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MOLECULAR CHARACTERIZATION OF BACTERIAL STRAINS ISOLATED FROM PESTICIDES CONTAMINATED SOILS OF DISTRICT GUJRAT, PAKISTAN

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Abstract

In the present study, bacterial strains were isolated from Fenitrothion and Diamethioate contaminated soils. Due to identical morphological characteristics only two strains, APDB9 and APDB10, were randomly selected for molecular identification through 16S rRNA gene sequence. These two strains were found closely related to *Bacillus megaterium* and *Bacillus thuringiensis*, respectively. The presence of 21 kb plasmid in all strains isolated suggests the involvement of this plasmid in pesticide resistance. However, none of the bacterial strains showed amplification of organophosphate degrading (*opd*) gene with gene specific primers, which suggested that the nucleotide sequence of the genes in these strains was different from the ones already reported. These results may help to characterize and investigate the new types of *opd* genes, so that they may be utilized in pesticides biodegradation in future.

Key words: Agricultural field soil, Pesticides, *OPD* genes, *Bacillus*, Pakistan.

Introduction

Pesticides are substances that are intended to control, prevent or kill any species which is designated as a pest, such as insects, rodents, weeds, fungi, bacteria, or other organisms. Improper use of pesticides has been reported to be harmful to non-target organisms by being lost from actual sites to non-target sites through many ways. As they are toxic, they have been reported to cause damage to different organisms e.g., damage to reproductive organs in birds, death in fishes and many diseases in humans (Greig-Smith, 1990). Among pesticides, the more commonly used are the organophosphorous pesticides. They have gained worldwide popularity by being effective over a wide range of pests (Choi *et al*., 2009). Presently, 140 different types of OPs are being used both as pesticides and plant growth regulators all over the world (Kang *et al*., 2006). It is evident from many studies that improper handling or accidental release of Ops can cause the contamination of terrestrial and aquatic ecosystems (Singh *et al*., 2006). Organophosphate compounds act by substituting the natural neurotransmitter acetylcholine and inhibit the natural degradation of it by acetylcholinesterase (Choi *et al*., 2009).

Biodegradation through microorganisms provides an efficient tool to remove organophophate pesticides and decontaminate the polluted environment (Surekha *et al*., 2008; Luo *et al*., 2018; Huang *et al*., 2018). Compared to conventional methods, the biological degradation strategies are much more acceptable and favorable because microorganisms decontaminate most of the pollutants without producing harmful metabolites (Furukawa *et al*., 2003). Numerous evidences reveal that microorganisms are responsible for degradation of pesticides in soil, as most of them reside in the topsoil and carry out aerobic biodegradation. The rate of biodegradation decreases as we move down the soil because the number of aerobic microorganisms decreases downwards (Dileep *et al*., 2008). Among Bacteria, work has been done on species of different genera, including *Flavobacterium, Arthrobacter, Burkholderia, Plesiomonas, Agrobacterium, Pseudomonas, Serratia, Ochrobactrum,* and *Bacillus* having ability to hydrolyze organophosphates (Liu *et al*., 2005; Pakala *et al*., 2006; Yang *et al*., 2007; Briceño *et al*., 2020).

Bacteria possess a number of enzymes responsible for pesticide degradation such as OPH, MPH, and OpdA which are encoded by *opd*, *mpd*, and *opdA* genes, respectively (Ali *et al*., 2012). The most widely studied enzyme is OPH (organophosphorus hydrolase). Encoded by the gene *opd* (organophosphate degrading) it has been studied extensively owing to its hydrolytic activity on organophosphates (Raushel *et al*., 2000; Rangasamy *et al*., 2018). The genes for pesticide degradation may either be present on plasmid DNA or chromosomal DNA. Most of the studies reported Opd gene to be plasmid based with similar sequences (Singh *et al*., 2004). However plasmid based Opd gene with different DNA sequence has also been reported which does not show any sequence homology with already reported *opd* genes (Kim *et al*., 2007; Kim *et al*., 2009). Some workers have reported an *opd* gene located on chromosomal DNA with similar or different sequence homology to existing bacterial *opd* genes (Horne *et al*., 2002; Singh *et al*., 2004). There are some other reports as well revealing that the *opd* gene sequences could be novel and different from those reported before (Mulbry , 2000; Zhongli *et al*., 2001; Choi *et al*., 2009).

Organophosphate pesticides are being used widely in Pakistan and improper use, little knowledge, mishandling and exposure to these chemicals results into a huge number of reported cases of poisoning in the hospitals (Farzana *et al*., 2020). From 2000 to 2007, 41% of all the cases reported in the National Poisoning Control Centre Karachi, Pakistan were of organophosphate poisoning (Ather *et al*., 2008).

To our knowledge, little work has been done on the molecular characterization of pesticides degrading bacteria or the strains living in pesticides contaminated soil in Pakistan. Therefore, this study was conducted to screen the soil of agricultural fields of District Gujrat for the organophosphate pesticides residues and the bacterial

strains living in this contaminated soil. Moreover, the presence of *opd* gene was analyzed to understand the pesticides degradation pathway in these bacteria.

Materials and Methods

Isolation of bacterial strains from the soil samples: Soil samples were collected from agricultural fields where pesticides were being used, in different areas of district Gujrat, Pakistan. The agricultural fields with pesticides application history of 5-10 years were selected for soil sampling. Total 21 soil samples were collected from the upper 15 cm layer of soil in sterile polyethene bags and stored at 4ºC till further use.

Bacterial strains were isolated from soil samples through enrichment technique, as described by Kim *et al*., (2009), with some modifications. Briefly, 10g of each soil sample was transferred to each 50-ml sterile beaker, treated with organophosphate pesticides (Sigma analytical grades) to a final concentration of 100 µg/g soil and thoroughly mixed. The treated soil was incubated with periodic mixing at room temperature. After three weeks treatment, 1g soil sample from each beaker was homogenized with 95 ml of sterile distilled water by shaking. Then 50µl of this suspension was spread on the nutrient agar plates and incubated at 37ºC overnight for growth. The isolates were transferred to the nutrient agar slants and were stored at 4ºC after growth.

Plasmid isolation and primer designing: The plasmid DNA was isolated by using plasmid DNA purification kit (Fermentas), following the manufacturer instructions. Primers for 16S rRNA and opd genes were designed using primers designing tool Primer 3 (version 0.4.0 - http://frodo.wi.mit.edu/). For 16S rRNA gene, universal primers were designed on the basis of multiple sequence alignment (clustalW) of different bacterial species 16S rDNA sequences on NCBI. Similarly for organophosphate degrading (*opd*) gene amplification, primers were designed using GenBank sequences, AY627034.1 (*Achromobacter* sp.), AY627039.1 (*Brucella* sp.), AY627035.1 (*Ochrobactrum* sp.) and AY627033.1 (*Pseudaminobacter* sp.). For oligoes synthesis, the services of National Center for Applied Molecular Biology (CAMB), Ministry of Science and Technology, Pakistan, were availed.

Gene amplification and DNA sequencing: Gene amplification was done by colony PCR. The bacterial colony was directly added to the PCR mixture and cells were lysed by high temperature in the start of PCR process to get out the DNA contents. After this PCR product was resolved on 1% of agarose gel and the 16S rDNA was purified from agarose gel through DNA Extraction Kit (Fermentas) according to the manufacturer's protocol. Sequence of the PCR amplified fragments was performed using both gene specific primers. Sequencing analysis was performed according to the manufacturer's instructions (Big Dye Deoxy Terminators; Applied Biosystems, Weiterstadt, Germany).

Sequence homology and phylogenetic analysis: "Nucleotides BLAST" was used for sequence homology analysis of the 16S rDNA sequences of the bacterial strains. The program used was BLASTN 2.2.26+ (Zheng *et al*., 2000) and database used was Bacteria and Archaea 16S rRNA sequences. For molecular identification of isolated bacterial strains, phylogenetic analysis, based on the 16S rRNA sequence was done by Molecular Evolutionary Genetics Analysis (MEGA6) (Tamura *et al*., 2011). The sequences were aligned by ClustalW using two parameters; Pairwise alignment and multiple alignments. The data was then processed for phylogenetic tree construction using Neighbor-Joining method (Saitou & Nei, 1987).

Detection and estimation of pesticides residues: Ten grams of soil sample was mixed with 20g anhydrous sodium sulphate and 2.5g of extra pure sodium chloride which were then dissolved in 100ml of solvent. Three solvent systems viz. methanol, ethyl acetate and acetone were used. After that, the sample was blended on high speed varying blender for 3 min and filtered through 0.4µm membrane filter. 25 ml of solvent was added in the residue and blended for 10 min. The blend was filtered, pooled and then concentrated on the rotary evaporator. The dried sample was dissolved in 100% solvent and analyzed on High Performance Liquid Chromatography (HPLC). The conditions applied to the HPLC process included the use of 100% methanol as mobile phase, a Flow rate of 0.5 ml/ min; ambient column temperature, using a C18 column for 7 minutes with an injection volume of 20 µl. A UV-Vis detector was used at a wavelength of 240nm.

Results

Total 21 pesticides applied soil samples were screened for pesticides residues through HPLC, out of which only 4 contained Fenitrothion and Diamethioate residues (Fig. 4). Bacterial strains were isolated from these Fenitrothion and Diamethioate contaminated soil samples and were subjected to plasmid profile analysis and screening of *opd* gene. Due to identical morphological characteristics only two strains were randomly selected for molecular identification through 16S rDNA sequence, which were found closely related to *Bacillus* species (Figs. 2 and 3).

Plasmid profile assay of the bacterial strains: The bacterial strain APDB4 and APDB9 showed same plasmid profile and had a single plasmid of 21 kb. Similarly, APDB10 and APDB11 were found to have identical plasmid profile with a total of three plasmids– 21kb, 1.1 kb and 0.7kb in size (Fig. 1). So, it was concluded that the 21 kb plasmid is common in all four bacterial strains.

Amplification of organophosphate degrading (*opd*) and 16S ribosomal RNA gene: The *opd* gene amplification was done with three primer sets but none of the strains showed *opd* gene amplification (Table 2). Similarly the bacterial strains isolated from Fenitrothion and Diamethioate contaminated soil were proceeded for 16s rDNA amplification and the required fragment of 769bp of 16S rDNA was obtained using primer set UPRS and UPRAS (Table 1).

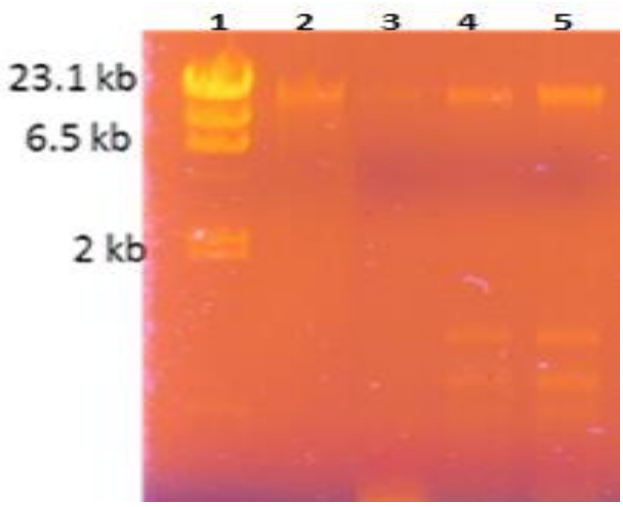

Fig. 1. Plasmid profile of the bacterial strains: lane 1 = λHind III DNA marker; lane 2 = strain APDB4; lane 3 = strain APDB9; lane 4 = strain APDB10; lane 5 = strain APDB11.

Table 1. Primer sequences for 16S rRNA gene.

Primer	Sequence (5'-3')	Length (nt)
UPRS	CTCCTACGGGAGGCAGCAGTAG	22
UPRAS	GCTCGTTGCGGGACTTAACCCAACA	25

Table 2. Primer sequences for *opd* gene.

Primer	Sequence (5'-3')	Length (nt)
ODS1	TTGCAATCTCGGTATACTCC	20
ODAS1	GAGAAAGGCTTGAACTTGC	19
ODAS2	CGAGAAAGGCTTGAACTTG	19
ODAS3	CCGAGAAAGGCTTGAACT	18

Sequencing & homology analysis: The PCR products of 16S rDNA were sequenced and nucleotide sequences of the two bacterial strains APDB9 and APDB10 were submitted to NCBI with accession numbers GenBank: JX274543 and GenBank: JX274544, respectively.

Sequence homology analysis was done for 10 BLAST Hits using sequences of bacteria and archaea 16S rRNA sequences databse. For APDB9, most of the hits were *Bacillus* spp. with Max Indent range of 94 - 98%. *Bacillus flexus* strain IFO15715 and *Bacillus megaterium* strain IAM 13418 revealed 98% homology (Table 3). Similarly, APDB10 showed homology with *Bacillus* spp. having Max Indent range from 96 – 99%. Four of these; *Bacillus thuringiensis* strain IAM, *Bacillus anthracis* strain ATCC 14578, *Bacillus weihenstephanensis* strain DSM11821 and *Bacillus mycoides* strain 273 showed 90% 16s rDNA sequence homology (Table 4).

The results of Phylogenetic analysis based on 16S rRNA gene sequence, showed that APDB9 is closely related to *Bacillus megaterium* (Fig. 2) while APDB10 is closely related to *Bacillus thuringiensis* (Fig. 3).

Detection of pesticide residues in soil samples: The soil samples were screened through HPLC technique for seven pesticides residues. These pesticides included Fenitrothion, Diamethioate, Endosulfan Chlorpyriphos, Mevinphos, Diazinon and Ethion. Among these, only Fenitrothion and Diamethioate were detected in the soil samples (Fig. 4). Fenitrothion was detected in three samples–APS4, APS10, APS11, while only APS9 showed Diamethioate residue. So 30% of the samples showed Fenitrothion residues while10% soil samples were found to be contaminated with Diamethoate residues.

Discussion

Organophosphates are the most widely used pesticides both in the developing and under-developed countries. Their non-targeted toxicity, especially in higher mammals poses a big challenge for its degradation and subsequent removal from the environment. The most widely studied organophosphate pesticide is Parathione while the bacterial degradation of Fenitrothion is less studied (Kim *et al*., 2009). In the present study we isolated *Bacillus* spp. from Fenitrothion and Diamethioate contaminated soil. Many bacterial strains have been reported to possess organophosphate pesticides degradation abilities (Horne *et al*., 2002; Tago *et al*., 2006; Kim *et al*., 2007; Briceño *et al*., 2020). Plasmid based gene for Diamethoate degradation was studied by Deshpande *et al*., (2001). In a recent study Awan *et al.*, (2012) analyzed top soil from cotton agricultural fields for commonly used pesticides in Pakistan, which included dichlorvos, diamethoate, methyl parathion, fenitrothion, endosulfan, mevinphos, chlorpyriphos and profenofos. The quantities found for Chloropyriphos, endosulfan and diamethoate were 0.486 mg kg^{-1}, 0.426 mg kg^{-1} and 0.555 mg kg^{-1} respectively. Similarly, fenitrothion residues ranged from 0.002 mg kg^{-1} to 0.86 mg kg^{-1} in soil samples. Many of the *Bacillus* species have been reported to degrade a variety of organophosphates e.g. a catabolic degradation of Parathion and Methyl parathion (Nelson & Yaron, 1982; Sharmila *et al*., 1989; Zhang *et al*., 2006). Moreover, *Bacillus cereus* degraded 2,4,6-Trinitrophenol (TNP), utilizing it aerobically as sole source of carbon and nitrogen (Singh *et al*., 2011). Similarly, *Bacillus pumilus* was reported to degrade *p*-nitrophenol actively and found to be able to grow in the presence of methyl parathion (Ali *et al*., 2012). *Bacillus magaterium* has been reported to degrade variety of organophosphates like, glyphosate (Quinn *et al*., 1989) and monocrotophos (Bhadbhade *et al*., 2002). *Bacillus magaterium* was also found to degrade dichloroaniline isomers, which are widely used in commercial synthesis of azo dyes, herbicides, paints, cosmetic and other industrial chemicals (Yao *et al*., 2011). Similarly the strain of *Bacillus thuringeinsis* was reported to catabolically degrade malathion (Zeinat *et al*., 2008).

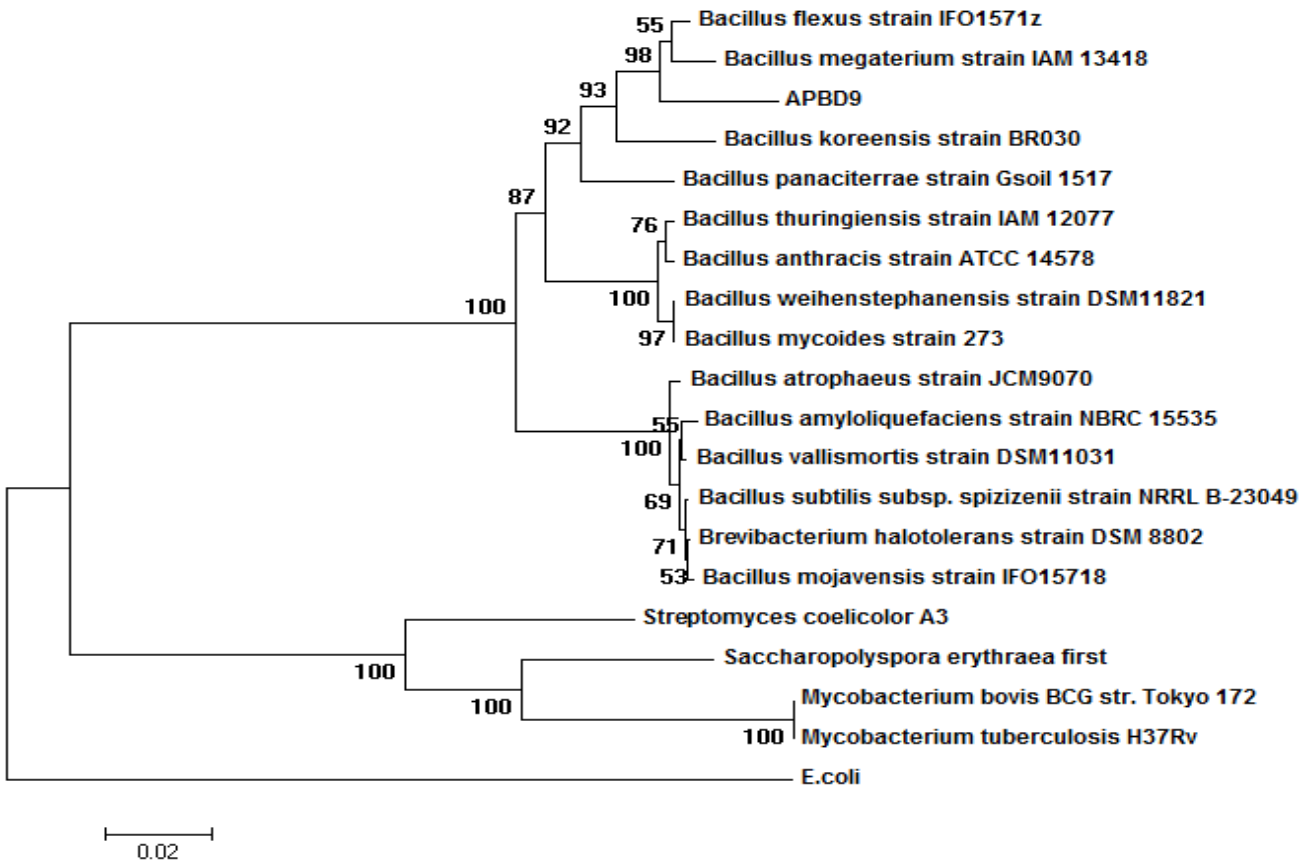

Fig. 2. Evolutionary relationships of strain APDB9. The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The optimal tree with the sum of branch length = 0.22094624 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al*., 2004) and are in the units of the number of base substitutions per site. The analysis involved 16 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 748 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (Tamura *et al*., 2011).

Table 3. Sequence homology of APDB9 strain (Zheng *et al*., 2000).

S #	Accession	Description	E value	Max indent
1.	NR024691.1	*Bacillus flexus* strain IFO15715 16S ribosomal RNA, partial sequence	0.0	98%
2.	NR043401.1	*Bacillus megaterium* strain IAM 13418 16S ribosomal RNA, partial sequence	0.0	98%
3.	NR043084.1	*Bacillus koreensis* strain BR030 16S ribosomal RNA, partial sequence	0.0	97%
4.	NR041379.1	*Bacillus panaciterrae* strain Gsoil 1517 16S ribosomal RNA, partial sequence	0.0	96%
5.	NR042286.1	*Bacillus herbersteinensis* strain : D-1,5a 16S ribosomal RNA, complete sequence	0.0	96%
6.	NR026144.1	*Bacillus halmapalus* strain DSM 8723 16S ribosomal RNA, partial sequence	0.0	96%
7.	NR026138.1	*Bacillus cohnii* strain DSM 6307 16S ribosomal RNA, partial sequence	0.0	96%
8.	NR028624.1	*Bacillus funiculus* strain NAF001 16S ribosomal RNA, partial sequence	0.0	96%
9.	NR028709.1	*Bacillus siralis* strain 171544 16S ribosomal RNA, partial sequence	0.0	96%
10.	NR042726.1	*Bacillus circulans* 16S ribosomal RNA, partial sequence	0.0	96%

Table 4. Sequence homology of APDB10 strain (Zheng *et al*., 2000).

S #	Accession	Description	E value	Max indent
1.	NR043403.1	*Bacillus thuringiensis* strain IAM 12077 16S ribosomal RNA, partial sequence	0.0	99%
2.	NR041248.1	*Bacillus anthracis* strain ATCC 14578 16S ribosomal RNA, partial sequence	0.0	99%
3.	NR024697.1	*Bacillus weihenstephanensis* strain DSM11821 16S ribosomal RNA, partial sequence	0.0	99%
4.	NR036880.1	*Bacillus mycoides* strain 273 16S ribosomal RNA, partial sequence	0.0	99%
5.	NR025511.1	*Bacillus luciferensis* strain LMG 18422 16S ribosomal RNA, partial sequence	0.0	97%
6.	NR043774.1	*Bacillus acidiceler* strain CBD 119 16S ribosomal RNA, partial sequence	0.0	97%
7.	NR025240.1	*Bacillus marisflavi* strain TF-11 16S ribosomal RNA, partial sequence	0.0	96%
8.	NR025373.1	*Bacillus shackletonii* strain LMG 18435 16S ribosomal RNA, partial sequence	0.0	96%
9.	NR041942.1	*Bacillus acidicola* strain 105-2 16S ribosomal RNA, complete sequence	0.0	96%
10.	NR026144.1	*Bacillus halmapalus* strain DSM 8723 16S ribosomal RNA, partial sequence	0.0	96%

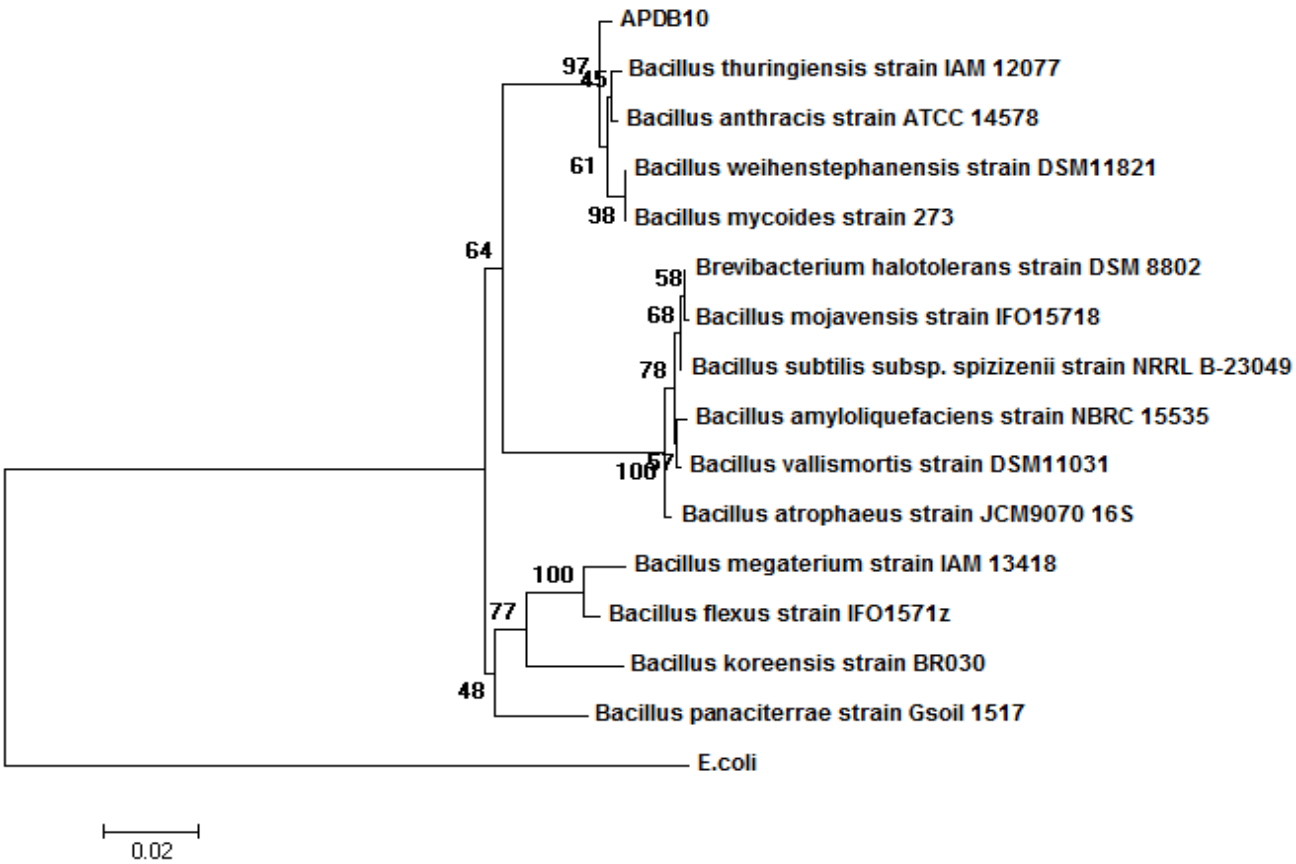

Fig. 3. Evolutionary relationships of strain APDB10. The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The optimal tree with the sum of branch length = 0.24932729 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al*., 2004) and are in the units of the number of base substitutions per site. The analysis involved 16 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 718 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (Tamura *et al*., 2011).

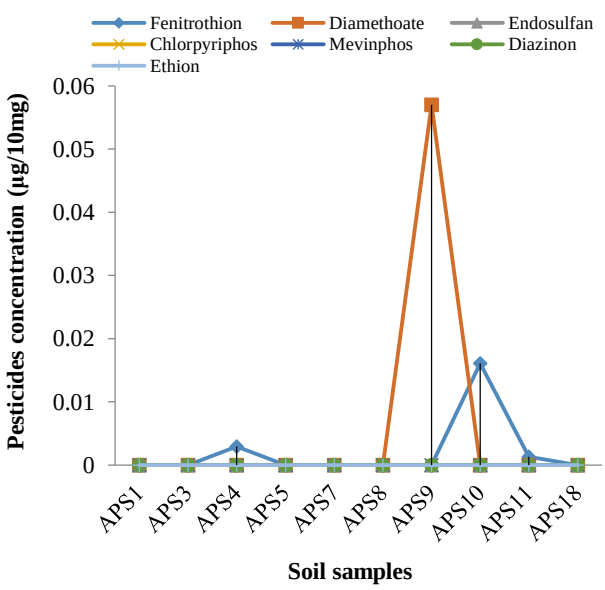

Fig. 4. Detection of pesticides in soil samples.

Genes for pesticides degradation may either be located on plasmid DNA or chromosomal DNA. In most of the studies, *opd* gene was observed on plasmid having similar DNA sequence. However, *opd* genes with different nucleotide sequence located on plasmid DNA have also been reported (Kim *et al*., 2007; Kim *et al*., 2009; Rangasamy *et al*., 2018). The bacterial strains of this study contained plasmids which ranged from 0.76 kb – 21kb in size (Fig. 1). All of the strains contained a large plasmid of 21kb, which suggests that it may be a supportive element for pesticides tolerance and degradation in these bacterial strains. However, Horne *et al*., (2002) reported a chromosome based *opd* gene in *Agrobacterium rediobacter* with similar sequence. Similarly Singh *et al*., (2004) also reported *opd* to be present on chromosomal DNA but with a different sequence from that of existing evidences. There are some other reports as well about *opd* genes having different nucleotides sequence (Mulbry, 2000; Zhongli *et al*., 2001). In present study none of the bacterial strains showed *opd* gene amplification through PCR, which may be due to different sequence than that of already reported ones. Choi *et al*., (2009), with almost similar results, reported bacteria with organophosphate hydrolase activity but the *opd* gene could not be amplified from those bacterial strains. Same results were found by Kim *et al*., (2009). They isolated fenitrothion-degrading bacteria from soil, related to *Burkholderia*, *Pseudomonas*, *Sphingomonas*, *Cupriavidus*, *Corynebacterium*, and *Arthrobacter* but none of isolates showed PCR amplification of organophosphorus pesticide hydrolase genes.

Conclusion

The data of the present study suggests the presence of a novel *opd* gene with different nucleotides sequence in the bacterial isolates. Therefore investigations on these new hydrolase genes would provide more information about the pesticides degradation process and would help to understand the degradation pathway as well.

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