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Isolation and Determination of Efficacy of Acephate Degrading Bacteria from Agricultural Soil

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Abstract: *Organophosphorus pesticides are heavily employed worldwide for their broad insecticidal spectrum and remains a major environmental pollutant. The degradation efficiency of bacteria isolated from pesticide contaminated agricultural paddy field soil was investigated against organophosphorus pesticide acephate. Five isolates identified as Lysinibacillus fusiformis ADI-01, Pseudomonas pseudoalcaligenes ADI-03, Pseudomonas species ADI-04, Pseudomonas pseudoalcaligenes ADI-06 and atypical VP negative Bacillus cereus ADI-10 were capable to degrade organophosphorus pesticide acephate up to 500 ppm concentration in mineral salt medium. The growth was evaluated by absorbance value, plating on mineral salt agar containing acephate. The degradation of pesticide was further monitored by HPLC analysis. The results reveal the potential of these bacteria on degradation of pesticide and lead to the development of bioremediation strategy to minimize pesticide contamination on land.*

Keywords: *Acephate, Organophosphorus pesticide, Biodegradation, Bacillus cereus, Lysinibacillus fusiformis, Pseudomonas pseudoalcaligenes.*

I. Introduction

All over the world, the use of pesticides is increasing because of the need to feed the world's ever expanding population while the amount of the land for the food production is diminishing. The increase in the use of the pesticides has increased both the hazards to human health as well as pollution of the environment [1]. Worldwide, deaths and chronic illness due to pesticide poisoning amount to about 1 million [2].

Among the various groups of pesticides that are being used the world over, organophosphorus group forms a major and most widely used group and accounts for more than 36% of the total world market [3]. The organophosphorus pesticides are used in agriculture, gardens and veterinary practices [4]. Despite their biodegradable nature, some are highly toxic and their residues are found in the environment [5].

Acephate (O,S-dimethyl acetylphosphoramidothioate) is an important systemic organophosphorus insecticide with toxicity attributed to mammalian metabolism and bioactivation to methamidophos which act as an acetyl cholinesterase (AchE) inhibitor [6]. Acephate was found to have longer half life than methamidophos in soil [7]. Acephate does not undergo photolysis and it's half life in pH 5 to 7 is 20 days at 40^{o} C and it is least stable in alkaline condition than acidic [8].

Acephate are not restricted to anticholinesterase action [6] and also as a genotoxic agent, it induces DNA damage in human lymphocytes [9]. Such serious health consequences signify a requirement for a better understanding of the fate of acephate in the environment and the development of safe, reliable and eco friendly technologies for the elimination of acephate and other organophosphorus compounds from contaminated areas [10]. Biodegradation is a natural process where the pesticide degradation done by microorganisms is primarily a strategy for their own survival [11]. Microorganisms from agricultural soil have the effect on persistence of most pesticides in soil [12]. Bioremediation is the cost effective process than conventional methods to clean up the hazardous chemicals on contaminated site using microorganisms and help in recovery of contaminated site [13]. In this present study, bacteria capable of degrading acephate were isolated from a pesticide contaminated site and identified. Their degrading efficiency was studied using various concentrations of acephate and compared.

II. Materials And Methods

2.1 Collection of soil sample: The rhizosphere soil of paddy field was collected from the agriculture land at Thiruvallur district, Tamil Nadu, India. The soil sample was collected at 10^{th} day after manifestation of pesticide on the field. The soil sample was stored at 4^{o}C till processing.

2.2 Pesticide used : Pesticide used in this present study was Acephate, an Organophosphorus pesticide.

2.3 Mineral salt medium: Mineral salt medium is a commonly used medium to check degrading ability of bacteria on specific compound. The composition of mineral salt medium is given in Table 1. Mineral salt medium contains basic minerals, trace elements and lack carbon source.

2.4 Isolation of pesticide degrading bacterial isolates

Five grams of rhizosphere soil sample contaminated with pesticide was inoculated into 50 ml of sterile mineral salt broth supplemented with acephate (50 ppm) as a sole carbon source in 100 ml Erlenmeyer flask [14]. The flask was incubated at 37^{o}C for 72 hours in shaker water bath (100 rpm). After 72 hours of incubation, 5ml of aliquots was transferred in to 45ml of fresh sterile mineral salt broth containing acephate (50 ppm). Aliquots from both flasks were plated on mineral salt agar containing 50 ppm of acephate using spread plate technique. The plates were incubated at 37^{o}C for 72 hours. Ten bacterial isolates were obtained after streaking to purity while fungal isolates were discarded and colony morphologies of bacterial isolates were noted. Among the ten isolates, five representative isolates ADI-01, ADI-03, ADI-04, ADI-06 and ADI-10 were selected for further analysis based on its growth on mineral salt agar as pure culture and stored on agar slant at 4^{o}C.

2.5 Identification of isolates

Identification of bacterial isolates were carried out by the routine bacteriological methods (i.e) by colony morphology, preliminary tests like Gram staining, Motility, Catalase, Oxidase, plating on selective media like Pseudomonas isolation agar, Skim milk agar, Cetrimide agar, Starch agar, special staining techniques like Endospore staining, Capsule staining and by performing biochemicals which includes Indole, Methyl red, Voges proskauer, Citrate, Urease, Nitrate, Triple sugar iron tests. The species level identification of four isolates [ADI-01, ADI-03, ADI-06, ADI-10] were done by 16S rRNA gene sequencing method.

2.5.1 Protocol of 16S rRNA sequencing

1. Bacterial Genomic DNA isolation kit – InstaGene TM Matrix genomic DNA isolation kit catalog #732-6030.
2. PCR machine - MJ research PTC-225 Peltier Thermal Cycler.
3. Sequencing kit - ABI PRISM ® BigDye TM Terminator Cycle Sequencing kit with AmpliTaq® DNA polymerase.
4. Sequencer - ABI 3730×1.

Bacterial genomic DNA was isolated by using the Insta Gene TM Genomic DNA isolation kit catalog #732-6030. Using 16S rRNA universal primers gene fragment was amplified using MJ research PTC-225 peltier thermal cycler.

2.5.2 Polymerase chain reaction

1 microlitre of template DNA was added in 20 microlitres of PCR reaction solution. 27F/1492R primers were used for bacteria and 35 amplification cycles were performed at 94^{o}C for 45 seconds, 55^{o}C for 60 seconds, 72^{o}C for 60 seconds. DNA fragments were amplified about, 1400 bp in the case of bacteria. Positive control (E.coli genomic DNA) and negative control were included in the PCR. Unincorporated PCR primers and dNTPs were removed from PCR products by using Montage PCR clean up kit (Millipore).

2.5.3 Sequencing

The PCR product was sequenced using the 518F/800R primers. Details of PCR and Sequencing primers are given in Table 2. Sequencing reactions were performed using a ABI PRISM ® BigDyeTM Terminator Cycle Sequencing Kits with AmpliTaq® DNA polymerase (FS enzyme) (Applied Biosystems). Single pass sequencing was performed on each template using 16S rRNA universal primers. Fluorescent labelled fragments were purified from the unincorporated terminators with an ethanol precipitation. The samples were re suspended in distilled water and subjected to electrophoresis in an ABI 3730×1 sequencer (Applied Biosystems). Sequenced data was aligned and analysed with their closely related sequences using NCBI BLAST tool after the 16S rRNA sequences were screened for chimeric fragments with the DECIPHER program version 1.10.0 [15]. The sequenced data have been submitted to the Genbank. The phylogeny analysis of our sequences with the closely related sequence of BLAST results was performed. CLUSTALW was used for multiple alignments of sequences and resulting aligned sequences were subjected to phylogeny analysis. The software MEGA 6.0 was used for constructing Neighbor-Joining Phylogenetic Tree and Maximum Composite Likelihood as substitution model [16].

2.6 Determination of efficacy of bacterial isolates on acephate degradation

The five bacterial isolates were inoculated into 5ml of sterile saline separately and aseptically. The tubes were incubated at 37^{o}C for three hours [17]. Six sets of 5ml mineral salt broth tubes were prepared by various concentration of acephate (50 ppm, 100 ppm, 200 ppm, 300 ppm, 400 ppm and 500 ppm). One set of mineral salt broth tubes was served as control. After incubation of 3 hours, 0.1 ml of bacterial cultures were inoculated in 5ml of sterile mineral salt broth tubes containing various concentration of acephate. The tubes were incubated at 37^{o}C for 7 days. At intervals of 1st, 2nd, 3rd and 7th day the optical density was checked at 620

nm and tubes were discarded. Using the 7th day broth culture, spread plating was done on the mineral salt agar in respective concentrations as duplicates to check the bacterial viability from 50 ppm to 500 ppm. The contents from the control tubes containing only mineral salt broth and acephate were also plated to ensure purity. The plates were incubated at 37°C for 72 hours.

2.7 Analysis of acephate degradation by high performance liquid chromatography

The aliquots containing mineral salt medium and acephate at 300 ppm concentration treated with individual isolates were taken and centrifuged at 14000 rpm for 10 minutes. The pellet was discarded and the supernatant was used for HPLC analysis. The cleared supernatants were diluted with methanol and 20 microlitre of aliquots was analyzed. The chromatographic separation was achieved on a reverse phase C18 column and ultraviolet detector set at 225 nm. The flow rate was adjusted at 1 ml/min [18]. The total elution time of analysis was set at 20 min for each run. The retention time of standard and all samples were compared for detecting the acephate degradation.

III. Results

3.1 Isolation and identification of pesticide degrading bacteria

Initial spread plating on mineral salt agar containing 50 ppm acephate using aliquots resulted in isolated colonies. At the completion of 24 hours the colonies were invisible and at 48 hours minute undifferentiated colonies were noted. At 72 hours completion, colonies were small, white to pale coloured, translucent to opaque, 1mm to 2mm in diameter. Well grown representative isolates ADI-01, ADI-03, ADI-04, ADI-06 and ADI-10 were selected and analysed for identification. On Gram staining and motility, isolates ADI-01 and ADI-10 were found to be Gram positive bacilli arranged in chains and motile meanwhile isolates ADI-03, ADI-04 and ADI-06 were identified as Gram negative bacilli and motile. The biochemical characteristics of five isolates are shown in Table 3. Plating on special media, Endospore and Capsule staining further revealed that isolated strains ADI-01 and ADI-10 belonged to the family *Bacillaceae*. The isolated strains ADI-03, ADI-04 and ADI-06 belonged to the family *Pseudomonadaceae*.

The gene sequences of four isolated strains ADI-01, ADI-03, ADI-06 and ADI-10 were analysed with their closely related sequences using NCBI BLAST tool and identified. The strains ADI-01, ADI-03, ADI-06, ADI-10 were identified as *Lysinibacillus fusiformis*, *Pseudomonas pseudoalcaligenes*, *Pseudomonas pseudoalcaligenes*, *Bacillus cereus* respectively based on the high degree of sequence similarity (99%). The sequences data were submitted to Genbank and can be accessible through the accession numbers KM349369, KM349370, KM349371 and KM349372 for the isolates ADI-01, ADI-03, ADI-06 and ADI-10 respectively. Phylogenetic tree exhibiting evolutionary relationship of four isolates are shown in Figs. 1, 2, 3 & 4. Atypical *Bacillus cereus* was found with negative voges proskauer test. The two *Pseudomonas pseudoalcaligenes* strains were differentiated by nitrate test. ADI-04 strain was confirmed as *Pseudomonas sp*.

3.2 Cell growth and acephate biodegradation

The five bacterial isolates were grown on mineral salt medium containing acephate as sole carbon source in the concentration of 50 ppm, 100 ppm, 200 ppm, 300 ppm, 400 ppm and 500 ppm without any additional carbon sources. Turbidity was observed in all concentrations of pesticide.The maximum absorbance at 620 nm of all isolates was observed at 7th day of incubation than day1, day2 and day3. As the day of incubation and concentration of acephate increases, the optical density gradually increases and indicates that acephate act as carbon source and helps in microbial growth. Fig.5 shows the growth of isolates on MSM containing 500 ppm concentration of acephate in day -1, 2, 3 and 7. Maximum optical density of 7th day culture at 500 ppm concentration was shown by *Bacillus cereus* ADI-10 (0.08), followed by *Lysinibacillus fusiformis* ADI-01 (0.07), *Pseudomonas pseudoalcaligenes* ADI-03 (0.06), *Pseudomonas species* ADI-04 (0.06), *Pseudomonas pseudoalcaligenes* ADI-06 (0.06).

The viability of bacterial isolates was checked through plating using spread plate technique. The undiluted sample of day7 of all isolates at all concentrations yielded too numerous to count (TNTC). This assures the cell viability on mineral salt medium containing acephate (50ppm to 500 ppm). The 500 ppm concentration of all isolates were serially diluted from 10^{-1} to 10^{-5}. 0.1 ml from 10^{-4} and 10^{-5} dilutions were plated onto mineral salt agar containing the same concentration (500 ppm). The maximum colony forming unit (cfu) was shown by *Bacillus cereus* ADI-10 (117×10^5 cfu/ml) followed by *Lysinibacillus fusiformis* ADI-01 (96.5×10^5 cfu/ml), *Pseudomonas pseudoalcaligenes* ADI-03 (87×10^5 cfu/ml), *Pseudomonas species* ADI-04 (77.5×10^5 cfu/ml) and *Pseudomonas pseudoalcaligenes* ADI-06 (76.5×10^5 cfu/ml).

3.3 High performance liquid chromatography

Based on the chromatogram obtained through HPLC analysis of the aliquots, it is inferred that all the samples subjected to HPLC analysis for checking acephate degradation showed degradation of acephate into various

other simple compounds. This was indicated by variation of peaks obtained in all the samples in correspondence to varying level of retention time. It is further supported by the variation in the area occupied by each compound in all the sample chromatograms when compared with the standard acephate chromatogram. Table 4 shows the HPLC report for the degradation of acephate at 300 ppm. It explores the retention time and percentage of area obtained in standard and all five samples after treating with respective isolates. Fig.6 reveals the retention time of standard (2.797) which was not found in the HPLC chromatogram of the treated samples. This indicates that the acephate is converted into other compounds by bacterial isolates. Figs.7, 8, 9, 10 & 11 shows the retention time peaks of samples after the treatment with individual isolates. The samples treated with respective isolates resulted in various peaks and differ from each other. Each isolates degraded acephate and converted in to different intermediate compounds in their own capacity and resulted in peaks of different retention times and area which do not correspond with the standard and with one another.

IV. Discussion

Acephate, an organophosphorus pesticide has been extensively used in world agriculture to manage pests or insects of many important crops [19]. The widespread use of organophosphorus pesticides over the years has resulted in problems caused by their interaction with the biological systems in the environment [3]. Microbial degradation is an eco-friendly, highly efficient approach and can be considered as a superior alternative to physical and chemical methods of pesticides degradation [20]. Though there are numerous studies on the biodegradation of other organophosphorus pesticides like chlorpyrifos, malathion, parathion, diazinon, ethion, etc [21] [22] [14], only few reports are available on acephate biodegradation by bacterial isolates [23] [10] [24]. In this present study, biodegrading ability of five bacterial isolates against organophosphorus pesticide acephate was reported and compared. The soil sample was analyzed for the detection of organophosphorus pesticide acephate degrading mesophilic (37^{o}C) bacteria.

In this present study, five best isolates were selected from several isolates obtained with ease from paddy field soil with the history of organophosphorus pesticide contamination. Based on microscopy, biochemical tests and special media, isolate ADI-04 was identified as *Pseudomonas sp.* and with the 16S rRNA gene sequencing analysis species of four isolates were identified as *Lysinibacillus fusiformis* ADI-01, *Pseudomonas pseudoalcaligenes* ADI-03, *Pseudomonas pseudoalcaligenes* ADI-06, *Bacillus cereus* ADI-10. Acephate can be used as a carbon source [10], phosphorous and energy source [23] [24]. No additional energy source added in this present study to assure the degrading capability of bacterial isolates. The process was carried out in neutral pH 7 to prevent the hydrolysis of acephate in alkaline pH, as the stability of acephate is lower in alkaline pH and at pH7 acephate remains for 20 days in mesophilic temperature [8]

Various strains of *Lysinibacillus fusiformis* have been widely employed in biodegradation of environmental contaminants such as hydrocarbon [25], sulfonated azo dyes [26], etc. *Lysinibacillus sp.* isolated from agricultural land has biodegrading activity on herbicide fomesafen and could degrade upto 81.32% within a week incubation [27]. But this is the first report on biodegradability of *Lysinibacillus fusiformis* against organophosphorus pesticide acephate.

B.cereus strains have been reported to degrade various pesticides like chlorpyrifos [28], profenofos [29], malathion [30], Synthetic pyrethoids fenvalerate [31] and acetanilide herbicides [32]. Phosphotonase enzyme secreting from *Bacillus cereus* is responsible for organophosphorus pesticide degradation [21]. *B.cereus* is generally a VP positive organism. VP test is considered as one of the important test for identification of this organism. But in this current study, *B.cereus* with negative VP reaction has been reported. Atypical VP negative *Bacillus cereus* had been isolated from raw milk sample [33], enterotoxigenic VP negative *Bacillus cereus* strains were isolated from *Fenneropenaeus indicus* (white shrimps) [34]. In this study, atypical VP negative *Bacillus cereus* was isolated from paddy field soil and proved its activity against acephate pesticide.

Pseudomonas aeruginosa strain IS 6 is capable to utilize acephate as carbon, phosphorous and energy source and could degrade acephate at 1000 ppm within 7 days of incubation [24]. *Pseudomonas acephalitica* Ind 01 strain can able to grow on acephate as high as 80 mM concentration [10]. Thermal resistance enzyme Phosphoesterase OPCH2 from *Pseudomonas pseudoalcaligenes* exhibited degrading ability against organophosphate compounds [35]. *Chryseobacterium sp.* XP-3 is found to utilize acephate as a nitrogen source along with carbon source and able to multiply and reproduce at 1500 ppm [36]. From these above studies, it has been concluded that acephate can be biodegradable and bacteria can utilize acephate as carbon, nitrogen, phosphorous sources. Previous researches involved bacterial consortium include *Rhodococcus sp.* and *Exiguobacterium sp.* [37], *Pseudomonadaceae* strains, *Chryseobacterium sp.* In this study, *Lysinibacillus fusiformis* ADI-01, *Pseudomonas pseudoalcaligenes* ADI-03, *Pseudomonas sp.* ADI-04, *Pseudomonas pseudoalcaligenes* ADI-06, atypical *Bacillus cereus* ADI-10 are reported against degradation of acephate upto 500 ppm. Nitrate reduction characteristic variation between *Pseudomonas pseudoalcaligenes* ADI-03 and *Pseudomonas pseudoalcaligenes* ADI-06 indicates the strain differentiation.

The mobility of acephate in soil is somewhat faster than that of methamidophos and thus acephate may lead to the contamination of ground water much more easily than methamidophos under normal condition [7]. These five bacterial strains can potentially degrade acephate and convert it in to other compounds and thus can prevent ground water contamination and environmental pollution.

V. Figures And Tables

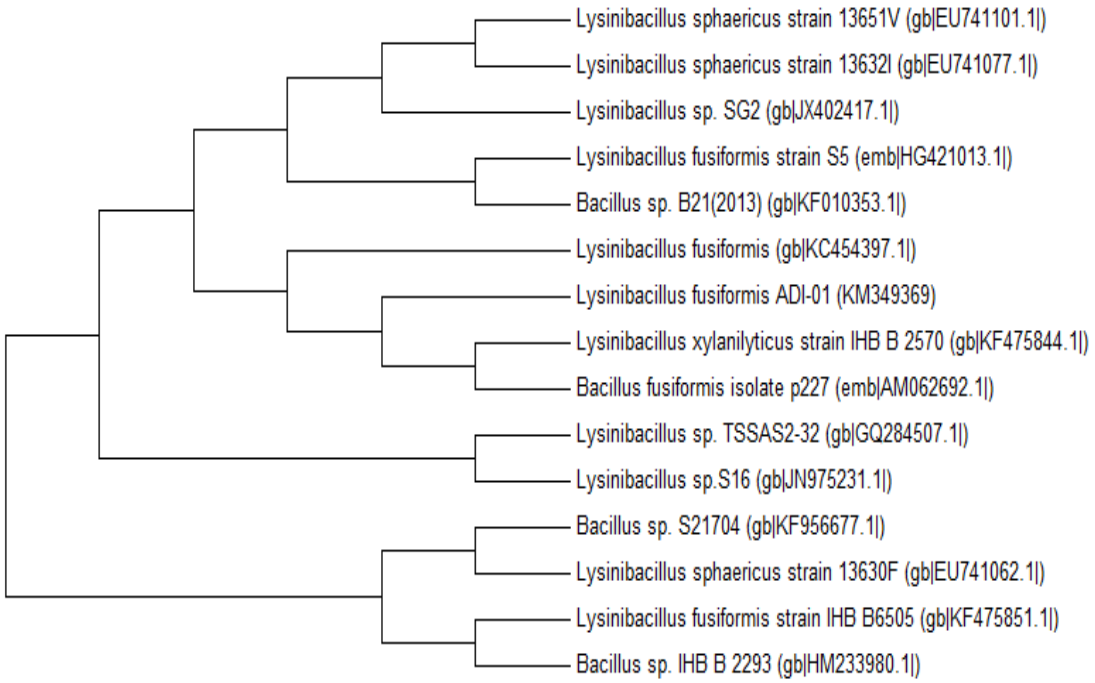

Fig.1. Phylogram of *Lysinibacillus fusiformis* ADI-01 based on 16S rRNA gene analysis and constructed by MEGA version 6.0.

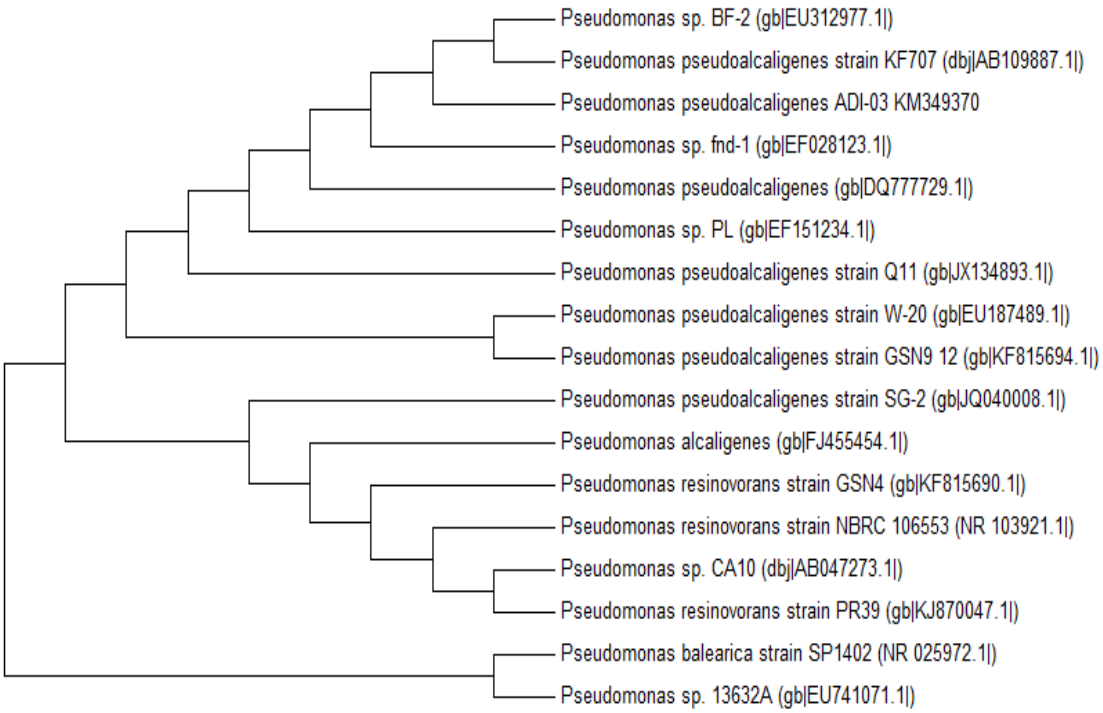

Fig.2. Phylogram of *Pseudomonas pseudoalcaligenes* ADI-03 based on 16S rRNA gene analysis and constructed by MEGA version 6.0.

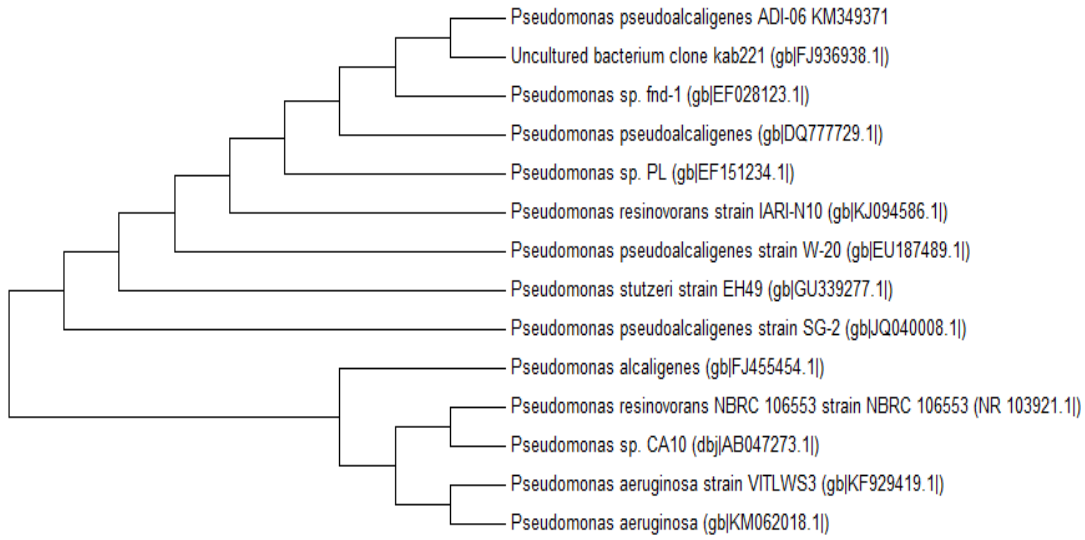

Fig.3. Phylogram of *Pseudomonas pseudoalcaligenes* ADI-06 based on 16S rRNA gene analysis and constructed by MEGA version 6.0.

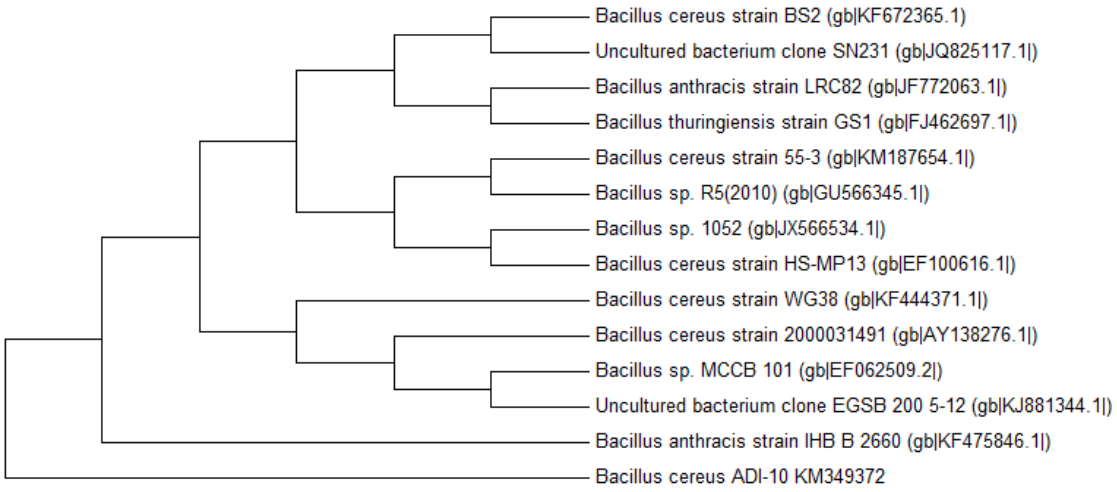

Fig.4. Phylogram of *Bacillus cereus* ADI- 10 based on 16S rRNA gene analysis and constructed by MEGA version 6.0.

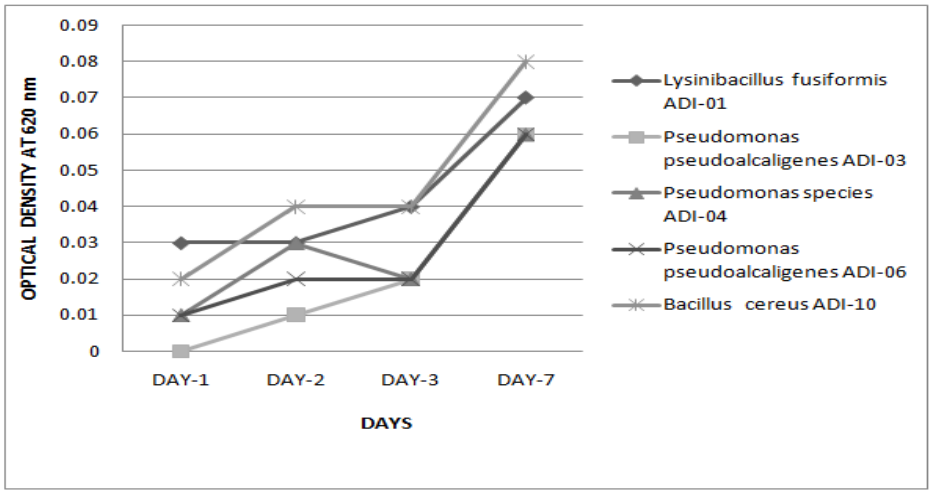

Fig.5. Growth of pesticide degrading bacterial isolates on mineral salt broth containing 500 ppm of Acephate

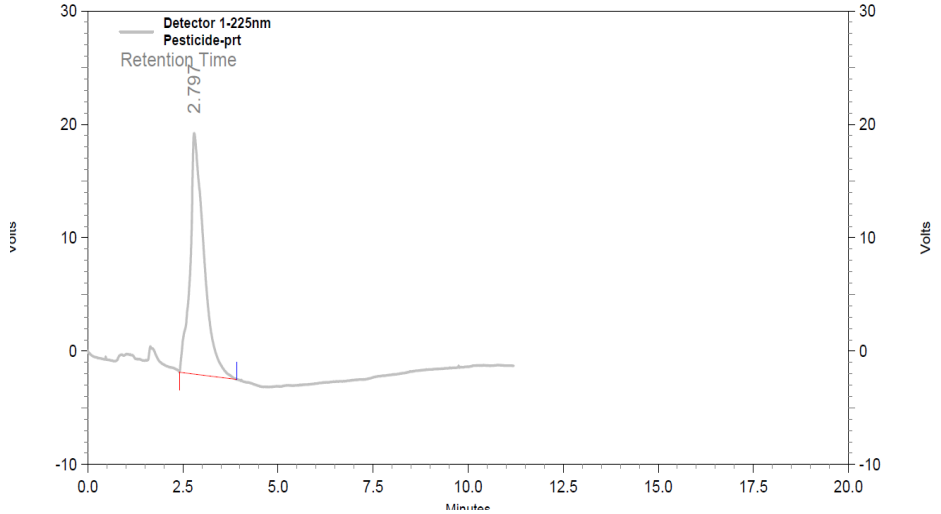

Fig.6. HPLC analysis - retention time peak 2.797 (540427) for standard (Acephate pesticide)

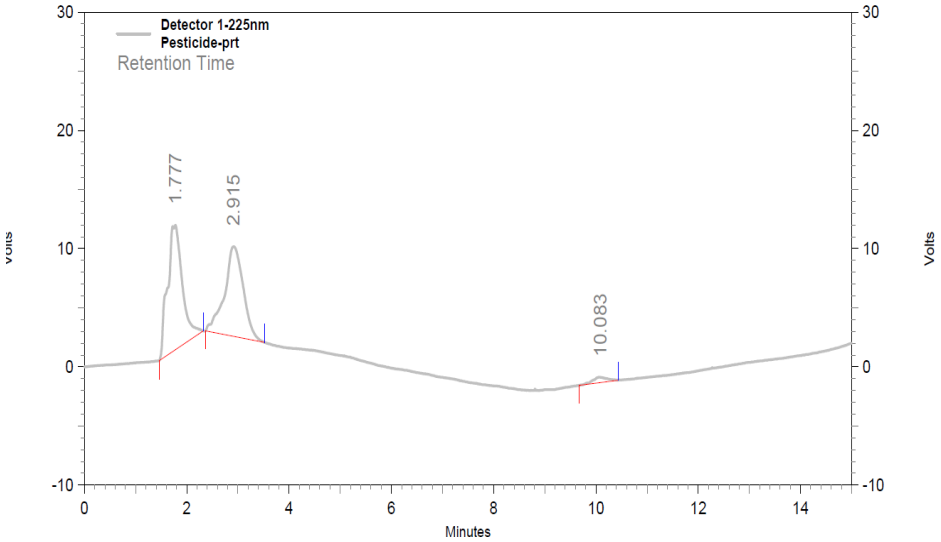

Fig.7. Profile showing peaks at different retention times 1.777 (212437), 2.915 (194323), 10.083 (9152) after a HPLC run for sample (7 th day) treated with isolate ADI-01.

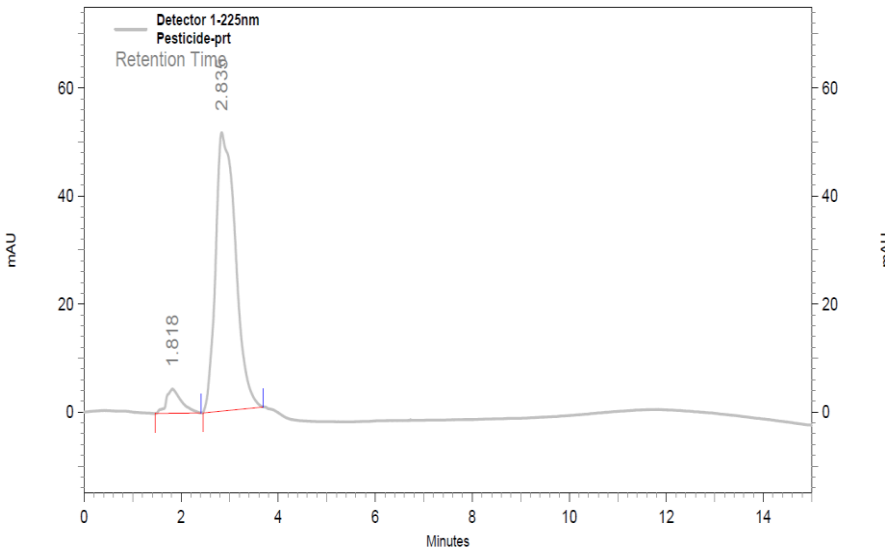

Fig.8. Profile showing peaks at different retention times 1.818 (96506), 2.835 (1472637) after a HPLC run for sample (7 th day) treated with isolate ADI-03.

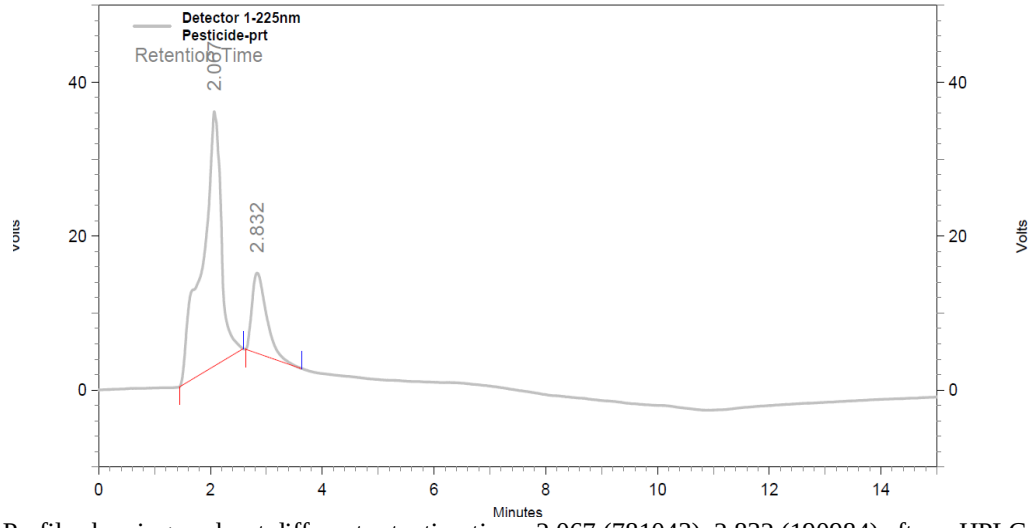

Fig.9. Profile showing peaks at different retention times 2.067 (781043), 2.832 (190984) after a HPLC run for sample (7 th day) treated with isolate ADI-04.

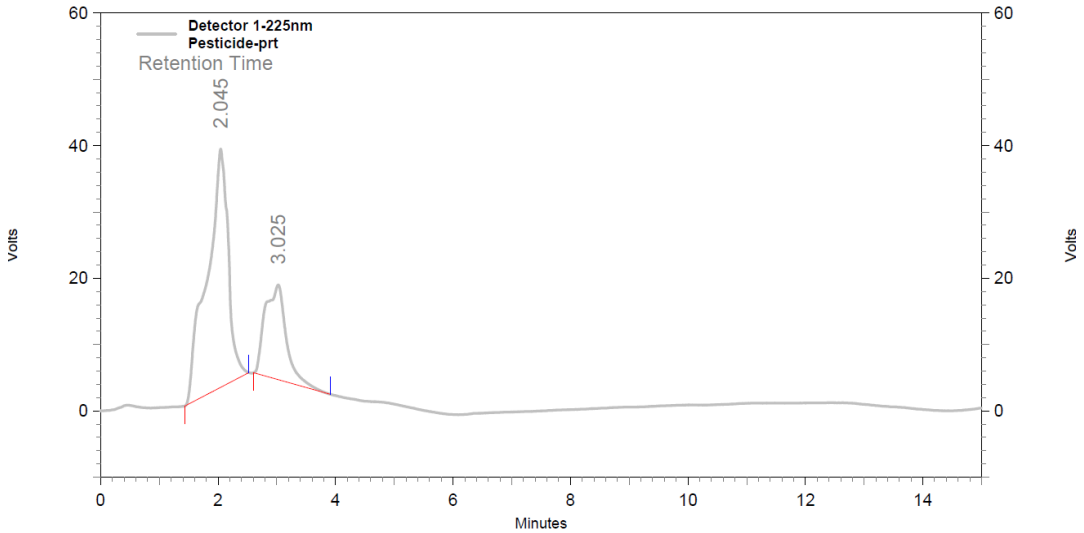

Fig.10. Profile showing peaks at different retention times 2.045 (897793), 3.025 (355990) after a HPLC run for sample (7 th day) treated with isolate ADI-06.

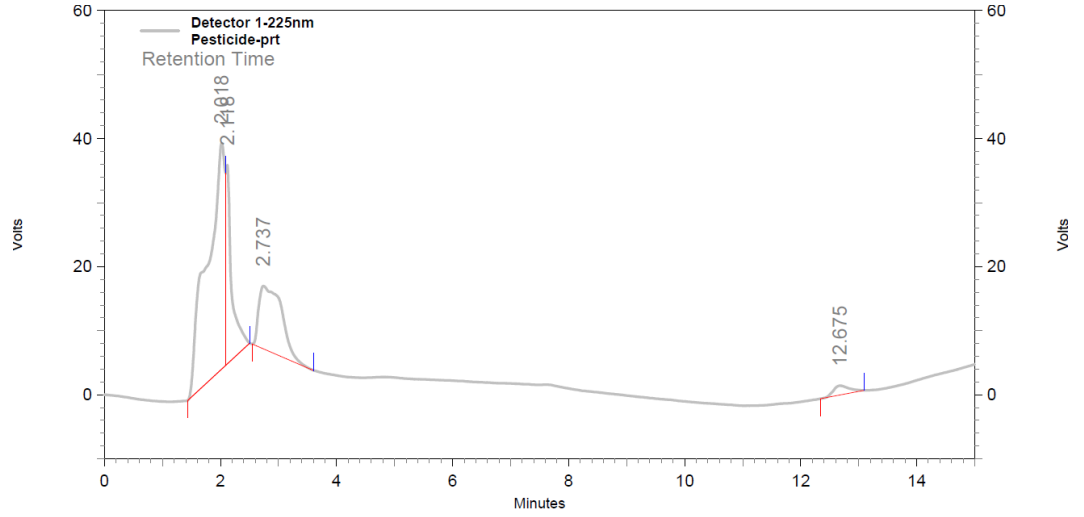

Fig.11. Profile showing peaks at different retention times 2.018 (736922), 2.118 (213737), 2.737 (280233) and 12.675 (27583) after a HPLC run for sample (7 th day) treated with isolate ADI-10.

Table 1. Composition of Mineral salt medium

Components	g/ l
Disodium hydrogen phosphate	4
Monopotassium phosphate	1.5
Ammonium chloride	1
Magnesium sulphate heptahydrate	0.2
Ferric ammonium citrate	0.005
Hoagland trace element solution	1 ml
pH	7.0

Table 2. Information of Primers name, Sequence details and Number of bases

Primer name	Sequence details	Number of base
27F	AGAGTTTGATCMTGGCTCAG	20
1492R	TACGGYTACCTTGTTACGACTT	22
518F	CCAGCAGCCGCGGTAATACG	20
800R	TACCAGGGTATCTAATCC	18

Note : Primers 27F & 1492R for PCR Amplification, Primers 518F & 800R for sequencing.

Table 3. Biochemical results of Pesticide degrading Bacterial isolates

Biochemicals	Isolate ADI-01	Isolate ADI-03	Isolate ADI-04	Isolate ADI-06	Isolate ADI-10
Catalase	+	+	+	+	+
Oxidase	+	+	+	+	-
Indole	-	-	-	-	-
Methyl Red	-	-	-	-	-
Voges proskauer	-	-	-	-	-
Citrate	+	+	+	+	+
Urease	+	-	-	-	-
Nitrate	-	+	+	-	+
Triple sugar iron	K/K	K/K	K/K	K/K	K/A

Note : (+) – Positive, (-) – Negative, (K) – Alkaline, (A) – Acid.

Table 4. HPLC analysis report of standard and all five samples after treating with pesticide degrading isolates for Acephate at 300 ppm.

Sample	Retention Time[Min]	Area %
Standard	2.797	100
Sample treated with isolate ADI-01	1.777	51.08
	2.915	46.72
	10.083	2.20
	Total	100
Sample treated with isolate ADI-03	1.818	6.15
	2.835	93.85
	Total	100
Sample treated with isolate ADI-04	2.067	80.35
	2.832	19.65
	Total	100
Sample treated with isolate ADI-06	2.045	71.61
	3.025	28.39
	Total	100
Sample treated with isolate ADI-10	2.018	58.56
	2.118	16.98
	2.737	22.27
	12.675	2.19
	Total	100

VI. Conclusion

From this present study, it has been concluded that the bacterial isolates *Lysinibacillus fusiformis, Pseudomonas pseudoalcaligenes, Pseudomonas sp., Bacillus cereus* (VP negative) have the capability of utilizing acephate as a carbon source. These five isolates are efficient to grow and degrade acephate upto 500 ppm without the addition of any extra carbon source. The pesticide degrading ability of *Bacillus* isolates are higher than *Pseudomonas* isolates and comparatively maximum growth exhibited by *Bacillus cereus* ADI-10 followed by *Lysinibacillus fusiformis* ADI-01, *Pseudomonas pseudoalcaligenes* ADI-03, *Pseudomonas sp.* ADI-04, *Pseudomonas pseudoalcaligenes* ADI-06. To the author's knowledge this is the first documentation of

bacterial isolates especially *Lysinibacillus fusiformis*, atypical strain of *Bacillus cereus*, *Pseudomonas pseudoalcaligenes* are involving in degradation of pesticide acephate. These bacterial isolates can be further analysed on degradation of higher concentration of pesticide and pathogenic aspects for bioremediation to clean up the pesticide contaminated land.

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