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Isolation, Characterisation and Screening of Acephate Degrading Bacteria from Paddy Field

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Abstract: The extensive usage of pesticides to meet the demand for food and feed has led to many environmental problems. Approximately 90% of agriculture pesticides never reach their target insects but are accumulated in the soil as soil pollutants. Owing to their toxicity and persistence in the environment, most of them are banned in the world. Bioremediation constitutes an attractive alternative to physico- chemical methods of remediation, as it is less expensive and can selectively achieve complete destruction of organic pollutants. Microorganisms play a major role in the breakdown and mineralization of these pollutants as bioremediation. For a successful bioremediation technique, an efficient bacterial strain that can degrade the largest pollutant to a minimum level is required. An enrichment culture technique was used to isolate bacterial strains from paddy soil infested with high concentrations of acephate. Sixteen pure cultures were isolated and subsequently processed to determine the growth activity with increasing concentration of acephate. Growth curve experiments showed that the bacterial isolates were able to grow in medium containing acephate as the sole source of carbon. Four strains were selected for further studies which showed more growth. Bio degradation studies were conducted by FTIR and GC-MS. The metabolites produced after acephate degradation were characterized and identified. The degradation conditions were optimized at a temperature of 37 ^{o}C and pH 7.5 with 200 ppm of acephate for the selected strain producing maximum number of metabolites. Thus, such findings may be useful in designing bacteria that can be used to return the altered environment to its original condition. Hence, this study is focused on the isolation of indigenous insecticidophilic bacteria from polluted soil.

Keywords: Bioremediation, Mineralization, Insecticidophillic, Pesticides, Acephate, Bacteria

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Introduction

The uncontrolled use of pesticides throughout the globe necessitates the need for detoxification of residues by novel methods as they can cause acute and chronic health hazards among consumers. Because of their broad spectrum specificity and great efficacy against insects and pests,

organophosphate chemicals are among the most commonly used pesticides. Acephate has been used extensively for many years to eradicate pests from rice, wheat, cotton, tea, tobacco, and vegetables. Methamidophos is a class IV "Highly toxic" pesticide, whereas acephate is a class II "Moderately dangerous" insecticide (WHO, 2009). Even though it is less effective, the decomposition of acephate, o, s-dimethyl acetyl phosphoramido-thioate results in the more hazardous metabolite methamidophos. Acephate has half-lives of less than 3 and 6 days in aerobic and anaerobic soils, groundwater, and plants, respectively, according to the US Environmental Protection Agency (EPA). Although their half-lives in stored rice can likewise be up to 23 and 40 days, respectively, acephate and methamidophos are not affected by the initial processing of food grains (Kong *et al*., 2012). Acephate has a high water solubility of 790 g L^{-1} and low organic carbon water partitioning co-efficient of log K_{oc} 0.48 and can easily contaminate ground water and soil which in turn is absorbed by plants and is accumulated in edible parts of plants (Mohopatra *et al*., 2011, Syed *et al*., 2014). Acephate and methamidophos have a low metabolic capacity in humans, which can lead to hyperglycemia, lipid metabolism issues, DNA damage, increased oxidative stress, and a higher risk of carcinogenesis with prolonged exposure (Chang *et al*., 2009). It will hurt the nervous and respiratory system, and cause eye and gastrointestinal problem in human (Tetsuo and Milan, 2014; Kumruzzaman and Sarker, 2017).

Physical and chemical techniques like ozonation, ultrasonication,UV/TiO_2,photo Fenton process (Zheng *et al*., 2016), can degrade acephate. Microbial degradation has been considered as the most efficient, environmentally friendly and promising remediation method (Zhang *et al*., 2020). The bacterial and fungal populations in soil can be impacted by the buildup and leaching of acephate and its metabolites, which may also cause DNA damage and encourage infertility in humans (Ozkan *et al*., 2009, Dhanushka and Peiris, 2017). Due of their enormous negative impacts on the animal race, they are major source of public worry.

Several bacteria have been identified from soils that have improved the biodegradation of organophosphorus insecticides, either in pure or mixed cultures (Dimitrios and Allan, 2000). It has been hypothesised that acephate in the environment can mineralize to CO_2, methyl mercaptan, and phosphoric acid via methamidophos or O-methyl N-acetyl phosphor-amidate, o,s-dimethyl phospho-rothioate (DMPT), and then to CO_2, methyl mercaptan, and phosphoric acid (Pinjari *et al*., 2012). Thus, the present study focused on the isolation, characterisation and screening of bacterial isolates from acephate polluted paddy fields.

Materials and Methods

Collection of soil sample: The soil sample was taken from three agricultural lands in Pandeeswaran, Thiruvallur District, Tamil Nadu, India. It came from a paddy field's rhizosphere. It was gathered on the tenth day following the appearance of pesticide in the field and kept at 4 ℃ until processing.

Pesticide tested: An organophosphorus insecticide called Acephate was employed in the current investigation.

Mineral Salt medium: Mineral Salt Medium (MSM) is typically used to evaluate a compound's capacity for breakdown by a bacteria. It lacks a carbon source but contains fundamental minerals and trace components. Table 1 lists the MSM's composition.

Physico–chemical parameters of soil sample:

Organic materials, air, water, inorganic components, and live organisms are found in soil. The fertility of the soil is determined by the interaction of the mineral and organic matter fractions. The following physico-chemical tests were run on the soil to analyse it for pH, electrical conductivity, organic carbon, and the availability of nitrogen, phosphorous, potassium, and sulphur.

Table 1: Composition of Mineral Salt Medium

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

Screening of pesticide degrading bacterial isolates (*Nivetha and Linnett, 2015*):

In 50 ml of sterile Minimal Salt broth with acephate (50 ppm) as the only carbon source, five g of rhizosphere soil sample was added. The flask was shaken for 72 h at 37 ℃ while being incubated (100 rpm). Following a 72 h incubation period, 5 ml of the aliquot was added to 45 ml of brand-new, sterile broth that contained 50 ppm of acephate. Spread plate technique was used to plate an aliquot from each flask onto mineral salt agar that contained 50 ppm of acephate. The plates were incubated for 72 h at 37 ℃.

Morphological and Biochemical characteristics of bacterial isolates (*Ibrahim et al., 2015*):

The common bacteriological techniques, such as colony morphology, microscopic examinations, and biochemical testing, were used to identify the bacterial isolates. Colony morphology was observed by streaking them on Nutrient Agar plates. Morphological characters viz. shape, surface, elevation, edge, color and cell morphology were determined by visual observation as well as using a light microscope. The purified colonies were stained with Gram stain and then examined under microscope. The biochemical tests conducted include Indole, Methyl Red, Voges Proskauer, Citrate, TSI, Urease and Oxidase tests.

Determination of efficacy of bacterial isolates on acephate degradation (*Ramu and Seetharaman, 2014*):

Separately and aseptically, the sixteen bacterial isolates were inoculated into 5 ml of sterile saline. Three hours were spent incubating the tubes at 37° C. We prepared four sets of 5 ml Minimal Salt Broth tubes with various acephate concentrations (50 ppm, 100 ppm, 150 ppm and 200 ppm). A single batch of mineral salt broth tubes containing 50 ppm acephate served as the control. After 3 h of incubation, 0.1 ml of bacterial culture was added to five sterile MS broth tubes with varying acephate concentrations. The tubes were incubated for three days at 37 ℃. The optical density was measured at 600 nm for every first, second, and third day, after which the tubes were discarded. Spread plating was carried out on the mineral salt agar in the appropriate concentration as duplicates using the third day broth culture to test the viability of the bacteria from 50 ppm to 200 ppm. To guarantee purity, the contents from the control tubes were also plate-tested and incubated at 37 ℃.

FTIR analysis (*Singh et al., 2017*):

FTIR analysis was performed on Shimadzu 8400s FTIR spectrophotometer using KBr pellets of the dry mass of technical grade acephate and extracted or decomposed metabolites. The FTIR analysis was performed in the mid IR region of 400 – 4000 cm^{-1} with a 16 scan speed.

GCMS Instrumentation (*Pinjari et al., 2012*; *Bhuvaneshwari and Rajendran, 2012*):

To perform GC – MS analysis, 50 ml of the spent medium was clarified by centrifugation at 13200 g, followed by filtration through a 0.2 µm filter.

Table 2: Physico– chemical parameters of Acephate infested soil

Parameter	Value	Unit
pH	7.44	-
Electrical Conductivity	0.38	dS/m
Organic Carbon	0.30	%
Available Nitrogen	165	Kg/ha
Available Phosphorus (Olsen's)	9	Kg/ ha
Available Potassium	186	Kg/ha
Available Sulphur	8.5	Ppm

The clarified medium was acidified with 0.09 N hydrochloric acid and then extracted thrice with an equal volume of ethyl acetate. The pooled organic phase was allowed to air dry, and the remaining residue was dissolved in a minimal volume (500 µl) of methanol and about 1 µl of it was taken for analysis. The samples were analysed in GCMS (Gas Chromatograph Mass Spectrophotometer) (QP 2010 Shimadzu Corp. Japan) equipped with capillary column DB – I (30 m long, ID 0.32 mm) and 5% methyl phenyl silicone. The limit of detection (LOD) of acephate was determined three times of the standard deviation of the blank. Before analysis, standards were run to check for the column performance peak height and resolution. The column, injection port and detector temperatures were maintained at 210, 230 and 250 ℃, respectively. Helium was used as the carrier gas and the flow rate was maintained at 1 mls.

Results and Discussion

Physico–chemical parameters of Acephate infested Soil:

The presence of various microbiological entities is greatly influenced by physico-chemical factors. Soil variables and their relationship to the microbial community must be studied. For the purpose of evaluating the microbial flora, some significant physico-chemical parameters of the soil were identified in the current study (Table 2). The soil sample had a pH of 7.4 and was brown in colour. The soil's texture was sandy loam, and its lime content was non-calcareous.

Screening of pesticide degrading bacteria:

Sixteen bacterial isolates have been obtained from the pesticide infested soil in MSM and named as A1 to A16 (Fig. 1).

Morphological and biochemical characteristics of bacterial isolates:

Colony characteristics on Nutrient Agar plates, cell morphology, gram staining results and biochemical results were shown in Tables 3 and 4.

Cell growth studies using UV –Visible spectrophotometric technique:

Following the discovery of sixteen bacterial isolates, their growth in MSM at various pesticide concentrations was examined. The minimal inhibitory concentration study was used to investigate the tolerance of acephate to various bacterial strains. Acephate was optimally concentrated at 200, 150, 100, and 50 ppm. Using UV-visible spectroscopy, the cell development of each strain was examined at 600 nm (OD=600). After 72 h of incubation, a rise in OD at 600 nm showed that acephate was being used as the only source of carbon and phosphorus. The results indicated that all the 16 isolates were able to utilize acephate in Minimal Salt medium with maximum OD in the following order A4>A2>A5> A11>A14 at an interval of 24 h, 48 h and 72 h with

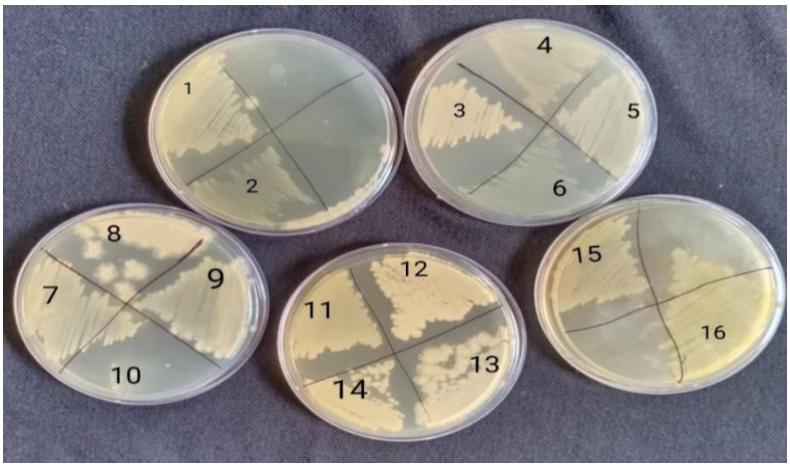

Fig 1: Isolates on Minimal Salt Medium.

Table 3: Colony Characterization of Acephate degrading bacterial isolates

Isolate No.		Colony morphology on Nutrient Agar plates				
	Size	Shape	Elevation	Edge	Surface	Color
PA1	Small	Circular	Raised	Entire	Moist	Pale yellow
PA2	Small	Circular	Raised	Entire	Mucoid	Pale yellow
PA3	Pin pointed	Circular	Flat	Entire	Dry	Cream
PA4	Large	Circular	Flat	Entire	Dry	Cream
PA5	Pin pointed	Circular	Flat	Entire	Dry	Cream
PA6	Large	Circular	Raised	Entire	Mucoid	White
PA7	Small	Circular	Raised	Entire	Moist	Cream
PA8	Small	Circular	Raised	Entire	Moist	Cream
PA9	Large	Circular	Flat	Entire	Dry	Cream
PA10	Small	Circular	Raised	Entire	Moist	Cream
PA11	Large	Circular	Raised	Entire	Moist	Cream
PA12	Small	Circular	Raised	Entire	Moist	Cream
PA13	Small	Circular	Raised	Entire	Moist	Cream
PA14	Small	Circular	Raised	Entire	Moist	Cream
PA15	Small	Circular	Raised	Entire	Moist	Cream
PA16	Small	Circular	Raised	Entire	Moist	Cream

concentration of 200 ppm. The results are shown in Figs. 2-5.

FTIR analysis:

Analysis of pure acephate and decomposed metabolites, obtained after treating with four bacterial isolates after 10 days, with FTIR, was performed to confirm the biodegradation of acephate. The FTIR spectra of standard acephate (Control) (Fig. 6a) and the samples with bacterial isolates infused with known amount of acephate (Figs. 6b, c, d and e for standard, A2, A4, A11 and A14 samples, respectively) were found to be significantly different from each other. The observed changes in peak pattern and shift of the functional groups indicated the conversion of

Table 4: Cell Morphology and Biochemical characteristics of bacterial isolates

Isolate No.	Gram staining	Indole	Methyl red	Voges proskauer	Citrate	TSI	Urease	Oxidase
PA1	G(-) rod	-	-	-	+	K/A	+	+
PA2	G(-) rod	-	-	-	-	K/A	+	+
PA3	G(-) rod	+	-	-	-	K/A	+	+
PA4	G(-) rod	+	-	-	-	A/K	-	+
PA5	G(+) rod	-	+	-	-	A/K	-	-
PA6	G(+) rod	-	-	-	-	K/A	+	+
PA7	G(-) rod	-	+	-	+	K/K	+	+
PA8	G(-) rod	-	-	-	+	K/A	+	+
PA9	G(+) rod	+	+	-	-	A/K	-	-
PA10	G(+)rod	-	-	-	-	K/K	+	+
PA11	G(+)rod	+	-	-	+	K/K	+	+
PA12	G(-) rod	-	+	-	-	K/A	+	+
PA13	G(-) rod	+	+	-	+	K/A, GAS+	-	-
PA14	G(-) rod	-	-	-	-	K/A	-	-
PA15	G(+) rod	-	+	-	-	A/K	+	-
PA16	G(+) rod	-	+	-	+	K/A, H2S +	+	+

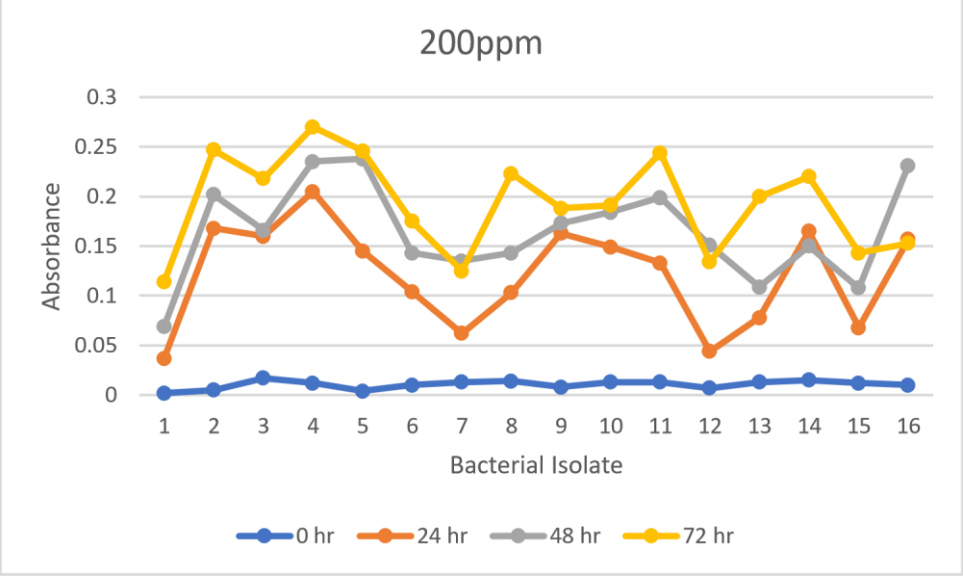

Fig 2: Growth of bacterial isolates in liquid media with 200 ppm of acephate at OD 600.

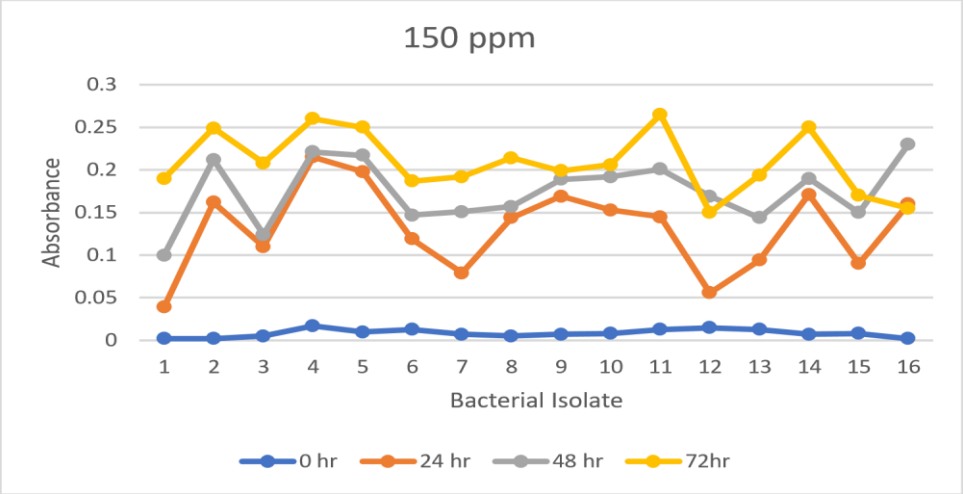

Fig 3: Growth of bacterial isolates in liquid media with 150 ppm of acephate at OD 600.

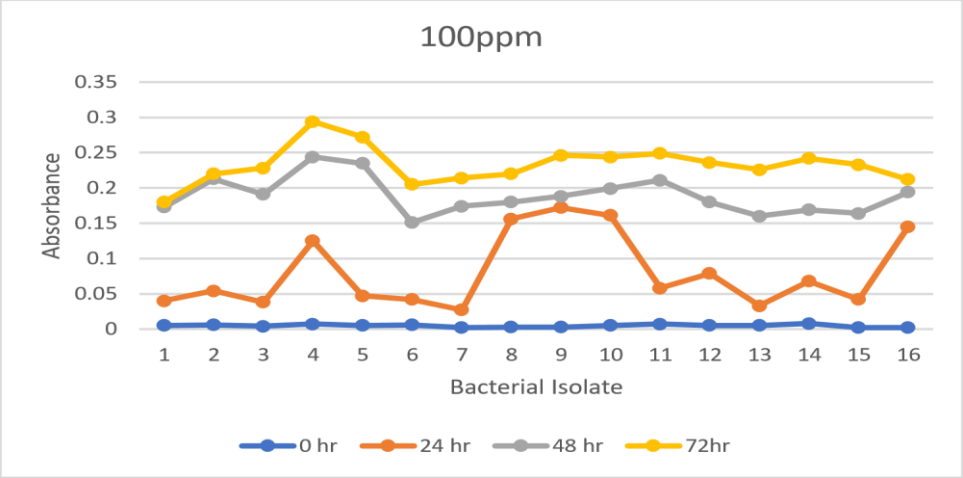

Fig 4: Growth of bacterial isolates in liquid media with 100 ppm of acephate at OD 600.

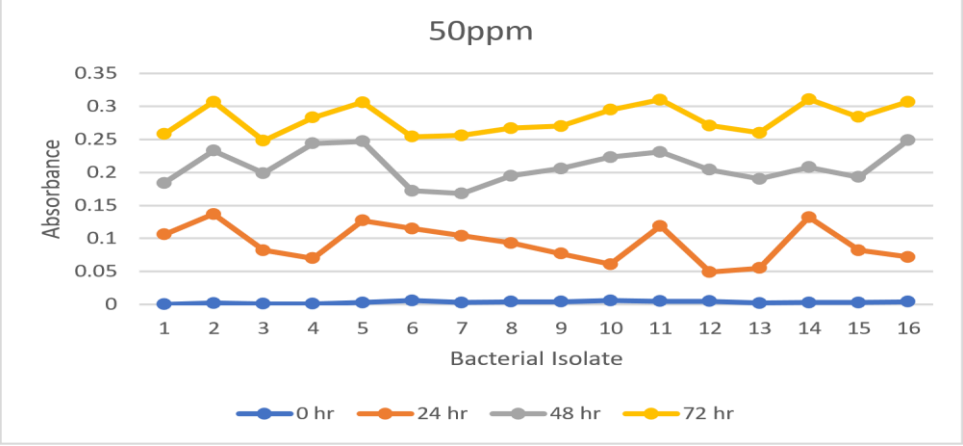

Fig 5: Growth of bacterial isolates in liquid media with 50 ppm of acephate at OD 600.

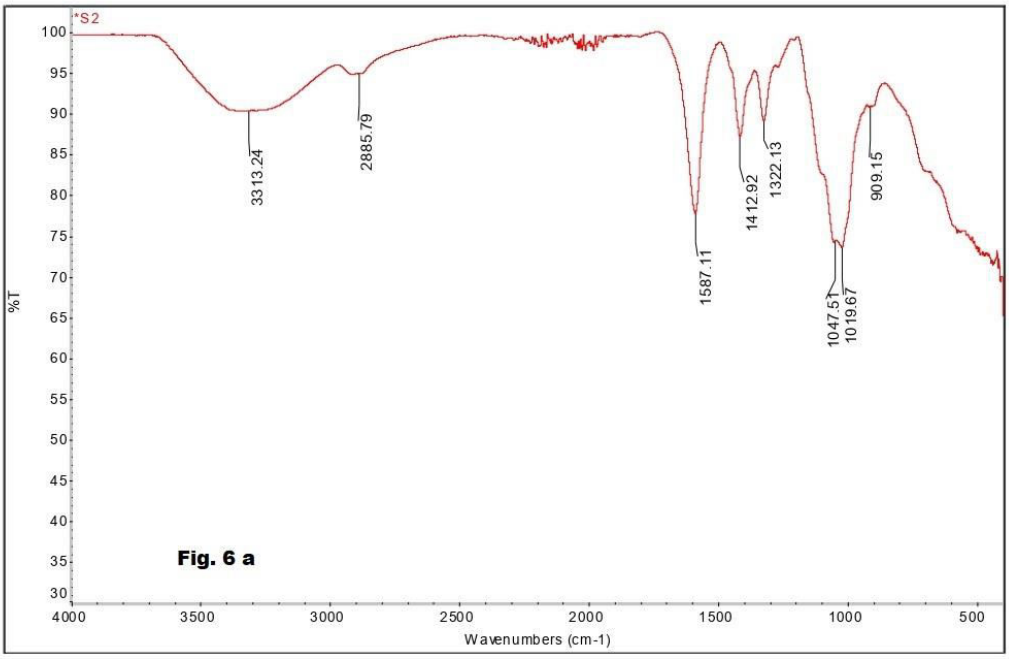

Fig. 6 a

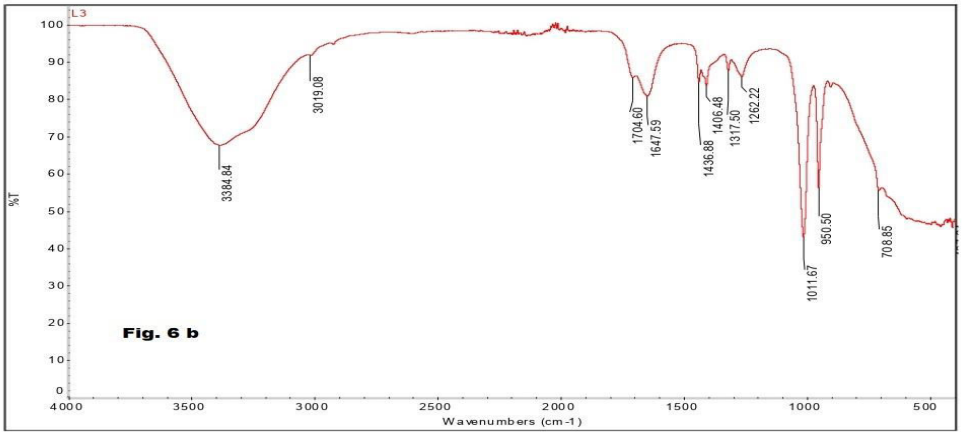

Fig. 6 b

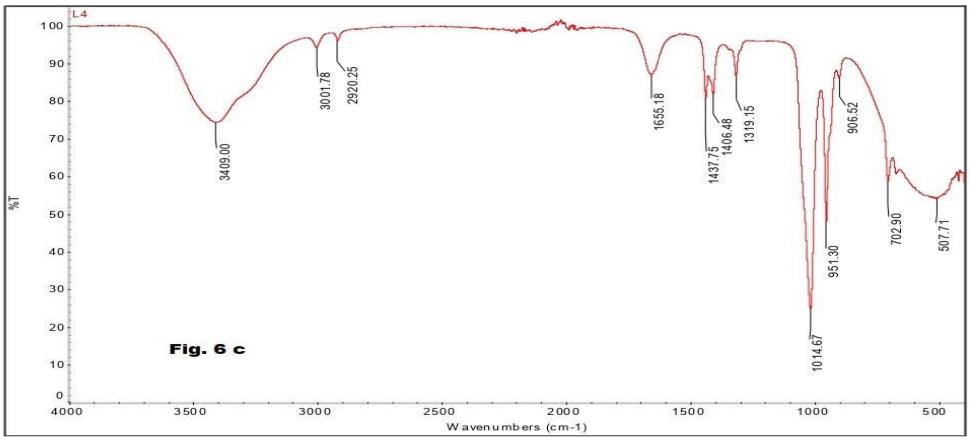

Fig. 6 c

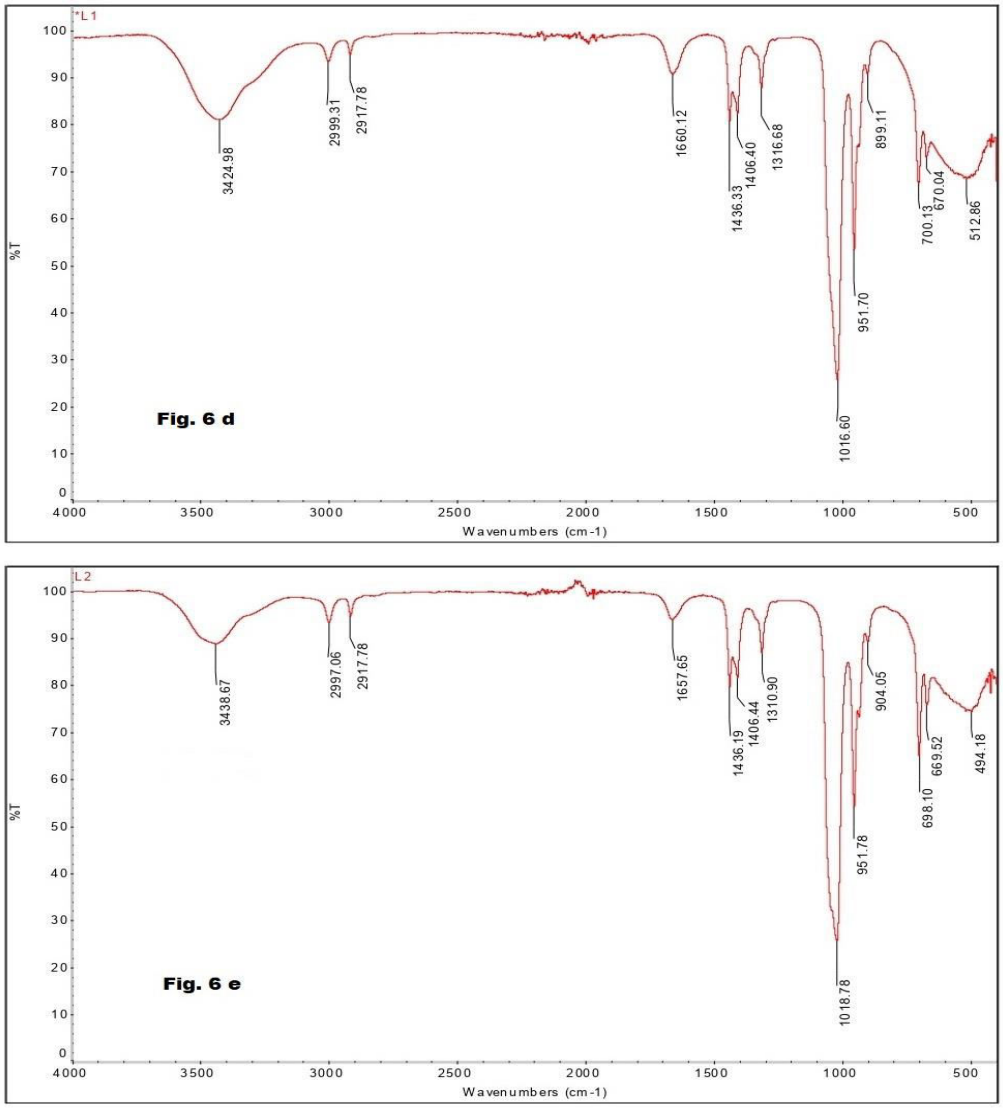

Fig. 6: FTIR analysis of acephate (standard) and four selected isolates PA2, PA4, PA11 and PA14.

acephate to different metabolites.

GC – MS analysis:

There are lots of reports available on the degradation of organophosphorus pesticides but only few reports are available for biodegradation of acephate (Singh *et al*., 2017, 2020). GC MS analysis was carried out to investigate the metabolites produced during the biodegradation process. The gas chromatograms of degraded acephate showed the presence of several peaks. Previous researchers (Pinjari *et al*., 2012; Prasad *et al*., 2013; Ramu and Seetharaman, 2014) have reported that acephate was biodegraded to form metabolites like methamidophos, O-methyl phosphoramidate, O,O- dimethyl phosphorothioate (DMPT). They have reported that acephate was decomposed to toxic metabolite methamidophos. Singh *et al*. (2017) have reported that *Pseudomonas pseudoalcaligenes* strain PS – 5 was capable of mineralization of acephate without formation of toxic metabolite methamidophos. Yuanyuan (2017) investigated and reported that acetamide was the metabolite produced on degradation of acephate and moreover, acephate was degraded more completely in nitrous oxide-

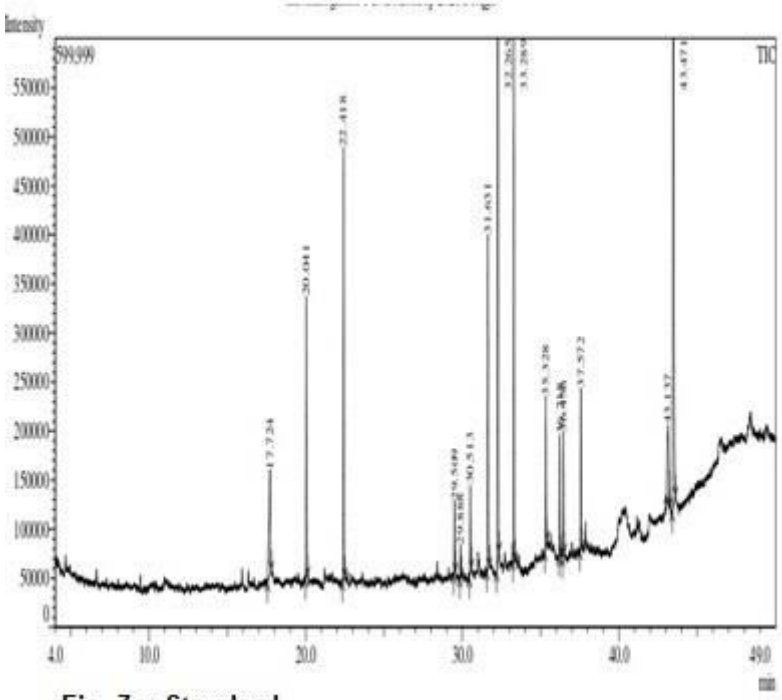

Fig. 7a: Standard

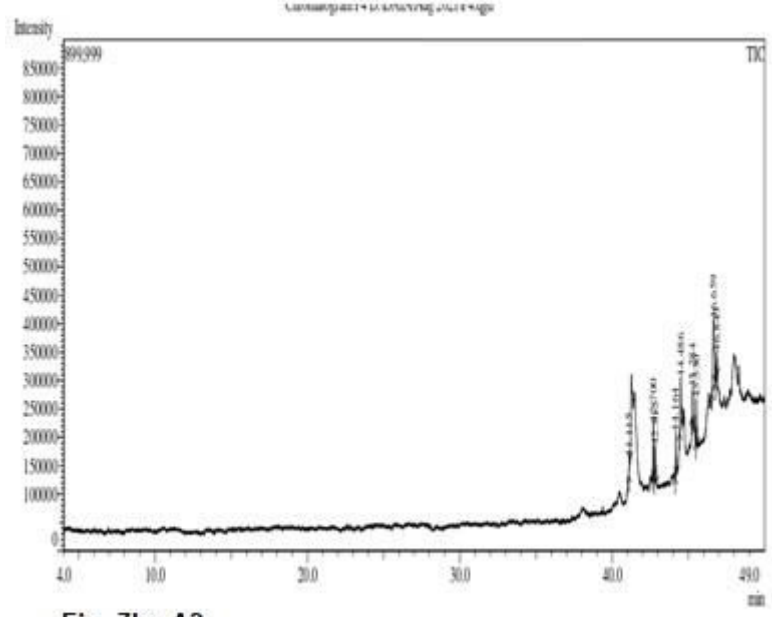

Fig. 7b: A2

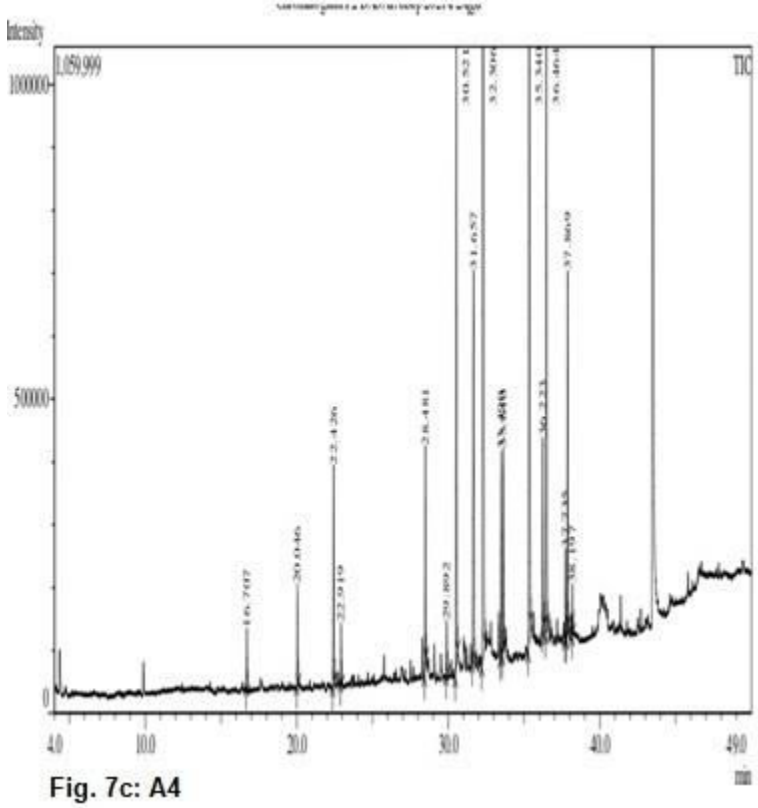

Fig. 7c: A4

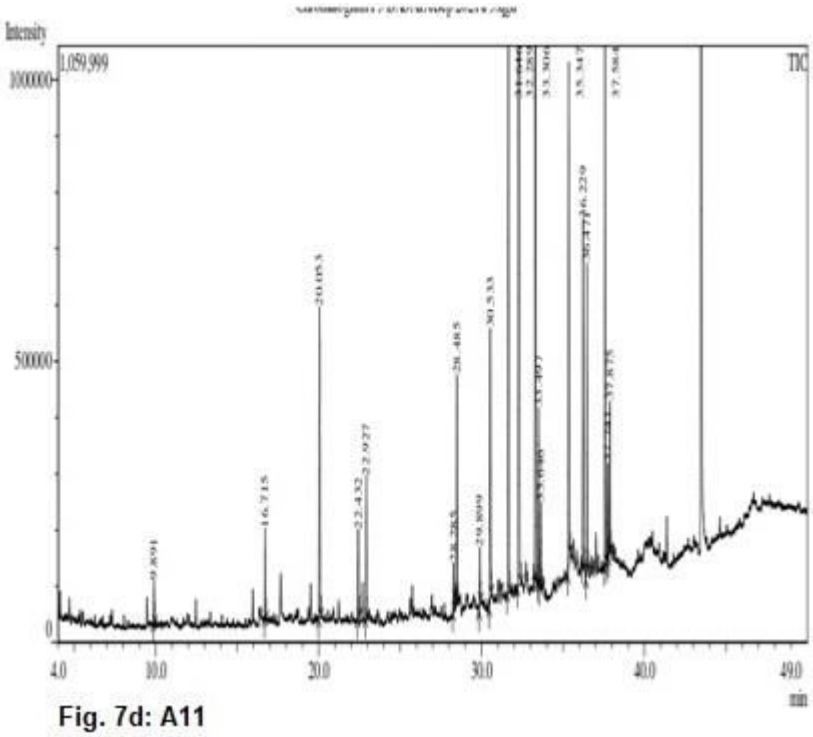

Fig. 7d: A11

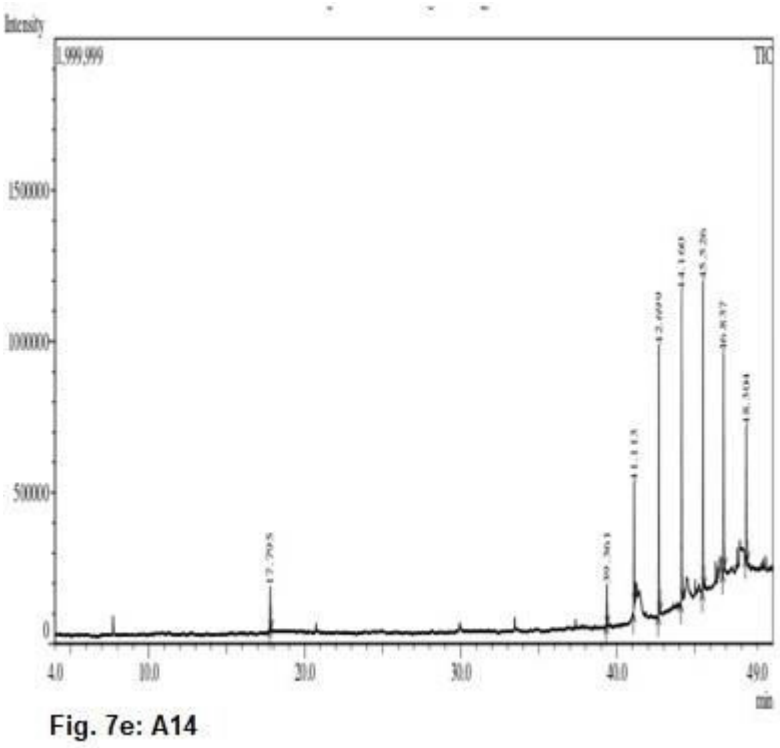

Fig. 7: GC-MS Results of the standard and selected four isolates showing the spectral properties of acephate degradation.

saturated solution than that in N_2-saturated solution containing tert-butyl alcohol. In the present study, A11 strain was found to be capable of metabolising acephate with maximum number of metabolites when compared to A2, A4 and A14 (Fig. 7 b, c, d, e). Figure 7 a depicts the peaks of standard acephate.

Conclusion

Air pollution, reduced biodiversity, altered biogeochemical cycles, leaching, deterioration of soil quality are few of the consequences of extensive infestation of pesticides in the environment. Bioremediators are the only best solution to reduce the pesticide contamination. In this study, 16 bacterial isolates were obtained which were subjected to degradation studies with varying concentrations of acephate. Morphological results showed the presence of Gram positive bacilli and Gram negative bacilli. Based on growth studies, the bacterial isolates A2,A4,A11 and A14 were found to have more capacity of utilizing acephate as a sole carbon source. These isolates were subjected to FTIR and GC MS studies.These bacterial isolates can be further analysed and thus can be used to cleanse the soil in a nontoxic manner.

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