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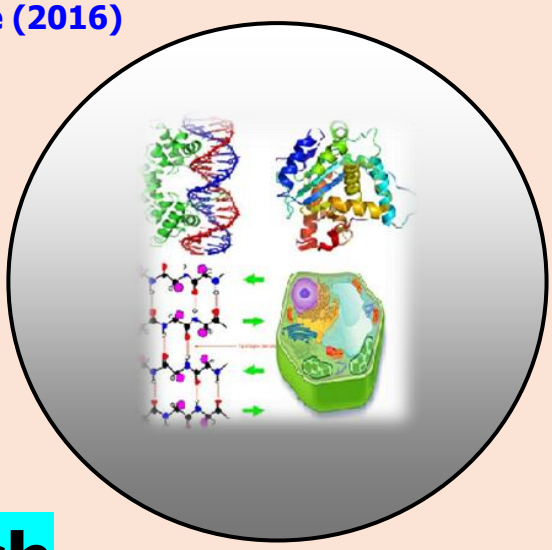

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RESEARCH PAPER

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Soil born *Pseudomonas fluorescens* and *Achromobacter pulmonis* Mediated Degradation of Dimethoate and its Bioassay

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ABSTRACT

Ability to degrade dimethoate by the soil bacterial isolates was investigated by microbial technique. As per sampling two isolates AM1 and AM2 identified by 16S rRNA gene sequencing found to be degrading dimethoate when confirmed by biochemical assays, GCMS and FTIR analysis. The % degradation of 1% dimethoate up to 10 days was 90.66 ± 0.57% and 83.66 ± 0.57% for Pseudomonas fluorescens strain AM1 and Achromobacter pulmonis strain AM2, respectively was recorded. Study highlighted that by GC-MS analysis of degradation metabolites, only one metabolite formed with P. fluorescens AM1 strain and by FTIR its confirmation was received. In conclusion, these two isolates reported to be useful in controlling the pollution caused by dimethoate with effective microbial degradation-based mechanism recorded.
Keywords: Dimethoate, Degradation, Bacteria, GC-MS and Pesticides.

INTRODUCTION

Insecticides Dimethoate classified in Organophosphorus insecticide category is mainly used in agriculture and urban areas as it possesses its high activity with early environmental linked degradation. It is mainly applied in fields to control wide range of insects such as flies, mites, aphids and plant hoppers which are major pests (Mirajkar, et. al. 2005). Application of Dimethoate is widely used in plants such as vegetables, fruit, grains and ornamental. In addition, it is also used in non-agricultural activities such as landscape maintenance and structural pest control. In one estimate, about 816,466 Kg of Dimethoate is applied annually over number of agricultural sites growing plants like wheat, cotton, corn and alfalfa (USEPA, 2008).

As per report of USEPA, (2008), Dimethoate begin an organophosphate insecticide remain soluble in water. It showcases low affinity towards soils and moderate affinity for organic matter. It showcases susceptibility to acidic conditions to undergo hydrolysis, little stable to microbial degradation and remains non-volatile as it possesses low vapour pressure since Dimethoate is less acceptable by soil it tends to shade itself in the receiving water and that possess the water, pollution problem. El Beit, et. al. (1977b) reported soil type which have different leaching feature of insecticide which was showcased below with increase in leaching clay < clay loam < loam < sandy clam loam < sand. El Beit's (1977a) also related increase in soil moisture resulted in an increase in dimethoate's ability to leach. They also related that reduction in organic matter content in soil take part in reducing the potential of biodegradation, but it leads to accelerate in pesticide loss such as evaporation and leaching.

Dimethoate hydrolysis under environmental conditions remains affected by pH of the water along with water hardness. It was observed that hydrolytic half-life of Dimethoate decreased when pH lowered from 8.0 to 7.5 in Thames river water and Irthing River water (Ruzicka, et. al. 1967). According to Druzina and Stegu (2007) in a ground water, at pH 6, Dimethoate half-life recorded to be 94.9 days that to at pH 8.5 it was 66 days. In a river water (pH 8), Dimethoate hydrolysis found to be more rapid and it remained dependant on the temperature (Increases hydrolysis from 4 to 25ºC) with $t_{1/2}$ = 169 days to $t_{1/2}$ = 74.5 days, respectively. Dimethoate also undergoes oxidation by photo catalytic activity as reported by Evgenidou, et. al. (2006). Microbial degradation of Pesticide is one of the strongest process of maintaining proper level of pesticide residue within environment Debmandal, et. al. (2008) investigated *Pseudomonas aeruginosa W171* (water isolate) and *Bacillus linchen iformis Flo2* (Source: *Labeorohita* intestine) and both strains able to degrade complete pesticide within three days. The bacterium *Raoultella sp. X1* remains featured with dimethoate degradation but remain dependent upon environmental and nutritional conditions. In a sole carbon source state dimethoate degradation resulted in poor state; however, 75% of initial concentration was successfully degradation via, co-metabolism (Liang, et. al. 2009). Worker Shinde et al., (2018) also reported the success of Pseudomonas species to degrade dimethoate by involving plasmid-based proteins, which was confirmed by transferring its plasmid in rhizobium species. Rhizobium transformed into dimethoate degrading strain once received plasmid from the *Pseudomonas* sp.Shinde et al., (2015) also stated the application of the dimethoate polluted soil which remain positive for the isolates capable of dimethoate such as *Pseudomonas* sp. A113.

Worker Li, et. al. (2010) put forward number of degradation products and suggested that *Paracoccus sp.* able to degrade dimethoate by involving decarboxylation, oxidation and hydrolysis. Similarly, isolates received from sewage and soil from cotton fields, reported the prevalence of fungi *Aspergillus niger ZHY256* able to degrade about 87% of the dimethoate via cleavage of phosphorus-sulphur (P-S) linkage (Liu, et. al. 2001). In a mass screening of 25 bacterial isolates able to degrade dimethoate; result highlighted that *Pseudomonas aeruginosa MCMB-427* and *Bacillus megaterium MCMB-428* able to degrade 95% of dimethoate and they also recorded the feature of *P. aeruginosa* remained linked with plasmid gene and hence proposed to be transferable to their bacterial species.

In the present study similar approach has been taken to screen the bacterial isolates obtained from potential soil source for its Dimethoate degradation capability under *in vitro* conditions. Promising isolates were checked for its identity and degradation compounds were detected by FTIR and GCMS up to identity level.

MATERIALS AND METHODS

Soil sampling and Inoculation

In the present study promising dimethoate degrading bacterial isolate aimed to be sampled from the dimethoate polluted soil of area Uruli Kanchan, Pune was sampled. In requirement, 250 g of soil from the depth of 20cm was collected in a sterile bag and it was sieved by 25µm sieve. 1g soil sample was then added into the saline (10ml) and shake well to obtain stock culture. One ml stock culture was again added in 9ml saline and from that dilution up to 10^{-4} and 10^{-5} was obtained. Diluted 4ml of sample was inoculated into 250ml of Davis Mengole's medium supplemented with dimethoate (1%). The preparation was kept on shaking at 150rpm for next 5 days at 37ºC for incubation.

By observing promising turbidity, loopful of culture was then plated on DM agar plates supplemented with 1% dimethoate. Inoculated plates were incubated at 37ºC for next 48 hrs. After incubation, colonies appeared on the plates were sub cultured on the same medium containing dimethoate and confirmed for its ability by flooding prepared replica plates with the addition of congo red solution showing the zone of degradation around the growth.

Identification of isolates

After incubation with Congo red, obtained positive isolates were checked from master plate biochemically. Those tests were Oxidase, Catalase, Methyl red, Voges Proskauers, Urease, Nitrate reduction, SIM, Glucose fermentation, Maltose fermentation, Mannitol fermentation, Lactose fermentation, Gelatinase, Motility and Gram staining.

Further isolates were targeted for 16S rRNA gene by using universal primer and conditions suggested by Rai et al. (2013). In result positive sequences of 16S rRNA gene was BLASTN analysed to show case the homology along with phylogenetic analysis by using MEGA6 software.

Dimethoate degradation

Ability to degrade dimethoate by microbial strains was tested in the nutrient agar medium at a variable % concentration by involving the standard N-Bromosuccinimide (NBS) assay at 557nm as per protocol suggested by Gouda et al., (2010). In a result, days wise effect on dimethoate degradation by isolates was recorded as per NBS assay along with variable % concentration in a comparative mode.

Detection of Dimethoate degradation:

Based on the promising result of isolates recorded in NBS assay for Dimethoate degradation; only isolate was considered into detection of metabolites which were positive in NBS assay. In requirement, Thin layer chromatography, FTIR and GC-MS techniques were implemented as per Debmandal et al., (2007) and Nagavardhanam and Zakkula, (2013) protocols.

RESULT

Isolation of 1% dimethoate tolerating isolates

Soil sample of Uruli Kanchan, Pune mainly remain exposed to pesticide dimethoate was considered to be positive for harbouring dimethoate tolerant bacterial isolates. In a response when soil sample was screened for bacterial presence on 1% dimethoate containing DM agar plate which was enriched earlier on liquid medium of same composition, found to be prominent in presence of bacterial population. Based on morphological, biochemical and molecular identification two isolates named as isolate AM1 and AM2 recorded to be promising when firstly isolated by primary screening as shown in Fig.1. It was then subcultured on low concentration Dimethoate (0.1%) on DM media which was grown profoundly as in Fig.2. The colony features of isolate AM1 and AM2 was recoded to be varying in number of aspects as shown in Table 1 and Gram stained as Gram negative short rods (Table 1). As per biochemical tests isolate AM1 found to be positive for all tests such as Oxidase, Catalase, Methyl Red, Voges Proskauers, Urease, Nitrate Reduction, SIM, Glucose, Maltose, Mannitol, Lactose fermentation and Gelatinase. Whereas isolate AM2 showcased positive test for Oxidase, Catalase, Nitrate Reduction, SIM, Glucose and Maltose fermentation only as in Table 2.

Based on 16S rRNA gene sequencing isolate AM1 identified as *Pseudomonas fluorescens*with 99% homology with *Pseudomonas fluorescens*and isolate AM2 was identified as *Achromobacter pulmonis* with 89% homology with *Achromobacter pulmonis* as results recorded in BLASTN and phylogenetic studies (Fig.3 and Fig.4).

Dimethoate degradation

As per NBS assay comparative response of isolate AM1 and AM2 was analysed for the 1% dimethoate degradation in a nutrient medium. Result highlighted that both of the isolates (AM1 and AM2) able to utilize the 1% dimethoate as recorded from 5 days onwards. At a 5^{th} day, significant degradation of 1% dimethoate was recorded with AM1 isolate (62.66 ± 0.57 %) which was significantly ($P< 0.0001$) higher than isolate AM2 degradation % (57 ± 1.00%). Further on day 10 also isolate AM1 dominated isolate AM2 with % degradation as 90.66 ± 0.57 % and 83.66± 0.5%, respectively which remain statistically significant in ($P< 0.0001$) in difference as given in Table 3 and Fig. 5.

Further these two isolates were tested to degrade higher % dimethoate concentration ranging from 1% and up to 19%, comparative result showcased that with the increasing concentration of %, degradation rate went on decreasing and among the isolates only isolate AM2 able to withstand highest concentration i.e., of 19% by degrading its content to 31.66 ± 2.082 % as compared to isolate AM2 which was not been able to grow at the given % concentration of dimethoate and resultant failed to degrade it at 19% of concentration as in Table 4 and Fig. 6.

GC-MS analysis

Upon exposure to 1% dimethoate it has been revealed that among two isolates, isolate AM1 (*P. fluorescens*) found to be better performing. Hence keeping in view, fermentation broth of testing set was compared with control set for their GCMS profile. In a standard set, with the introduction of pure (99%) Dimethoate in GCMS set, the only standard peak of Dimethoate was appeared and confirmed for its purity and presence as in Fig. 7. When isolate *P. fluorescens* allowed to grow in presence of 1% dimethoate till five days, sampled nutrient broth found to be containing the by-product Phosphorodithioic acid, O,O-dimethyl S-[2-(methylamino)-2-oxoethyl]esteras a result of degradation of Dimethoate which confirms isolate AM1 ability to bio-convert the pesticide as in Fig. 7.

FTIR analysis

As per FTIR analysis, the availability of alkene, amine, alkane and another defined functional group for dimethoate was obtained as in Fig. 8. In contrast, with the metabolism of pesticide by *Pseudomonas fluorescens* different functional group pattern was recorded which has confirmed the degradation of dimethoate by this isolate. The details are present in Fig. 9 and 10 and Table 5.

DISCUSSION

In the present study bacterial isolates capable of degrading pesticide Dimethoate was sampled successfully from the agriculture soil from Uruli Kanchan, Pune and found to be possessing positive strains for the said feature. In the study, it was assured that pesticide exposed soil possess micro flora capable to degrade to degrade the related pesticides as evident in the present study. In a similar study, Ishag, et. al. (2016) reported the presence of pesticide polluted soil in Sudan. They characterized them as *Bacillus subtilis subsp. inaquosorum strain KCTC13429T, Bacillus cereus strain ATCC14579T, Bacillus safensis strain FO-36bT.* They reported the degradation of Dimethoate as half-lives as α and β (days) such as 9.5, 11.0; 9.53, 15.11 and 4.16, 9.27 for *B. safensis, B. subtilis, B. cereus* respectively. As per GCMS they detected few metabolites after degradation. Along with the agricultural soil, Sewage sludge or waste water isolate also been the source of bacterial strains reported with Dimethoate degrading *Paracoccus sp.* reducing dimethoate (100mg/L) within 6 hours (Li, et. al. 2010).

In the present study based on the primary screening isolate AM1 and AM2 able to degrade 1% Dimethoate in DM agar and those were identified as *Pseudomonas fluorescens strains AM1* and *Achromobacter pulmonis strain AM2.* In a similar report, worker Debmandal, et. al. (2008) report the success of *Pseudomonas aeruginosa* isolated from water able to degrade dimethoate and confirmed to form four metabolites which confirm that genus *Pseudomonas* posses some features for effective dimethoate degradation. Deshpande, et. al. (2001) also mentioned the dimethoate degradation ability in *Pseudomonas aeruginosa MCMB-427* and *Bacillus megaterium MCMB-428.* Similar to the present study with *Pseudomonas species.* Since the Dimethoate found to be adversely affecting lifecycle of many organisms it is important to degrade it once its use is over in agricultural practices.

In the present study, when both the isolates AM1 and AM2 tested positive for Methoate degradation promising use of these isolates as a soil inoculant could be proposed to reduce the pesticide pollution in coming time. Result showcased that isolate AM1 (*P. fluorescens*) found to be better performer for 1% Dimethoate degradation with 90.66 ± 0.57% degradation recorded up to 10 days and in comparison, 83.66 ± 0.57% recorded with isolate AM2 (*A. pulmonis*).

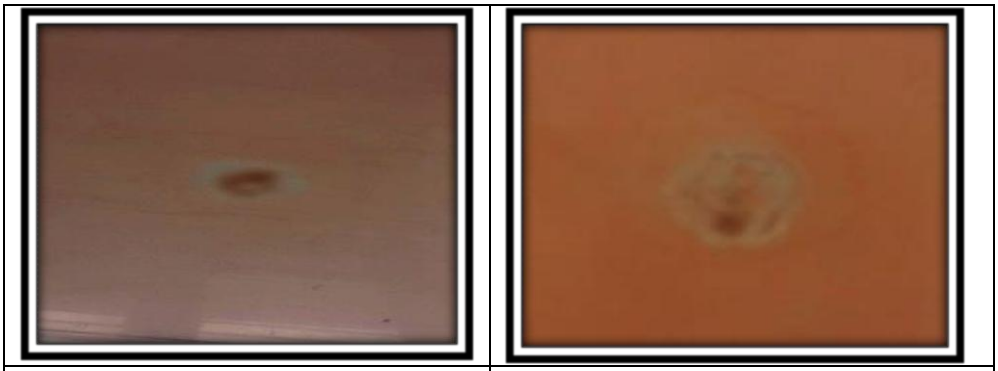
In a range of Dimethoate concentration (1%, 5%, 10%, 15% and 19%) degradation screening with isolate AM1 reported better result and sustained up to 19% of concentration to showcase 31.66 ± 2.08% degradation which was not evidenced with isolate AM2. In a similar report, by involving Dimethoate as a sole carbon source by involving Dimethoate as a sole carbon source bacterium *Raoultella sp. X1* able to degrade 75% of dimethoate via co-metabolism (Liang, et. al. 2009).

Pseudomonas putida also been reported to degrade Dimethoate 2g per litre with 50% and 100% degradation rate after 48h and 96h, respectively which displayed similar result to the present study (Nazarian 2007).

In the present study as per GCMS analysis, dimethoate able to metabolize by the isolate AM1 to give the only one metabolite (Phosphorodithioic acid, O,O-dimethyl S-[2-(methylamino)-2-oxoethyl]ester) and FTIR analysis also reported the same that with different functional group as compared to control (Dimethoate) ascertained the formation of metabolites. Similar to the present study Gas chromatography-Mass spectra used successfully to detect metabolites formed during Dimethoate degradation in other studies (Priya, et. al. 2011; Zhou, et. al. 2012; Ortiz- Hernandez, et. al. 2003).

CONCLUSION

The insecticide dimethoate as an organophosphate able to control insects such as mites, flies, aphids and many plant hoppers. The Dimethoate needs to degraded once reach to the soil as a pollutant. Present study successfully reported the possibility to degrade the dimethoate by involving two isolates *P. fluorescens* and *A. pulmonis.* These isolates originated from dimethoate polluted soil able to degrade up to 15% of dimethoate polluted soil with great success. These isolates could be used in fast track degradation of dimethoate once applied to polluted soil and recommended to add in dimethoate used areas.



AM1	AM2

Figure 1. Soil borne isolates able to grow on DM agar in presence of 1% dimethoate.

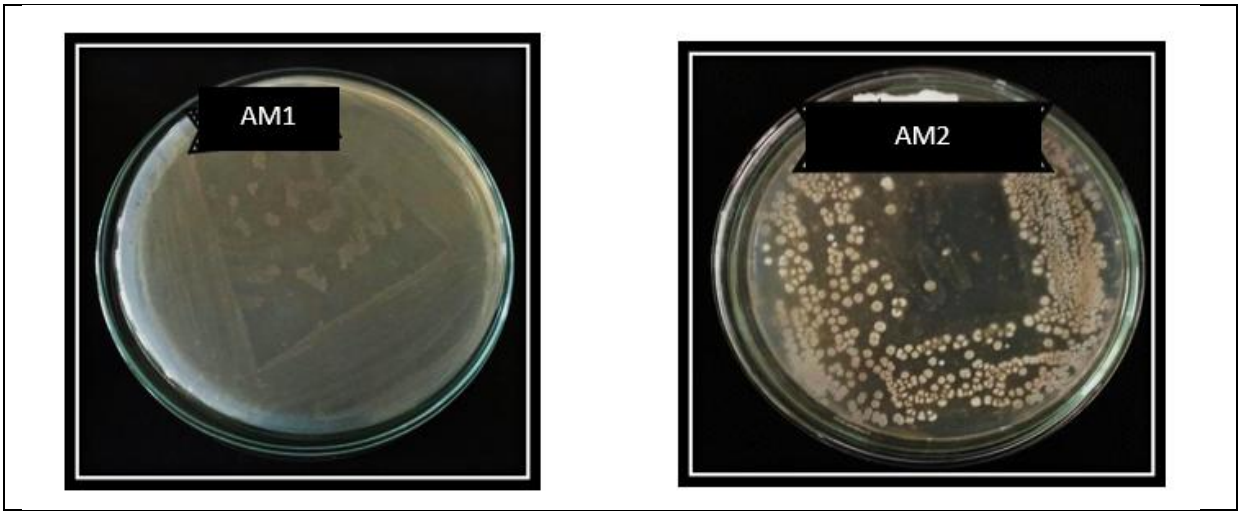


Figure 2. Promising isolates able to survive on the 0.1% dimethoate supplemented DM agar.

Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Pseudomonas fluorescens strain Bp-15 16S ribosomal RNA gene, partial sequence	1203	1203	97%	0.0	99%	KJ888143.1
Pseudomonas fluorescens strain Bp-14 16S ribosomal RNA gene, partial sequence	1201	1201	97%	0.0	99%	KJ888142.1
Uncultured gamma proteobacterium clone PD-09 16S ribosomal RNA gene, partial sequence	1199	1199	97%	0.0	99%	EF491549.1
Pseudomonas lactis YT4 gene for 16S ribosomal RNA, partial sequence	1197	1197	96%	0.0	99%	LC333862.1
Pseudomonas azotoformans strain JZ RK-57 16S ribosomal RNA gene, partial sequence	1197	1197	96%	0.0	99%	MH119657.1

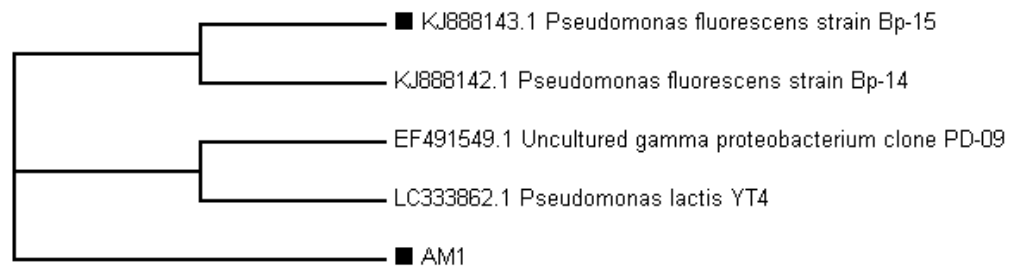


Figure 3. Sequence homology recorded for 16S rRNA gene of isolate AM1 with *Pseudomonas fluorescens.*

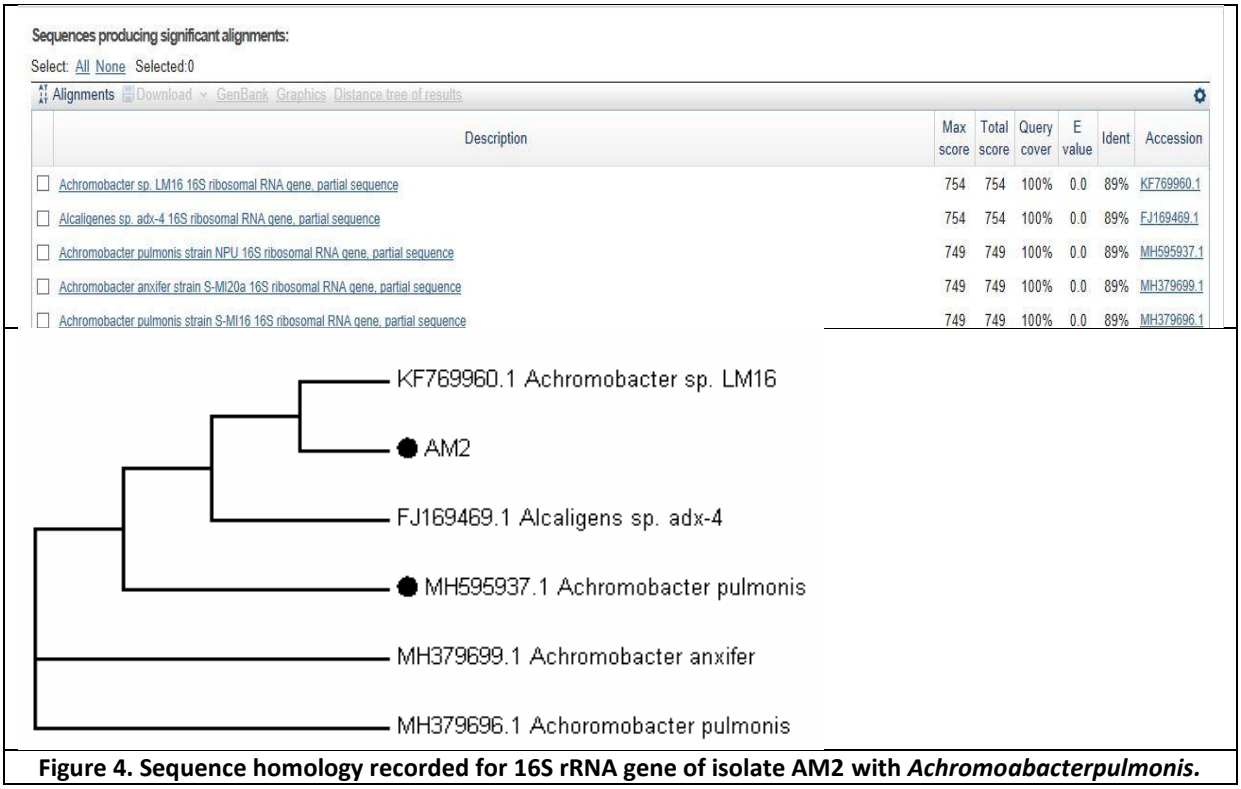


Figure 4. Sequence homology recorded for 16S rRNA gene of isolate AM2 with *Achromoabacterpulmonis*.

Table 1. Colony and cell morphology features of isolates AM1 and AM2.

Characters	AM1	AM2
Size	1mm	2mm
Shape	Circular	Circular
Colour	Whitish	White
Margin	Entire	Irregular
Elevation	Slightly elevated	Convex
Opacity	Opaque	Opaque
Consistency	Smooth	Butyrous
Gram character	Gram Negative Short rods	Gram Negative Rods
Motility	Motile	Motile

Table 2. Biochemical features of isolate AM1 and AM2.

Test	AM1	AM2
Oxidase	Positive	Positive
Catalase	Positive	Positive
Methyl red	Positive	Positive
Voges Proskauers	Positive	Negative
Urease	Positive	Negative
Nitrate reduction	Positive	Positive
SIM	Positive	Positive
Glucose fermentation	Positive	Positive
Maltose fermentation	Positive	Negative
Mannitol fermentation	Positive	Negative
Lactose fermentation	Positive	Negative
Gelatinase	Positive	Negative

Table 3. Degradation of dimethoate by microbial isolates estimated by NBS assay.

Time (Days)	*Pseudomonas fluorescencs*	*Achromobacter pulmonis*
5	62.66 ±0.57***	57±1.00
10	90.66±0.57***	83.66±0.57
Two-way ANOVA set at P <0.05; *** P<0.0001 highly significant, row wise comparison		

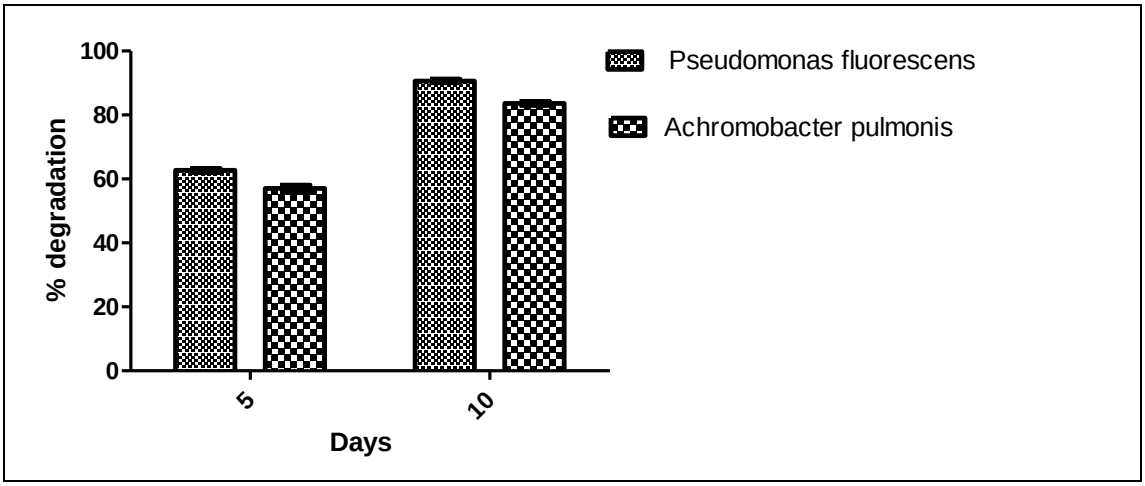


Figure 5. Increase in % degradation was observed from 5 days and up to 10 days in both the isolates.

Table 4. Degradation of different % dimethoate by microbial isolates estimated by NBS assay.

Concentration of dimethoate (%)	*Pseudomonas fluorescens*	*Achromobacter pulmonis*
1	63±1***	58.66±1.52
5	52±1	51±1.52
10	42.33±1.52	45±1
15	39.33±0.57***	45±.57
19	31.66±2.082***	-
Two-way ANOVA set at P <0.05; *** P<0.0001 highly significant, row wise comparison		

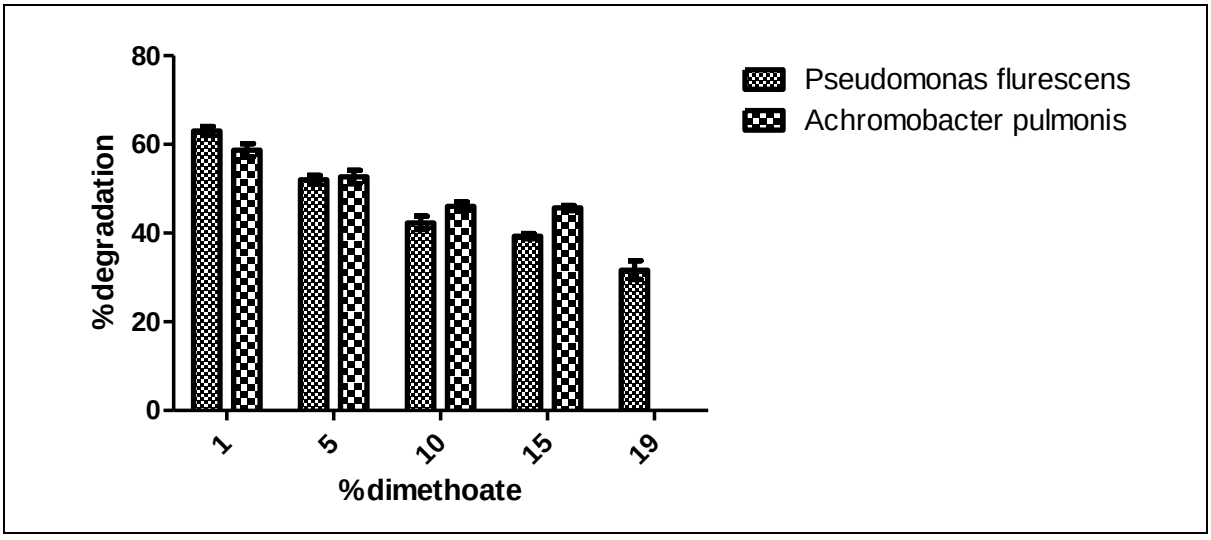


Figure 6. Decrease in % degradation was observed as % concentration of dimethoate increased from 1% to 19% for both the isolates.

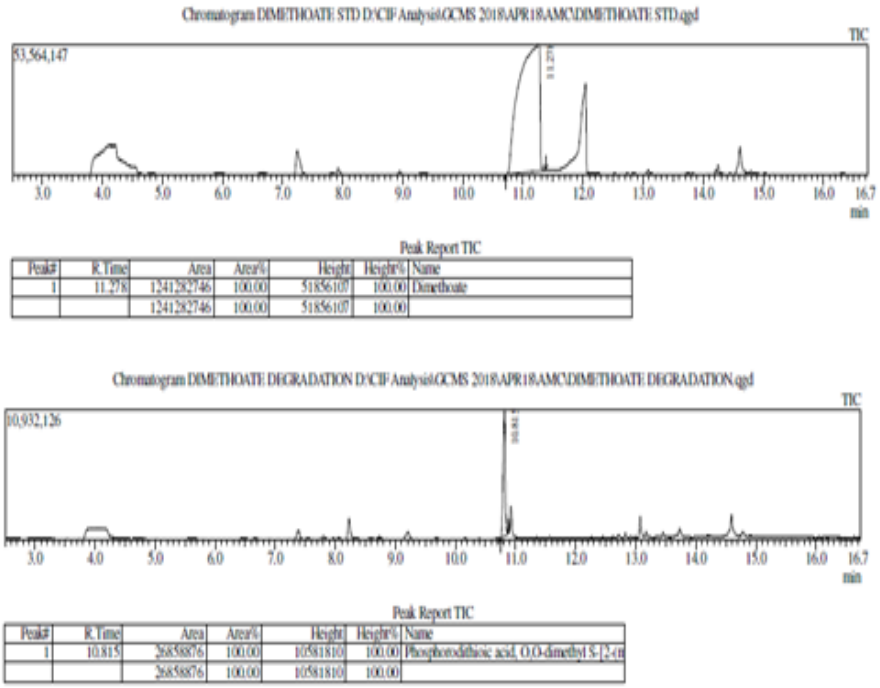


Figure 7. GCMS profile of the Pure 1% dimethoate (Top) showcasing the only peak of the compound and GCMS profile of the broth degrading dimethoate to Phosphorodithioic acid, O,O-dimethyl S-[2-(methylamino)-2-oxoethyl]ester by isolate *P. Fluorescens* also indicated that only secondary compound formed after activity of the isolate.

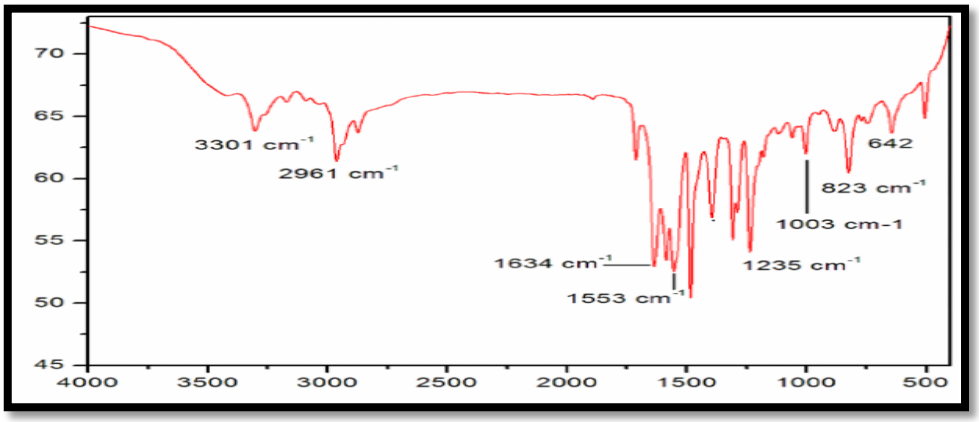


Figure 8. Standard FTIR of dimethoate.

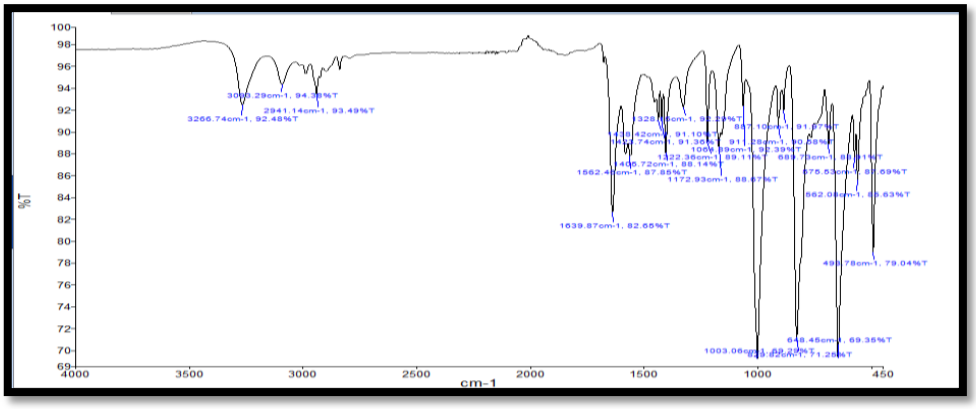


Figure 9. FTIR of dimethoate metabolites produced by *Pseudomonas fluorescens*.

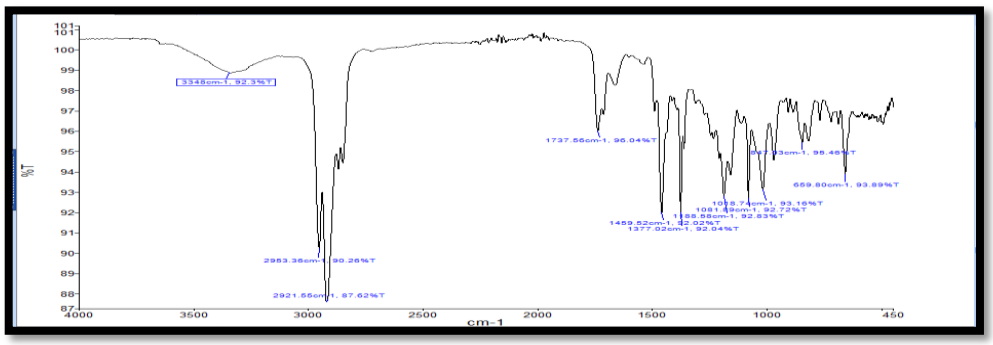


Figure 10. FTIR of dimethoate metabolites produced by *Achromobacter pulmonis.*

Table 5. FTIR spectrum of Dimethoate, *P. fluroscens* and *A. pulmonis* metabolites.

Dimethoate		*P. fluorscens* metabolite		*A. pulmonis* metabolite	
Standard (Frequency in cm-1)	Bond	Frequency in cm-1	Bond	Frequency cm-1	Bond
3098	=C-H,-C-H	2954	-C-O-H,=C-H	3348	-NH
3266	-N-H	2920	-C-H	2953	-C-H,=C-H
2941	-C-H	2852	-C-H	2921	-C-H
1639	R-CO-NH	1459	-COO	1737	R-CO-R
1003	C-O	1377	-COO	1377	-C-O
829	RCH=CR2	1098	C-C	1081	C-O
648	=CH	1013	C-C	1018	C-O
493		841	RCH=R2	847	R-CH=R2

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