

# Intra species variation among *Bacillus subtilis* isolated from monocrotophos contaminated agricultural soil

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## Abstract

Five (05) bacterial strains, which are capable of degrading monocrotophos, were isolated from monocrotophos contaminated soil samples from agricultural fields of Heggadadevana Kote, a place in Mysuru District, Karnataka, India. The ability of the strains to degrade monocrotophos was investigated under different culture conditions. Potential strains degrading monocrotophos was selected and named Ja-HD-1, Hom-HD-6, Ba-HD-2, Ho-HD-2 and Ho-HD-3. The strains were identified as *Bacillus subtilis* on the basis of the results of its cellular morphology, physiological, chemotaxonomic characteristics and phylogenetic analysis of 16S ribosomal DNA (rDNA) gene sequences. Organophosphate hydrolase (*opdA* gene) involved in the biodegradation of monocrotophos in *Bacillus subtilis* strains was quantitatively expressed.

RT-qPCR data revealed that the strains harboring *opdA* gene were observed significantly down regulating from *opdA* gene in a degradation stage. Growth and degradation kinetic studies proved that the strains were able to grow in minimal salt medium containing 1000 ppm monocrotophos as the only carbon source. Hence, Ja-HD-1, Hom-HD-6, Ba-HD-2, Ho-HD-2 and Ho-HD-3 culture strains of *Bacillus subtilis* have great potential utility for the bioremediation of agriculture soils contaminated monocrotophos insecticide.

**Keywords:** Soil contamination, Bioremediation, Monocrotophos, *Bacillus subtilis*, *opdA* gene, gene variation, SNP.

## Introduction

Single-nucleotide polymorphisms (SNPs) are recognized as important markers for detecting and subtyping bacterial gene variation<sup>19</sup>. Nucleotide alterations in bacteria are associated with response to tolerance to toxic pesticides which include nucleotide substitutions, insertion, duplications and fusions. In single-nucleotide substitutions, a nucleotide is replaced with any of the other three kinds of nucleotides. This phenomenon is commonly referred to as single-nucleotide polymorphisms (SNPs) or single-nucleotide mutations<sup>6,18</sup>. Many soil bacteria have been isolated and characterized which can degrade organophosphorus compounds as a

source of phosphorus and carbon<sup>7</sup>. Monocrotophos (dimethyl (E)-1-methyl-2-(methylcarbamoyl) vinyl phosphate-MCP) is extensively used organophosphorus insecticide against aphides, scale insects, leaf hoppers, mites and other foliage pests in agricultural operation persisting as soil residue and seeps into ground water<sup>14</sup>.

In growing relevance and bio-remedial measures in treating toxic pesticide residual wastes, attempts have been made to identify these organophosphate degrading (*opd*) genes<sup>17,20</sup>. Pesticide degrading soil bacteria have been isolated from diversified geographical regions and *opd* plasmids have shown considerable diversity with respect to the size and genetic information<sup>20</sup> and subsequently, the plasmid borne *opd* gene has been cloned and successfully expressed in heterologous hosts<sup>5,12</sup>.

In the present study, we have isolated *Bacillus* strain that could use monocrotophos as the sole carbon source. A large indigenous plasmid, pBC9 was found to be involved in degradation of organophosphate pesticides and has been used as a reference plasmid identified in other soil bacterial isolate<sup>10,18</sup>.

In the present study, we have reported PCR amplification, cloning and sequencing of *opd* gene from *Bacillus subtilis* with a high potential to degrade monocrotophos. Monocrotophos degradation by *Bacillus subtilis* isolates was found as maximum at 2-3 ml inoculum size of 48-h old culture used as sole carbon source in Minimal salt medium. Five *Bacillus subtilis* isolates were able to degrade monocrotophos to the extent of 50-75% at a concentration of 1000 ppm monocrotophos in 7 days under shake culture condition at 35 °C.

## Material and Methods

Monocrotophos resistant *Bacillus subtilis* isolates and *E. coli* (MTCC 5891) used in this study were grown in LB medium<sup>13</sup>. Restriction enzyme and other enzymes used in this study were purchased from Sigma Aldrich, India and were used according to the manufacturer's protocol.

**Polymerase chain reaction (PCR):** Based on the reported sequence information, highly conserved regions of *opd* gene were identified to design primers<sup>22</sup>. The oligos were purchased from Sigma Aldrich, India. PCR reaction 50 µl consisted of 100 ng of plasmid pBC9, 5 µl of 10X PCR buffer, 5 p moles of each forward primer and reverse primer, 2 µl of 4 mM MgCl<sub>2</sub>, 1.5 µl of 200 µM d NTP mix and 0.5 U of Taq polymerase. A similar reaction mixture without

template DNA was taken as negative control<sup>2</sup>. The reaction mixture was overlaid with 50 µl of mineral oil and subjected to 35 temperature cycles using Thermal cycler<sup>8</sup>. Each cycle consisted of denaturation at 94°C for 90 sec, primer annealing 53°C for 90 sec and extension at 72°C for 1 min. After completion, 8 µl of reaction mixture was analysed on agarose gel to detect the amplified DNA fragment<sup>9</sup>.

**Cloning of PCR product:** The vector pUC18 and PCR product were independently digested with EcoRI<sup>15</sup>. The digested DNA was independently extracted with phenol: chloroform and ethanol precipitate. The ligation of linearized vector and PCR product was used to perform following the standard procedures<sup>4</sup>. The ligation mixture was used to transform *E. coli* and the colonies containing the recombinant plasmid were selected. The recombinant plasmid was purified and digested in EcoRI to assess the presence of insert<sup>21</sup>. The fragment having the right sized insert was then selected for determining the detailed restriction map.

**Sequencing of PCR product:** After establishing the detailed restriction map, the PCR fragment was digested with various restriction enzymes to obtain overlapping fragments. These fragments were sub-cloned in pUC18 and the sequence was determined<sup>18</sup>.

**OpdA gene expression:** Bacterial cultures were grown for 24 h in MSM supplemented with 1000 ppm of monocrotophos. Total DNA was isolated from cell pellets of 14 day sample<sup>1</sup>. Complementary DNA (cDNA) was prepared from the total RNA samples using H minus First Strand cDNA synthesis kit (Thermo Scientific, India) using random hexamer primer.

The reaction mixture composed of total RNA template 4 µl, primers 1 µl, DEPC-treated water 7 µl, 5X reaction buffer 4 µl, RiboLock RNase inhibitor (20 U/µl) 1 µl, 10 mM dNTP mix 2 µl and MMuLV reverse transcriptase (20 U/µl) 1 µl. After mixing gently, it was centrifuged and incubated for 5 min at 25 °C followed by 60 min at 42 °C. The reaction was terminated by heating at 70 °C for 5 min with temperature at 4°C<sup>4</sup>.

The prepared cDNA samples were amplified using gene specific primers. Light Cycler Probe Design Software 2.0 was used for designing the primers to Aryldialkylphosphatase (opdA) gene and then synthesized using Biolytic synthesizer<sup>1,21</sup>. The sequences of the opdA primers were OPDA F5'-TGTTCCGGTAACCACTCACA-3' and OPDA R5'-CACT CTCAGAGGGACGAAGG-3'. The reaction mixture composed of nuclease free water 9.0 µl, cDNA 1 µl, primers (10 p-mole/µl) 1.0 µl and 2× PCR master mix 13 µl<sup>23</sup>.

Denaturation and annealing were carried out at 95 °C and 58 °C for 30 s respectively. Extension temperature was set as 72 °C for 45 s and final extension was 75 °C for 7 min in 40 cycles. Nuclease free water 9.5 µl, cDNA 1 µl, primers (10 p-mole/µl) 1 µl and Light Cycler 480 SYBR Green I Master 12.5 µl were included in the reaction mixture. PCR product was electrophoresed along with 100-bp DNA molecular weight marker (Thermo Scientific, India). Electrophoresis was carried out at 95 V for 30 min<sup>3</sup>.

## Results and Discussion

Identical opd genes have been identified in *Bacillus* sp. A homology was expected in organophosphate pesticide degrading gene of *Bacillus subtilis* accordingly, appropriate primers were designed to amplify it using PCR. A 21 mer oligo (5' ACCCGGCATTGACATCTGAC 3') corresponding to 5' end of the gene and a 21 mer reverse primer with a sequence of 5' CTGGCTGGAAGGAT CCAGATG 3' corresponding to 3' end of the gene were designed using the two primers and plasmid pBC9 was used as template. Resulting recombinant plasmid was digested and each fragment was then cloned to generate clones with overlapping fragments.

Comparison of amino acid sequence deduced from nucleotide sequence of PCR amplified fragment with sequence of *Bacillus subtilis* isolates showed considerable homology with the deduced amino acid sequence. Five *Bacillus subtilis* isolates which showed maximum degradation of monocrotophos at 1000 ppm also were analyzed for opdA gene expression which showed presence of SNP as depicted in fig. 1-6.

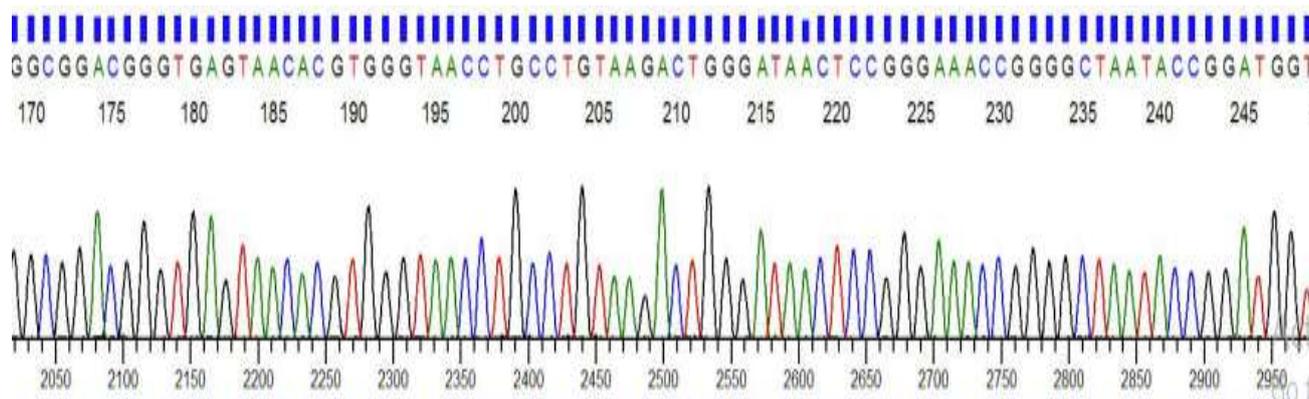


Fig. 1: Fasta format of MTCC *Bacillus subtilis* strain

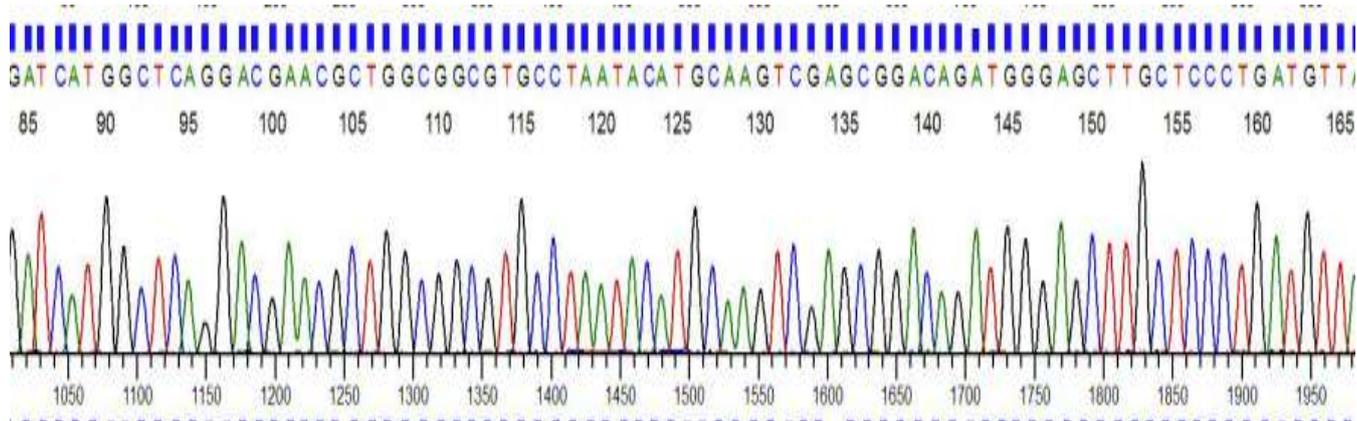


Fig. 2: Overlapping observed in the isolate *Bacillus subtilis* PKM1

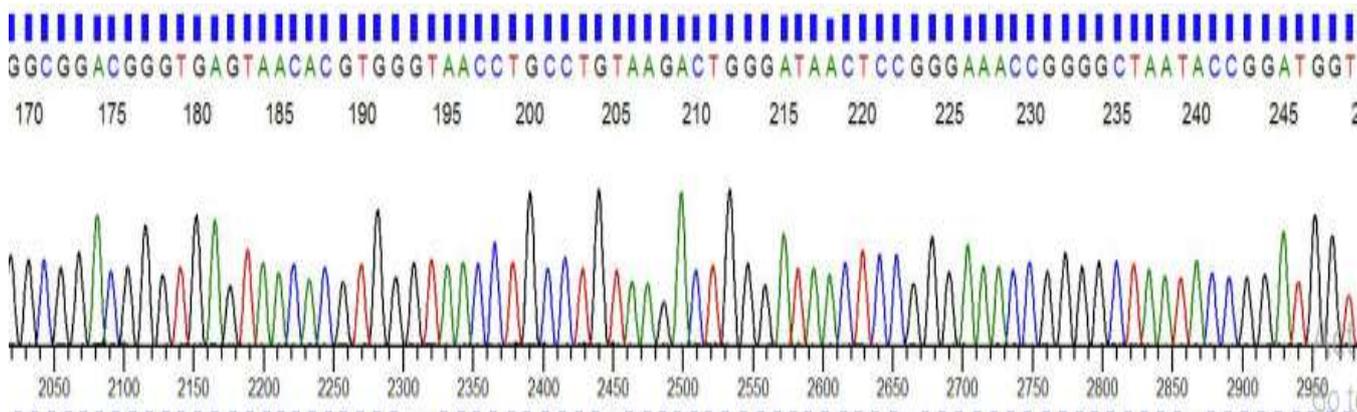


Fig. 3: Overlapping observed in the isolate *Bacillus subtilis*-5

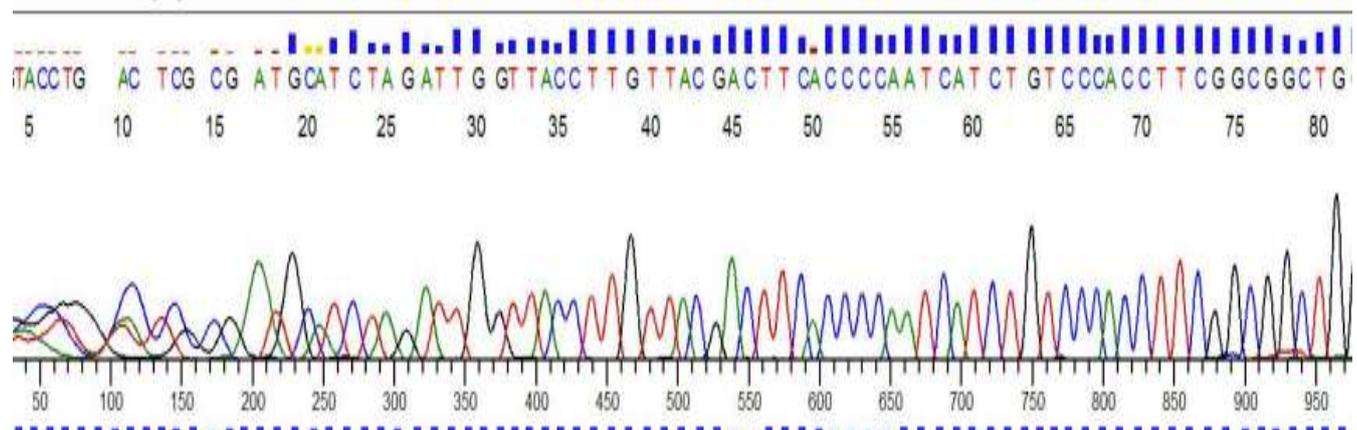


Fig. 4: Overlapping observed in the isolate *Bacillus subtilis*-9

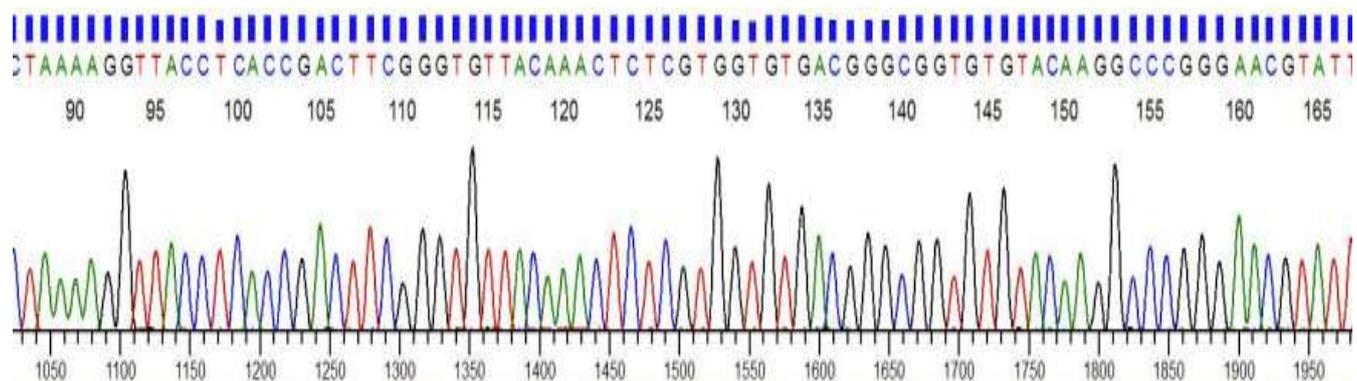


Fig. 5: Overlapping observed in the isolate *Bacillus subtilis*-17

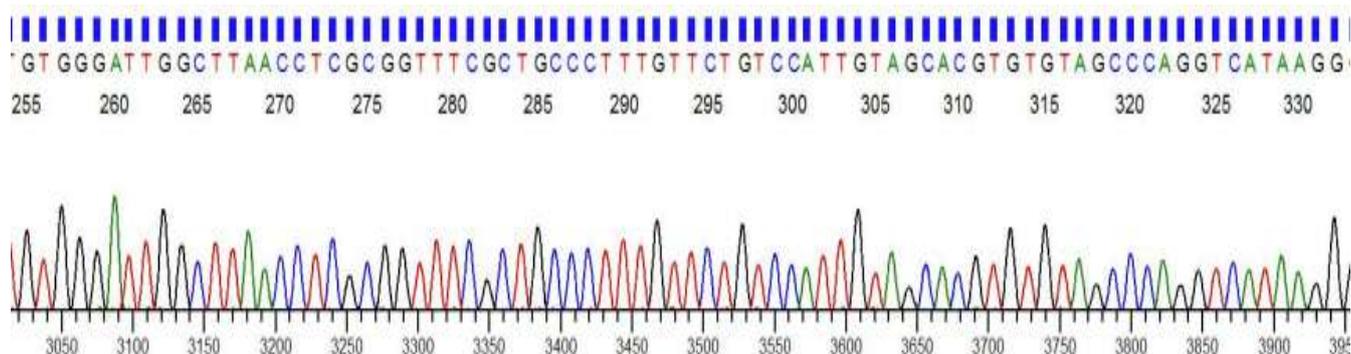


Fig. 6: Overlapping sequence observed in the isolate *Bacillus subtilis*-20

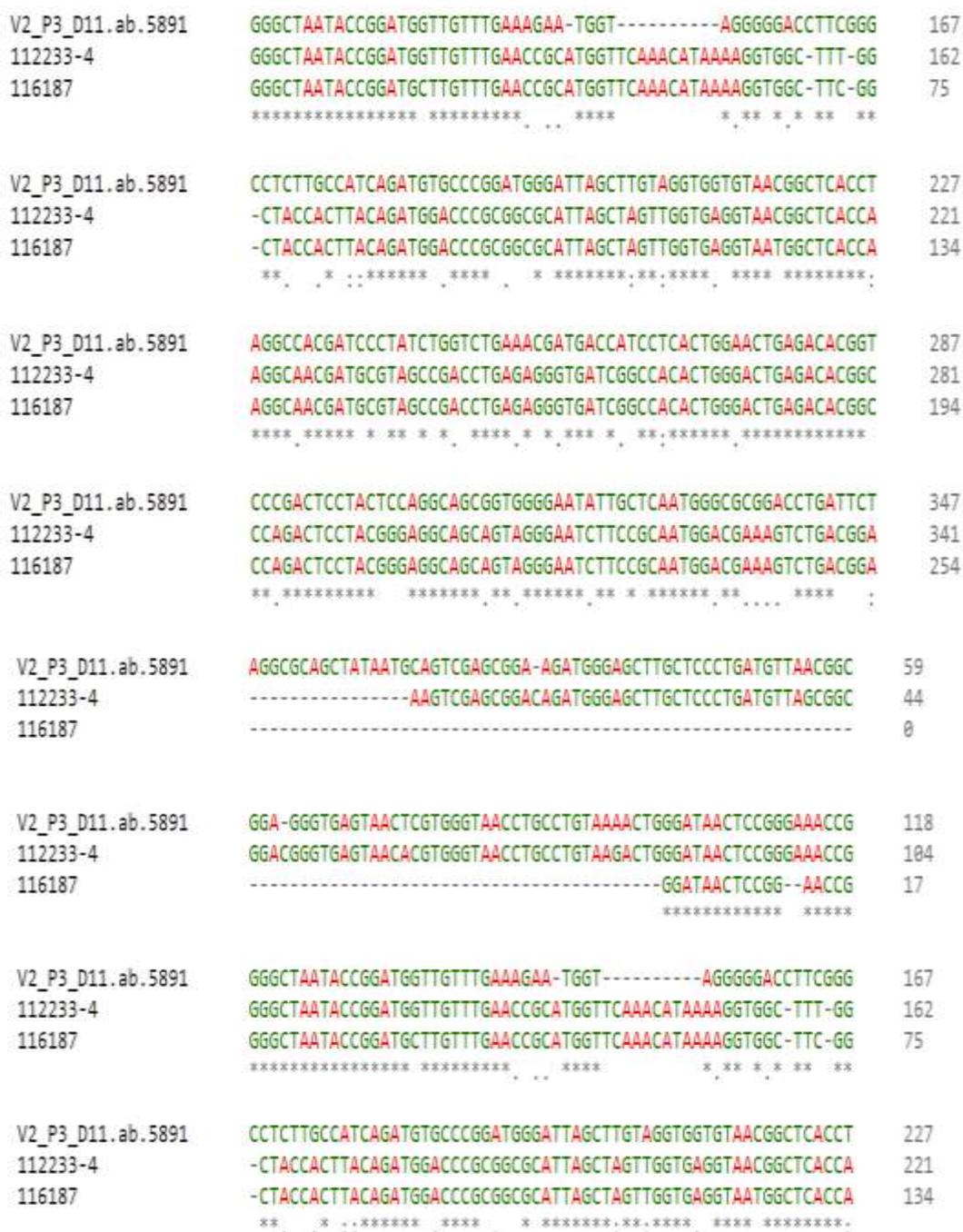
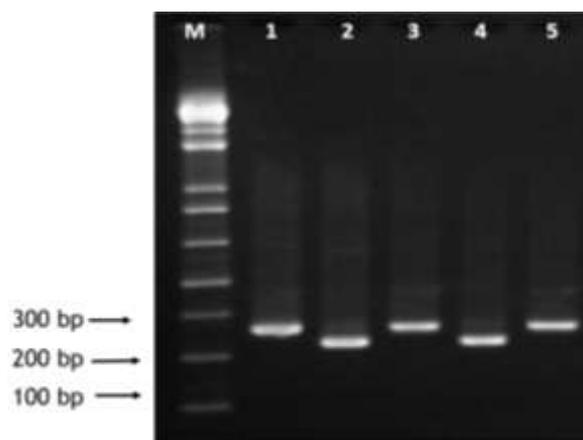


Fig. 7: Multiple sequence alignment showing intra species variations among monocrotophos degrading *Bacillus subtilis* isolates



**Fig. 8: PCR amplification indicating the presence of organophosphate hydrolase (opdA) enzyme Lane 1- marker (1000 bp), Lane 2- Isolate Ja-HD-1, Lane 3- Isolate Hom-HD-6, Lane 4- Isolate Ba-HD-2, Lane - Isolate Ho-HD-3, Lane 6- Isolate Ho-HD-3**

Five (05) *Bacillus subtilis* isolates from the present study (Ja-HD-1, lane 3- Hom-HD-6, lane 4- Ba-HD-2, lane - Ho-HD-3, lane 6- Ho-HD-3) exhibited maximum degradation at 1000 ppm carried out in *in vitro* degradation studies previously conducted in our laboratory<sup>16</sup>. PCR amplification result (fig. 8) indicates the presence of organophosphate hydrolase (opdA) enzyme of five *Bacillus subtilis* isolates and its constitutive expression in cytoplasm upon the exposure of monocrotophos. A study of the degradation characteristics of the isolates made us to confirm the soil bacterium involved in the degradation of monocrotophos insecticide.

## Conclusion

Bacteria play an important role in the bioremediation of monocrotophos insecticide. Five *Bacillus subtilis* isolates from monocrotophos contaminated agricultural soil were able to perform biodegradation of monocrotophos insecticide. Organophosphorus pesticides degradation gene opdA sequence was obtained in isolated strains by PCR amplification.

A whole-cell bioreporter assay was evaluated from genotoxicity of monocrotophos biodegradation which is a rapid, cost-effective and simple approach for genotoxicity assessment of environmental samples. This work unraveled indigenous *Bacillus subtilis* monocrotophos degraders with OP-degrading genes leading to monocrotophos degradation process, providing possible candidates for the bioremediation of monocrotophos contaminated soil.

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