), Parathion, Chlorpyrifos, Malathion, Dimethoate, and Methyl Parathion. Among these pesticides, Monocrotophos is less studied but widely used.

Pesticide residues persist in the soil for extended periods. The lack of knowledge regarding application of these pesticides has led to over exploitation of these pesticides causing short and long term health hazards. These pesticides interact with the biological systems in the environment due to their widespread use over the years; hence give rise to numerous problems related to environment pollution (Despotovic *et al*., 2019).

In response to the negative implications of pesticides, many alternative strategies have been developed to remove them from the environment, such as chemical treatments, landfills, and incineration. Although landfills are functional, there is a serious worry over the leakage of pesticides into nearby soil and groundwater supplies. The chemical treatment methods presents several challenges because they result in the generation of significant amounts of acids and alkalis, safe disposal of these hazardous chemicals is a big issue. Since incineration has been considered to be the most reliable approach to dispose of these substances, there has been strong public opposition because of the possibility of harmful emissions and financial constraints (Kumar *et al*., 2018)

The most suitable method for pesticide degradation is bioremediation. The potential of microbes to degrade pollutants from contaminated sites is an ecofriendly, effective, economical, versatile, and less hazardous strategy. Work on the microbial degradation of

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organophosphates has been reviewed, Within 24 hours at 37°C, *Pseudomonas aeruginosa* and *Clavibacter michiganense* subsp. *Insidiosum* were able to degrade technical MCP up to 79 and 80%, respectively (Subhas and D.K. Singh, 2003). *Pseudomonas moraviensis*, *Enterobacte rludwigii*, and *Serratia marcescens*, were isolated from agricultural soil contaminated with monocrotophos and were found to be capable of degrading MCP up to the 1000 mg/l (Abraham *et al.*, 2014).

Methodology Media and Chemicals

Monocrotophos (analytical grade), was purchased from Sigma Aldrich. The commercial formulation was dissolved in sterile distilled water to be added at desired concentrations to both soils and media. Himedia- HPLC grade Acetonitrile, Methanol and water was used. All the reagents utilized in this investigation were of exceptional purity and analytical grade. All reagents employed in this study were of analytical grade and met exceptional purity standards. The minimal salt media contains g/l of 1.5g KH_2PO_4, 0.5g K_2HPO_4, and 1g $(NH4)_2SO_4$, 0.2g $MgSO_4$, 0.02g $FeSO_4$ and 0.5g NaCl with pH 7.2 amended with monocrotophos analytical grade as a carbon source. Nutrient agar and nutrient broth is used for bacteria culturing.

Isolation and Enrichment of monocrotophos degrading bacterial isolates

Soil samples were collected from agricultural fields in Haryana, India, where there has been prolonged exposure to monocrotophos. A 20 g portion of soil sample was thoroughly mixed with monocrotophos and maintained at 37 ± 2°C for 5-7 days. Following the incubation period, the soil samples were serially diluted, and these soil samples were spread onto MSM agar plates containing monocrotophos. Afterward, the plates were placed in an incubator at 37°C for 5 to 7 days. Subsequently, unique colonies were identified, isolated, and conserved on identical plates and MSM agar slants for further examination and use.

Degradation of Monocrotophos

All bacterial strains obtained from soil samples underwent screening for their tolerance to monocrotophos using a plate assay method. MSM agar plates with varying concentrations (ranging from 100 to 500 ppm) of monocrotophos were employed for this purpose. Among the bacterial strains tested, designated as HR-29, HR-100, and HR-112, those demonstrating the highest tolerance to monocrotophos were selected for further experimentation as individual pure bacterial inoculants. The investigation into pesticide degradation was conducted using sterile minimal salt broth containing: 0.5 g KH_2PO_4, 0.2 g MgSO4, 1.5 g NH_4NO_3, 0.5 g NaCl, 1.5 g K_2HPO_4, per litre and having pH 7.0 (as outlined by Srinivasulu *et al*., 2017). The MSM broth was supplemented with filter-sterilized monocrotophos as a carbon source, added at a concentration of 500 ppm.

Identification of monocrotophos degrading bacterial isolates

Bacterial strains with the ability to degrade monocrotophos were identified through a process involving morphological characteristics, followed by molecular identification using 16s rRNA gene sequencing conducted by Macrogen Korea. The resulting sequences were then submitted to the NCBI gene bank.

Analytical Methods for Degradation Study

The confirmation of monocrotophos degradation was carried out using high performance liquid chromatography. To prepare samples, the MSM cultures were centrifuged at 9000 rpm for 10 minutes at 10°C using a refrigerated centrifuge. The resulting cell-free supernatant was utilized for estimating residual monocrotophos content, which was filtered through a 0.2 µm pore size syringe filter for subsequent analysis. HPLC analysis utilized a Reverse phase C18 column with dimensions of 4.6×250 mm and a particle size of 5 µm. The mobile phase, operated in isocratic mode, comprised a blend of acetonitrile and high-performance liquid chromatography (HPLC) grade water in an 80:20 ratio. The sample was injected at a flow rate of 1 ml/min, with a volume of 20 µl. Monitoring degradation was achieved using a UV detector scanning within the range of 214 nm. (Abraham *et al*., 2014).

Results Enrichment and Isolation of Monocrotophos Degrading Soil Bacteria

A total of 15 bacterial strains were isolated by enrichment of soil samples utilizing monocrotophos as energy and carbon source. The most potent bacterial isolates namely HR-29, HR-100, and HR-112 were found to be capable of utilizing monocrotophos up to the concentration of 500 ppm. After 120 h of incubation at culture condition of 37˚ C temperature and 90 RPM, up to 73.14%, 73.43%, and 69.11%,

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degradation was achieved by these 3 bacterial isolates.

Identification of Isolates

Molecular identification of isolated cultures were done using 16s rRNA gene sequencing from Macrogen Korea. Sequencing results revealed that the cultures labelled HR-29, HR-100, and HR-112 were *Bacillus weidmannii* (MRCJ-1017), *Bacillus subtilis* (MRCJ-1018), and *Microbacterium sacchrophilum* (MTCC-13005), respectively, and two bacterial strains, *Bacillus weidmannii* and *Bacillus subtilis*, were submitted at the Microbial Resource Center, Jammu (MRCJ), whereas *Microbacterium sacchrophilum* was submitted at the Microbial Type Culture Collection Center (MTCC), Chandigarh. The sequences of the 16s rRNA gene for bacterial strains are depicted in Figure 1 (a), (b), and (c) given below.

HR 29

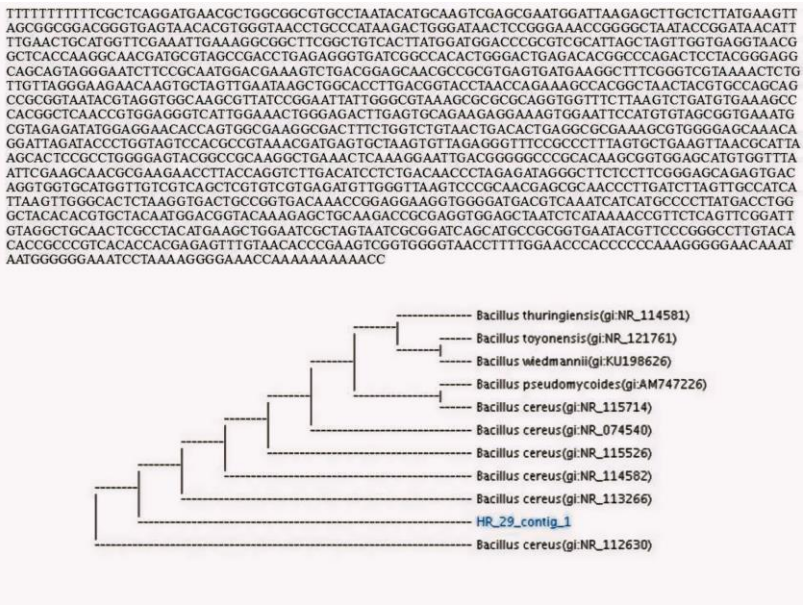

Figure 1(a): Sequences of the 16s rRNA gene for bacterial strain HR-29.

HR 100

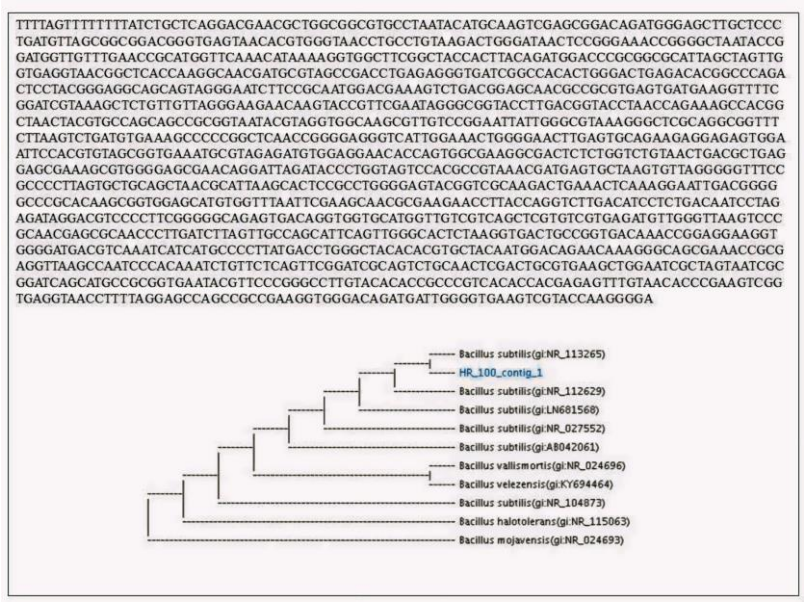

Figure1 (b): Sequences of the 16s rRNA gene for bacterial strain HR-100.

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HR 112

TTTTTTAGATTTTTTGCACTGCTCAGGATGAACGCTGGCGGCGTGCTTAACACATGCAAGTCGAACGGTGAAAGAGAGCTTGC
TCTCTGGATCAGTGGCGAACGGGTGAGTAACACGTGAGCAACCTGCCCCGGACTCTGGGATAACAGCTGGAAACAGCTGCTAA
TACCGGATACGAGCTGCGAAGGCATCTTCAGCAGCTGGAAAGAATTTCGGTCCGGGATGGGCTCGCGGCCTATCAGCTAGTTGG
TGAGGTAATGGCTCACCAAGGCGTCGACGGGTAGCCGGCCTGAGAGGGTGACCGGCCACACTGGGACTGAGACACGGCCCAG
ACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATGCAGCAACGCCGCGTGAGGGATGACGGCCT
TCGGGTTGTAAACCTCTTTTAGCAGGGAAGAAGCGAAAGTGACGGTACCTGCAGAAAAAGCGCCGGCTAACTACGTGCCAGCA
GCCGCGGTAATACGTAGGGCGCAAGCGTTATCCGGAATTATTGGGCGTAAAGAGCTCGTAGGCGGTTTGTCGCGTCTGCTGTGA
AAACCCGAGGCTCAACCTCGGGCCTGCAGTGGGTACGGGCAGACTAGAGTGCGGTAGGGGAGATTGGAATTCCTGGTGTAGCG
GTGGAATGCGCAGATATCAGGAGGAACACCGATGGCGAAGGCAGATCTCTGGGCCGTAACTGACGCTGAGGAGCGAAAGGGT
GGGGAGCAAACAGGCTTAGATACCCTGGTAGTCCACCCCGTAAACGTTGGGAACTAGTTGTGGGGTCCATTCCACGGATTCCGT
GACGCAGCTAACGCATTAAGTTCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGACCCGCACA
AGCGGCGGAGCATGCGGATTAATTCGATGCAACGCGAAGAACCTTACCAAGGCTTGACATATACGAGAACGGGCCAGAAATGG
TCAACTCTTTGGACACTCGTAAACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGA
GCGCAACCCTCGTTCTATGTTGCCAGCACGTAATGGTGGGAACTCATGGGATACTGCCGGGGTCAACTCGGAGGAAGGTGGGG
ATGACGTCAAATCATCATGCCCCTTATGTCTTGGGCTTCACGCATGCTACAATGGCCGGTACAAAGGGCTGCAATACCGCAAGGT
GGAGCGAATCCCAAAAAGCCGGTCCCAGTTCGGATTGAGGTCTGCAACTCGACCTCATGAAGTCGGAGTCGCTAGTAATCGCA
GATCAGCAACGCTGCGGTGAATACGTTCCCGGGTCTTGTACACACCGCCCGTCAAGTCATGAAAGTCGGTAACACCTGAAGCC
GGTGGCCCAACCCTTGTGGAGGGAGCCGTCGAAGGTGGGATCGGTAATTAGGACTAAGTCGTACAAGGGTAACCAAATC

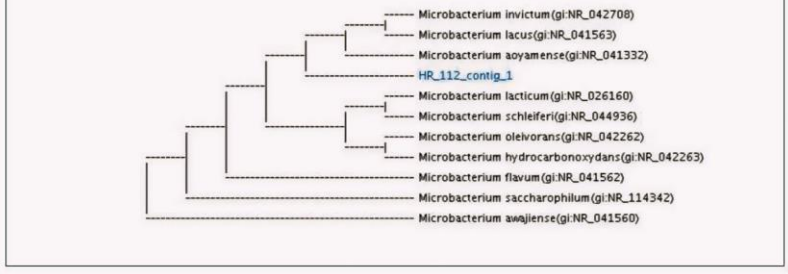

Figure 1 (c): Sequences of the 16s rRNA gene for bacterial strain HR-112.

Monocrotophos Degradation

HPLC was employed to validate the degradation study of monocrotophos. Figure 2 (a), (b), (c) and (d) illustrates a comparison of HPLC profiles between standard and degraded monocrotophos.

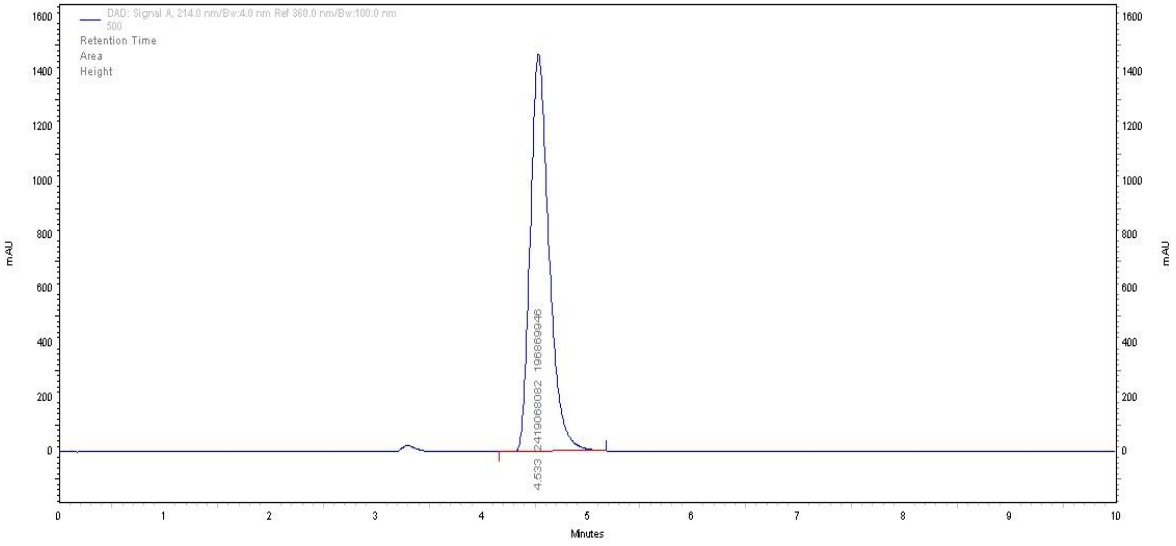

Figure 2 (a): Represents chromatogram of standard monocrotophos at 500ppm concentration.

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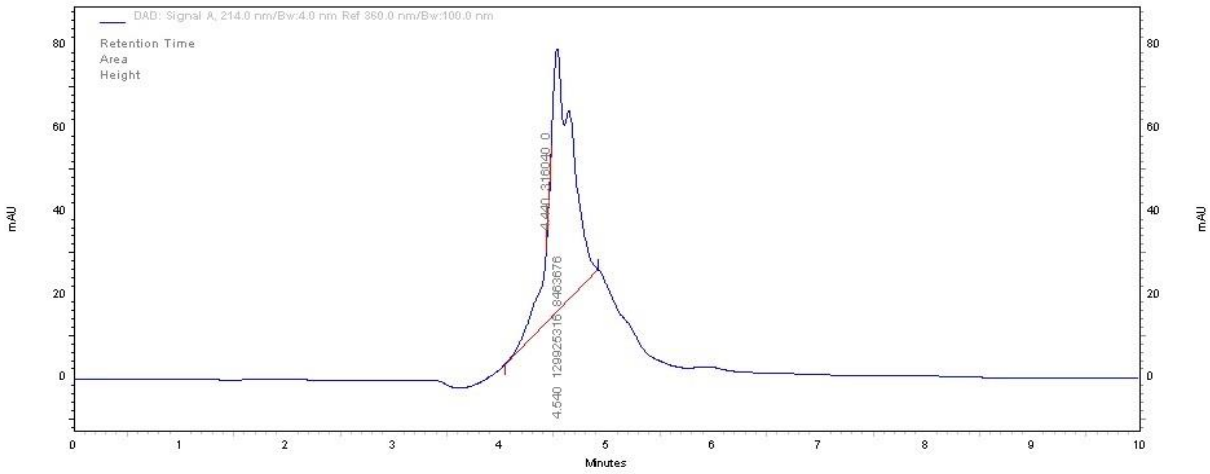

Figure 2 (b): Monocrotophos in minimal salt media treated with isolate HR- 29

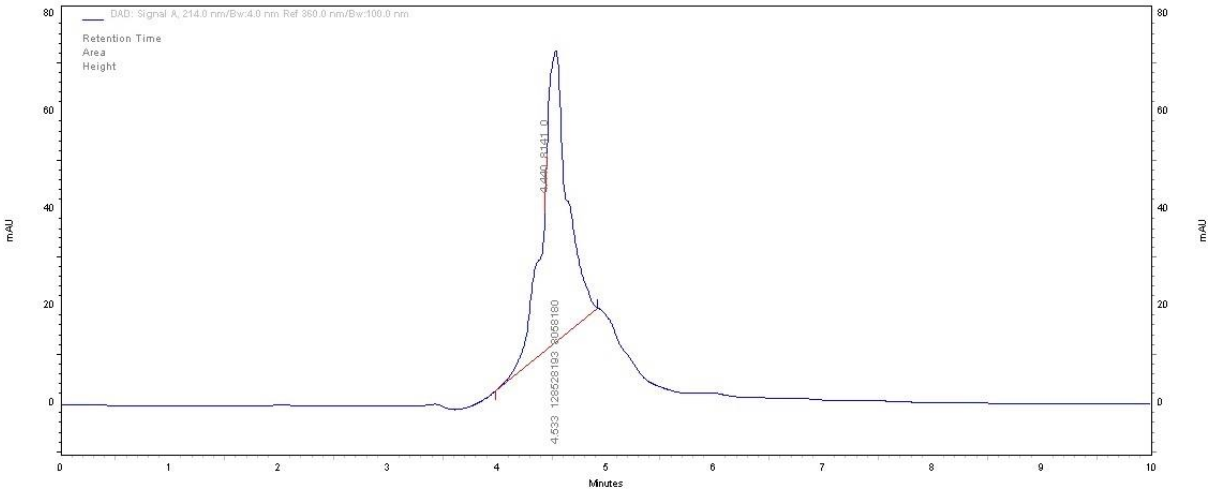

Figure 2(c): Monocrotophos in minimal salt media treated with isolate HR- 100

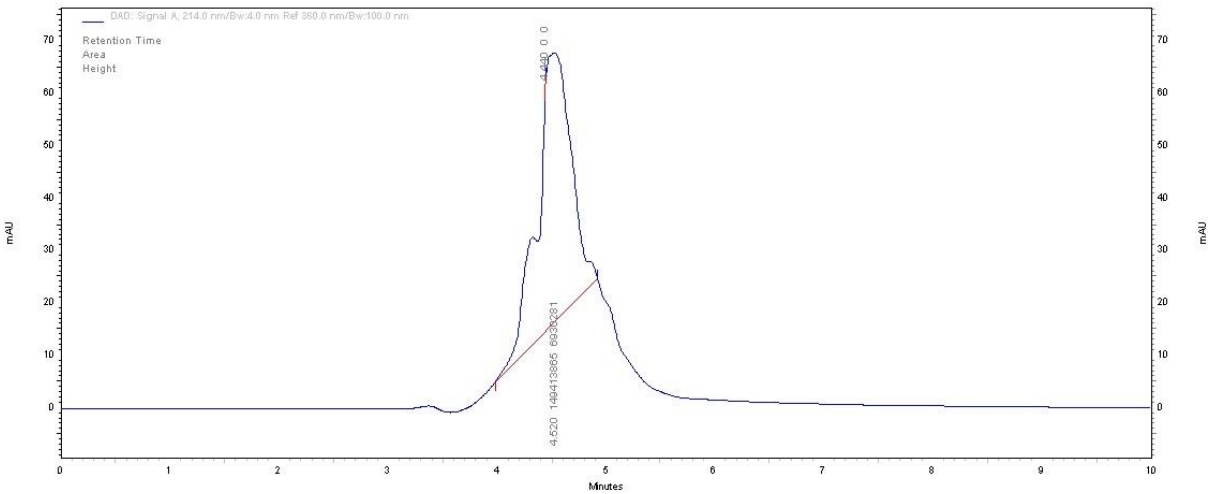

Figure 2 (d): Monocrotophos in minimal salt media treated with isolate HR- 112

The recorded retention time for monocrotophos was 4.5 minutes. The diminishing peak area and height of the extracted samples from Table 1 validates the degradation of monocrotophos over time.

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Table1: Showing Peak area, height and Retention time of the bacterial strains that confirmed the degradation of monocrotophos analyzed in HPLC.

Sr. no.		Strain	Retention Time	Peak Area	Peak Height
1.	1.	Standard MCP	4.5	2419068082	196869946
2.	2.	*Bacillus weidmannii*(HR-29)	4.5	129925316	8463676
3.	3.	*Bacillus subtilis*(HR-100)	4.5	128528193	8058180
4.	4.	*Microbacteriumsacchrophilum* (HR-112)	4.5	149413865	6930281

Thus, degradation analysis revealed that bacterial isolates for monocrotophos degradation were found capable of degrading MCP upto 73.14 %, 73.43 % and 69.11 % respectively in standard conditions at 37˚ C temperature, shaking at 90 RPM in 5 days of incubation time. Some other studies found that *Arthrobacter atrocyaneus* and *Bacillus megaterium*, were capable of degrading monocrotophos to the level of 93% and 83%, respectively, from growth medium containing monocrotophos at the concentration of 1g/l) , within eight days (Bhadbhade *et al.*, 2002), *B. subtilis* KPA1 is capable of breaking down 1000 ppm of monocrotophos within a span of 6 days. (Acharya *et al.*, 2014). Srinivasulu *et al.*, (2017) observed that, when monocrotophos was provided as a carbon source in a mineral salts medium, *R. phenolicus* strain MCP1 and *R. ruber* strain MCP-2 degraded 13%, 20%, 24%, and 30% of the compound by the end of the 1st, 2nd, 3rd, and 4th day, respectively. After 17 days of incubation, degradation percentages of 67.8%, 16.58%, and 6.67% were reported by Sidhu *et al.*, (2015) for isolates *P. synxantha*, *B. subtilis*, and *S. enterica* respectively.

Conclusion

The bioremediation of MCP has undergone extensive research, leading to the isolation of numerous bacterial strains with potential for degrading various pesticides. This study provides information about the monocrotophos-degrading bacterial strains isolated from pesticide-contaminated soils. Based on the sequence characterization and analysis, the bacterial strains were identified as *Bacillus weidmannii* (MRCJ-1017), *Bacillus subtilis* (MRCJ-1018), and *Microbacterium sacchrophilum* (MTCC-13005). These bacterial strains were isolated from soil samples collected from different agricultural fields in Haryana. Among all of these bacterial strains, *Bacillus weidmannii* (MRCJ-1017) and *Bacillus subtilis* (MRCJ-1018) show almost similar degradation potential, followed by *Microbacterium sacchrophilum* (MTCC-13005) when analysed using HPLC.

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