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By

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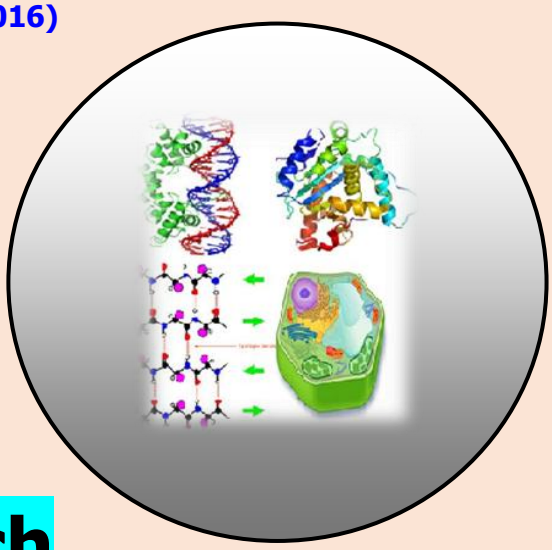

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Screening the potential of *Pseudomonas fluorescens* and *Achromobacter pulmonis* as safe dimethoate degrader and plant growth promoting agents

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ABSTRACT

Ability to degrade pesticides by biological approach remains the priority for better eco-systems, once it becomes the pollutant of soil and water. In the present study, dimethoate degrading bacterial isolate Pseudomonas fluorescens and Achromobacter pulmonis found to be producing metabolites after degrading dimethoate which are safe to the environment when screened for mutagenicity assay (Ames test), toxicological studies against fish and earthworm and hence recommended safe for soil inoculation to control thepollutant level of dimethoate by bio-degradation. These isolates also been checked positive for the plant growth promoting features when tested for indole acetic acid production, hydrogen cyanide production, siderophore and others. Study highlighted the introduction of two bacterial isolates capable of doing pesticide degradation as well as plant growth promotion if inoculated to soil contaminated with dimethoate.

Keywords: Dimethoate, Degradation, Plant Growth Promotion and Ames test.

INTRODUCTION

Organophosphorus pesticides are the major pesticides used in agricultural production and now it has become a major pollutant to the ecosystem (Zhang et.al. 2012). In a view, focus has been made to degrade dimethoate by using approach like pulsed electric field with 20 $KVcm^{-1}$ for 260 µs. Result showcased reduced toxicity of the treated sample with reduction in pesticide level (Zhang et.al. 2012). Dimethoate degradation has also been achieved by UV irradiation using TiO_2/Polymer films. It has been achieved when under optimum loading of TiO_2 of 4g/l at a UV irradiation time of 180 min was given. The degradation products were then analysed by gas chromatography-mass spectra (GCMS) (Priya et.al. 2011).

In another approach pesticide degrading microorganisms such as *Bacillus safensis FO-36bT, Bacillus subtilissubsp.inaquosorumKCTC13429T* and *Bacillus cereus strain ATCC14579T* were found to be degrading dimethoate with α and β-half-lives (days) recorded to be 9.5, 11.0, for *B. safensis*, 4.33,9.99 for *B.cereus* and 4.16,9.27 for *B.subtilis*. They also recorded by-products of degradation by GC and GC-MS (Ishaget.al.2016).

Liang et. al. (2009) reported a bacterium *Raultella sp.X1* able to degrade 75% of dimethoate when used in co-metabolism approach.

Attempt has been made to degrade the dimethoate by transferring genes available in plasmid (engineered) of Rhizobiumto *Pseudomonas sp.* by which positively cloned and expressing *Pseudomonas sp.* showcase better dimethoate degradation (Shinde et.al.2018).

In a search ofbetter isolates, natural sources like the exposed soil (e.g.: dimethoate containing soil) also been screened by implementing microbial techniques in vitro (Shinde et.al. 2015).

Besides that, microbial community has also been added to the soil to improve plant productivity by supplying nutrient and growth factor derived from microbes. For example, *Rhizobacteria* (Bhattacharya and Jha, 2012); *Bacillus lichenformis* (Singh et.al. 2014), *Bacillus megaterium* (Porcel et.al. 2014) *Phyllobacterium brassicacearum STM 196*(Brestonet.al. 2013); *Proteus vulgaris, Proteus penneri*, and *Proteus hauseri* (Dr. Zewjecka, 2016).

In the present study, two isolate *P. fluorescens* and *A. pulmonis* were screened for their dimethoate degradation derived metabolites for their biosafety nature as well as for their plant growth promotion activity in together. Result can direct to use these isolates in dimethoate exposed soil for better dimethoate degradation as well as a candidate of plant growth promotion.

MATERIAL AND METHODS

In the present study dimethoate degrading *P. fluorescens* and *A. pulmonis* isolate's (Shinde and Hamde, 2018) metabolites were tested for the biosafety parameters by using Ames test and toxicity assay with fresh water fish and earthworm under laboratory conditions. Further both these isolates were screened for the plant growth promoting parameters under laboratory conditions to screen second important aspect of these isolates. The details are given below:

Bacterial Isolates

Isolates *P. fluorescens* and *A. pulmonis* were successfully grown in nutrient broth for 48 hours and further screened in Ames test, Acute fish toxicity, acute earthworm toxicity test and PGPR screening.

Biosafety Parameters

- **Ames test**

In a given test, metabolite formed after dimethoate degradation by the isolate *P. fluorescens* and *A. pulmonis* were tested as per Ames Salmonella/microsome mutagenicity assay. This test is a widely used to screen mutagenic properties of new chemicals and drugs. In a test, Salmonella tester strains were checked for mutation profile as suggested by Mortelmanset.al. (2000) protocol. During the test, metabolites of *P. fluorescens* named as AM1 and that of *A. pulmonis* as AM2 were tested at three concentrations (1mg/ml, 10mg/ml and 100 mg/ml) along with positive control having sodium azide and negative control added with sterile water. In a result, for positive sets, revertant colonies were observed with increased number of colonies formed which remain dependent on the doses given and in case of negative mutagenic set, no revertant colonies were apparent.

- **Toxicity of metabolites to fresh water fish**

This study was carried out as per OECD guidelines for testing of chemicals, section 203, adopted 17thJuly 1992.

The objective of the study was to determine the 96 hours LC_{50} value of "metabolites (AM1 and AM2)" to fresh water fish (*Poecilia reticulate*). This method of testing for the toxicity to fish is used in identifying the toxicological properties of a substance and its toxicological properties to the eco-system. This study was designed to determine the LC_{50} of the test material or to establish a non-lethal dose level of the test material in mg/L and to study the toxic effect of the chemical with onset, severity and reversibility.

Dose preparation: The test material was prepared shortly before dosing by dissolving 100 mg of the "Metabolite" per litre water.

Study Design: Fourteen fish, acclimatized for 08 days, and were assigned to the control and test groups.

Group I served as control group which was not treated with test sample.

Group II served as test group which was treated with test sample at a concentration of 100 mg/L.

The test material was administered once in each fish tank to known measured amount of diluent water. The fish were deprived of feed for 3 days prior to the study. Seven fish per dose were transferred into each fish tank without causing any injury to the fishes. All the fish were observed for 96 hours for any abnormal behaviour, signs of toxicity and death.

- **Acute toxicity of "metabolites" to earthworm**

This study was carried out as per OECD guidelines for testing of chemicals 207, adopted 4th /April/1984.

The objective of the study was to determine the 7 days LC_{50} of "Metabolites AM1/AM2" to Earthworm. This method of testing for the toxicity to Earthworm is used to determine 7 days LC_{50} of the "Metabolites AM1/AM2" or to establish a non-lethal dose level of the test material in parts per million.

Dose preparation: The test material was prepared shortly before dosing. The test article was used at concentrations of 100 mg/kg, 500 mg/kg and 1000 mg/kg, respectively.

Study Design: 750 gm of artificial soil was prepared by mixing 10 % sphagnum peat, 20% kaolin clay and 70% industrial fine sand. The pH was adjusted to 6.5. The mixture was kept moist by adding deionised water. For each test, 750 gm of the soil was taken into a container and ten earthworms were conditioned for 24 hrs in an artificial soil and then washed quickly before use. Earthworms were assigned to the Control, Test 1, Test 2 and Test 3 groups (10 earthworms per group). Control group did not receive any treatment. Test 1 group was treated with "PM1/PM2" at 100 mg/kg dose. Test 2 group was treated with "AM1/AM2" at 500 mg/kg dose. Test 3 group was treated with "AM1/AM2" at 1000 mg/kg dose. The containers were covered with perforated plastic film to prevent the test medium from drying and kept under the test conditions for 07 days. The temperature was maintained at 22±2^{o}C. Testing was carried out in continuous light during 07 days study period and earthworms were observed for the assessment of mortality daily after treatment.

- **Plant Growth Promoting (PGP) activities of dimethoate degraders**

Bioremediation is a method that exploits the potential of microbial degradation for providing a cost effective and reliable approach to pesticide abatement. Pesticide degrading bacteria found in soil are known to have multifarious abilities such as mineral solubilization, Indole acetic acid production, siderophore production, ammonia production and hydrogen cynide production. These activities are extremely crucial for promotion of plant growth.

- **Indole acetic acid production (Ahmad *et al,* 2008)**

Bacterial cultures were grown for 48 hrs. in DM medium broth at 30°C.Fully grown culture were centrifuged at 3000 rpm for 30 min. The supernatant (2ml) was mixed with two drops of orthophosphoric acid and 4 ml of the Salkowasky's reagent. Development of pink colour indicates IAA production. Concentration of IAA produced by cultures was measured with the help of standard graph of IAA.

- **Ammonia Production (Ahmad *et al,* 2008)**

McCartney bottles containing 10 ml peptone water along with dimethoate were inoculated with bacterial strains and incubated at 35°C for 96 hrs. The NH3 production was detected by observing the formation of yellow colour in the bottle upon addition of Nessler's reagent.

- **Phosphate solubilization (Ahmad *et al,* 2008)**

The ability of bacteria to solubilize phosphate was determined by plating the bacteria on Pikovaskya's agar medium containing dimethoate. The presence of clear zone around the colonies following one week of incubation at 35°C indicates phosphate solubilization.

- **Hydrogen Cynide production (Ahmad *et al,* 2008)**

Nutrient broth was amended with 4.4 g glycine/l, dimethoate and bacteria were streaked on modified agar plate. A Whatman filter paper no.1 soaked in 2% sodium carbonate in 0.5% picric acid solution was placed in the top of the plate. Plates were sealed with parafilm and incubated at 30°C for 4 days. Development of orange to red colour indicates hydrogen cyanide production.

- **Siderophore Production (Ahmad *et al,* 2008)**

Bacterial cultures were grown for 48 hrs. in Kings B medium containing dimethoate at 30°C.Fully grown cultures were centrifuged at 3000 rpm for 30 min. The supernatant was used for detection of siderophore production**.**

A. Arnow's assay (For Catechol type siderophore)

Supernatant of bacterial culture taken in test tube. 1 ml 0.5 N HCl and 1 ml nitrite molybdate reagent was added to it. After development of yellow colour, 1 ml of 1 N NaOHwas added. Development of red colour indicated positive test.

B. Tetrazolium salt assay (For Hydroxamate type siderophore)

Pinch of tetrazolium salt and 2 drops of 2 N NaOH added to 0.1 ml of cell free supernatant. Development of deep red colour indicated positive test**.**

C. Vogel's chemical test (For Carboxylate type siderophore)

The 5 ml water,3 drops of 2 N NaOH and 1 drop phenolphthalein was added in together. Light pink colour is developed. To that 2 drops of supernatant was added. Disappearance of pink colour indicate positive test.

RESULT

Utility of dimethoate degrading isolate,*P. fluorescens* and *A. pulmonis* for betterdegradation of soil mixed dimethoate with parallel plant growth promoting ability has been witnessed positive for both the isolates as evidenced with results obtained below: the metabolite of degradation (AM1/AM2) of isolates also found to be safe for the fish and Earthworm along with that it has not shown any mutagenic effect as described below:

- **Ames test**

Isolate *P. fluorescens* metabolite AM1 and *A. pulmonis* metabolite AM2 when tested on the *Salmonella typhimirium TA100* at 1mg/ml, 10mg/ml by plate incorporation assay (Mortelmanset.al. 2000), result highlighted that both the metabolites found to be non-mutagenic in nature as evident with no revertant colonies appeared and hence recorded as safe metabolites.

- **Toxicity test with fresh water fish**

Metabolites (AM1 and AM2) when tested at 100mg/L in an individual set for induced toxicity against fish, *Poecilia reticulate* in an experimental set, up to 90 hours of recording for LC50 value in both the experimental set metabolite induced was not recorded. Hence, both the metabolites recorded safe at (100mg/L) to the fresh water fish as in Table 1 and LC50 remains higher than the said concentration (100mg/L).

- **Toxicity test with Earthworm**

The test material was administered to 10 earthworms per dose, at different dose levels. The earthworms were observed for the assessment of mortality daily up to 7 days of post administration. Control group was not treated with test sample. All the earthworms appeared normal visually and showed no behavioral or pathological symptoms after dosing till the end of the study. Test 1 group was treated with 100 mg/kg of "Metabolites AM1 /AM2". The test material caused NO mortality. All the earthworms appeared normal visually and showed no behavioral or pathological symptoms after dosing till the end of the study. Test 2 group was treated with 500 mg/kg of "Metabolites AM1 /AM2". The test material caused NO mortality. All the earthworms appeared normal visually and showed no behavioral or pathological symptoms after dosing till the end of the study. Similarly, Test 3 group which was treated with 1000 mg/kg of "Metabolites AM1 /AM2" also caused no mortality. All the earthworms appeared normal visually and showed no behavioral or pathological symptoms after dosing till the end of the study.Therefore, on the basis of results, it can be said that "Metabolites AM1 /AM2" did not cause mortality at the dose of 1000 mg/kg to the Earthworms. The 7 days LC_{50}value of "AM1 /AM2" for earthworm was found to be greater than 1000 mg/kg by artificial soil test method (Table 2).

- **PGP activities of Isolates**

Both the isolates (*P. fluorescens* and *A. pulmonis*) found to be better performer in dimethoate degradation and remain safe metabolite producers. Further these isolates evidenced to be the strains with plant growth promoting features with adds the dual function in these bacterial strains as given below:

- **Indole acetic acid production**

As per standard formula calculation, both of the isolates (*P. fluorescens* and *A. pulmonis*) able to produce indole acetic acid as 48.75µg/ml and 56.25 µg/ml, respectively as shown in Fig. 1 and Table 3.

- **Ammonia production**

After addition of dimethoate to the bacterial peptone water promising ammonia production was evident in both the strains (*P. fluorescens* and *A. pulmonis*) as in Fig.2.

- **Phosphate solubilisation**

Pikovaskys agar plate supplemented with dimethoate when inoculated individually with *P. fluorescens* and *A. pulmonis* strains and as per formula given below about 75% of solubilisation was recorded for *A. pulmonis* isolates only with solubilisation zone (3.5mm) as well as colony diameter of 2mm (Fig. 3). Isolate *P. fluorescens* remained negative for the test.

SE (%) = [solubilisation zone (mm) – colony diameter (mm)]/ colony diameter x 100

= [(3.5-2)/2] x 100

SE = 75%

- Hydrogen cyanide production

Hydrogen cyanide production found to be positive only in isolate *P. fluorescens*while isolate *A. pulmonis* remained negative as in Fig. 4.

- Siderophore production

As per number of tests such as Arnow's assay, Tetrazolium salt test and Vogel's chemical test isolate *P. fluorescens* showed production of carboxylate and hydroxamate type siderophore was evident. In case of isolate *A. pulmonis* production of only hydroxamate type of siderophore was recorded as in Fig (5.1-5.3).

DISCUSSION

In the present study isolate *P. fluorescens* and *A. pulmonis* capable of degrading dimethoate produces metabolites AM1 and AM2, respectively and these metabolites found to be safe for the ecosystem as per Ames test, toxicology studies on fish and earthworm. Based on these results, it has been confirmed that both these isolates could be recommended for the pesticide degradation in an eco-friendly approach to regulate the level of soil pesticides. According to Pant K et.al. (2016) bacterial mutation assay (Ames test) established as a routine test for the safety assessment of new chemicals as in our case of the degradation of metabolites were tested. They reported the importance of Ames test by using plate incorporation and pre-incubation methods as the most preferred one which was also been used by us in the present study to decipher non-mutagenic features both metabolites (AM1 and AM2). According to Ihsan et.al. (2013) Ames test also been used to screen genotoxic potential of Quinocetone and Cyadox which are derivatives of heterocyclic N-oxide quinoxaline which is an antimicrobial compound. According to report, Ames test indicated Quinocetone with higher level of genotoxicity than Cyadox. While, in our case both of the metabolites (AM1and AM2) found to be weak or no mutagenic potential to bacterial cells as Ames test confirmed them as safe metabolites in nature (AM1 and AM2). Number of reports given the success of Ames test to check chemical genotoxicity against bacterial cells such as Vinyl chloride (de-Messteret.al. 1980) and Macrocyclic musk compounds (Abramsson-Zetter berg and Sianina, 2002). In the present study by using 100 mg/litre of metabolites (AM1/AM2) in a treated water, ability of fish to survive has been demonstrated with 100 % survival rate for both of the metabolites tested which ensures their safety with them. In another report by using NMR technology toxicity of insecticides such as methomyl (methyl-(1E)-N- (Methylcabamoyloxy) ethanimidothioate was assessed which brought about altering patterns of whole-body metabolism, energy metabolism, oxidative stress and muscle maintenance (Yoon et.al. 2016). In reverse, our study did not showcase any physiological variation, change in metabolism and even mortality which has indicated towards safe dose rate (100mg/litre) of the metabolites (AM1/AM2) for aqua-system especially the fish. Narraet.al. (2011) when investigated *in vivo* about toxicity of monocroptophos on fresh water fish *Clarias batrachus,* they observed that upon 28 days of treatment, fish developed with high level of Urea and glutamine level in gill, kidney and muscle tissues. They also reported high level for enzymes such as protease phosphatase, transaminase upon exposure to chemical. Once again, this type of phenomenon was not evidenced in studied fish as no physiological effect was seen with these treated fish with metabolites (AM1 and AM2). In another set of present study, earthworm also remained survived with metabolite (AM1/AM2) level kept at 1000mg/kg in the medium which ensured that high level of metabolites might be lethal to the earthworm but not the given one. In another study, Mudiamet.al. (2013) while studying on carbofuran-a common agricultural pesticide effect on earthworm, they put forward that when carbofuran given as a treatment at level 0.15, 0.3 and 0.6mg/kg about seventeen metabolites were detected which affect the metabolism of earthworm when detected by GCMS. Similar to this study, when we inoculated earthworm with more fold high dose (1000mg/kg) we did not find any cytotoxic effect or any resultant mortality with both metabolites (AM1/AM2) indicated its safe nature for sure.

Since no mortality was recorded in the present study with high dose (1000mg/kg) indicated its safe and non-toxic features that certainly we can recommend to use these isolates in soil inoculation for dimethoate degradation. In a one report, of Caceres et.al. (2011) they indicated the toxicity of organophosphate insecticide as low as 10 to 200mg/kg towards earthworm (*Eisenia fetida*) which was not evidenced at all in our study even when the dose as high as 1000mg/kg was given. In the next set of study, isolate *P. fluorescens* and *A. pulmonis* were individually screened for PGP features and found promising in producing indole acetic acid, phosphate solubilisation, ammonia production, hydrogen cyanide production and siderophore production which promises to utilise these two isolates not only as pesticide degrader but also as a plant growth promoter when applied in soil. As in the present study, isolate *P. fluorescens* found to be promising isolate similarly in number of reports *Pseudomonas aeruginosa* remained PGPR in feature for example, *Triticum aestivum L* (wheat) stress evaluation by *P. aeruginosa strain CPS B1* (Rizvi and Khan, 2017). Khare and Arora, (2010) reported the IAA produced by *P. aeruginosa* remained involved in suppression of charcoal rot disease occurs in chickpea by inhibiting *Macrophomia phaseolina.* They also put forward the synergy between IAA and siderophore along with HCN and its overall role in disease suppression in chickpea.

Similar to our study, number of plant growth promoting bacteria were reported till date which can produce IAA, ammonia, HCN and siderophore such as *Pseudomonas sp.* (Chang et.al. 2014); *Orchrobactrum sp.* and *Enterobacter sp.* (Tariq et.al. 2014); *Rhizobium sp.* and *Bacillus sp.* (YU et.al. 2014) and many others.Here study introduced two more bacterial isolates becoming ready to tackle the problem of pesticide pollution as well as to promote plant growth in coming time which are named as isolate *P. fluorescens* and *A. pulmonis*.

Table 1. Mortality rate recorded for AM1 and AM2 metabolites against fresh water fish

Group	Dose (mg/L)	Time in hours									No. of Died/ No. of Treated
		1/2	1	2	4	6	24	48	72	96	
		AM1									
Control	**0**	0	0	0	0	0	0	0	0	0	**00/07**
Test	**100**	0	0	0	0	0	0	0	0	0	**00/07**
		AM2									
Control	**0**	0	0	0	0	0	0	0	0	0	**00/07**
Test	**100**	0	0	0	0	0	0	0	0	0	**00/07**

Table 2. Mortality rate recorded for AM1 and AM2 metabolites against Earthworm.

Group	Dose (mg/kg)	Time in hours									No. of Died/ No. of Treated
		1/2	1	2	4	6	24	48	72	96	
		AM1									
Control	**00**	0	0	0	0	0	0	0	0	0	**00/10**
Test 1	**100**	0	0	0	0	0	0	0	0	0	**00/10**
Test 2	**500**	0	0	0	0	0	0	0	0	0	**00/10**
Test 3	**1000**	0	0	0	0	0	0	0	0	0	**00/10**
		AM2									
Control	**00**	0	0	0	0	0	0	0	0	0	**00/10**
Test 1	**100**	0	0	0	0	0	0	0	0	0	**00/10**
Test 2	**500**	0	0	0	0	0	0	0	0	0	**00/10**
Test 3	**1000**	0	0	0	0	0	0	0	0	0	**00/10**

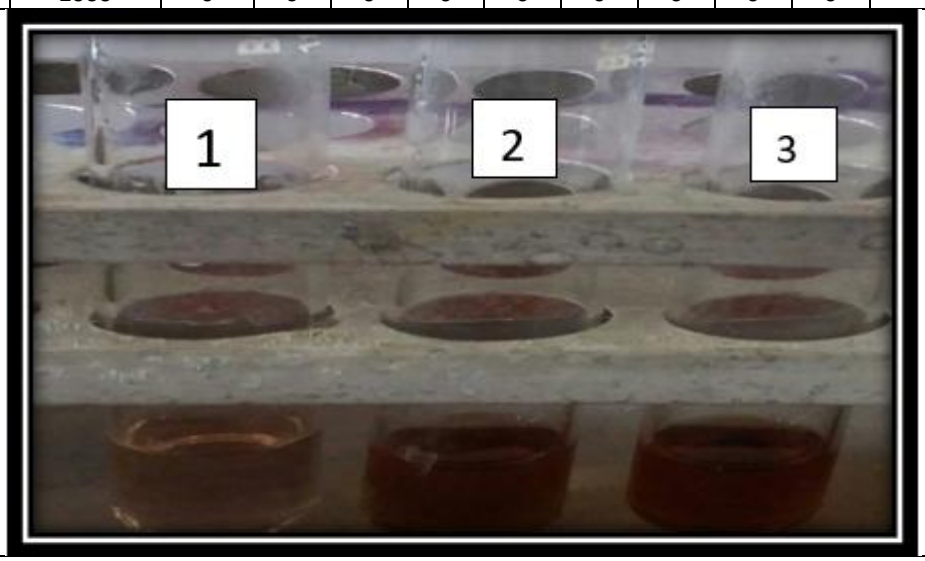

Figure 1. Salkowasky's assay for the estimation of IAA production. Set 1.Sterile tryptone water; 2. Fermented tryptone broth by *Pseudomonas spp.A1113 and 3. Achromobacter spp.*

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Table 3. IAA production by isolate *Pseudomonas fluorescens* and *Achromobacter pulmonis.*

Test organisms	Abs. at 530 nm	Concentration (µg/ml) of IAA
Pseudomonas spp A1113	0.39	48.75
Achromobacter spp 93	0.45	56.25

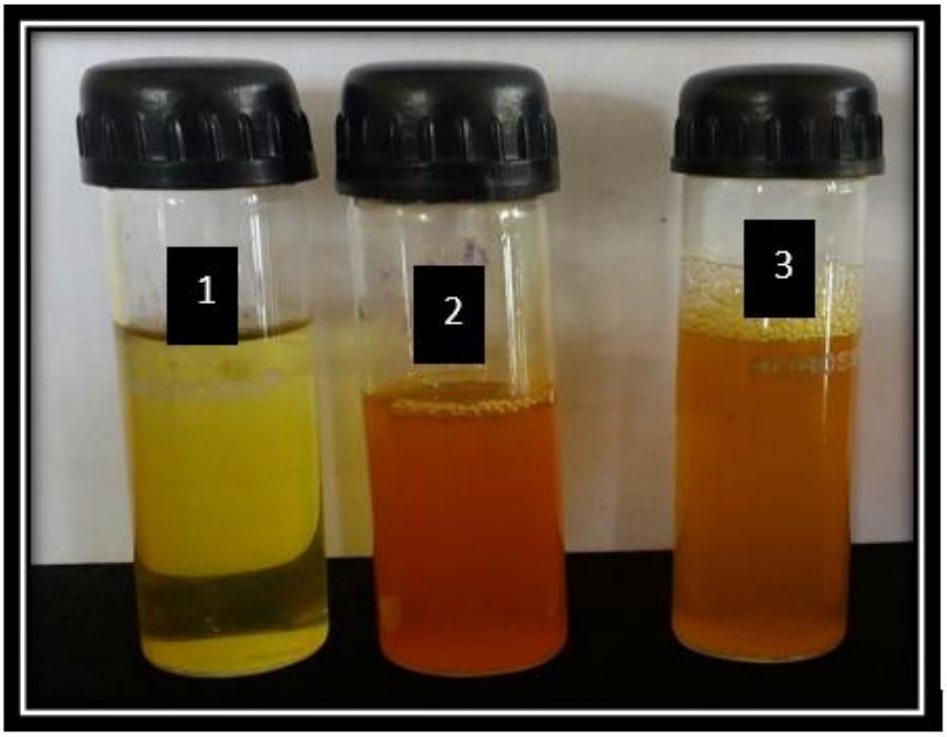

Figure 2. Ammonia production. 1. Control: 2. *Pseudomonas spp.A1113;* 3. *Achromobacter spp. 93*

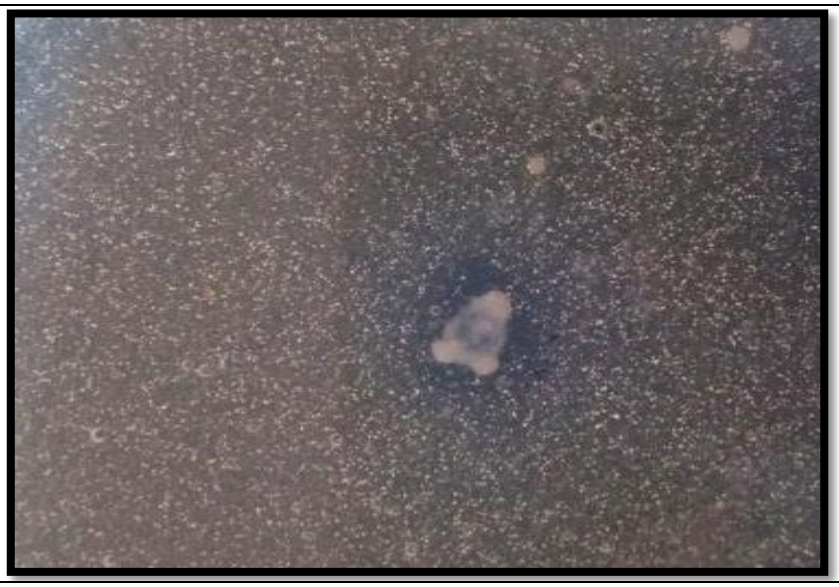

Figure 3. *Zone of phosphate solubilization by Achromobacter spp. 93.*

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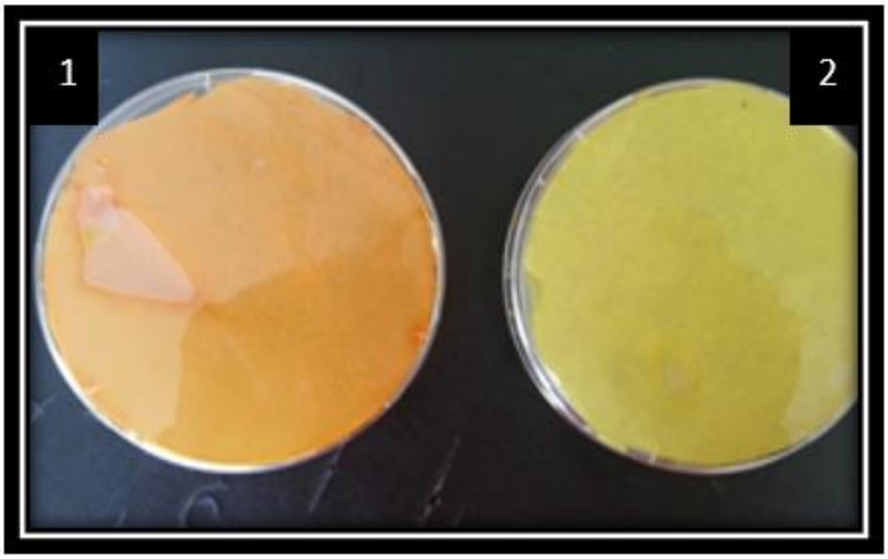

Figure 4. Positive HCN production by 1. *Pseudomonas spp.A1113*and negative result for 2. *Achromobacter spp. 93*

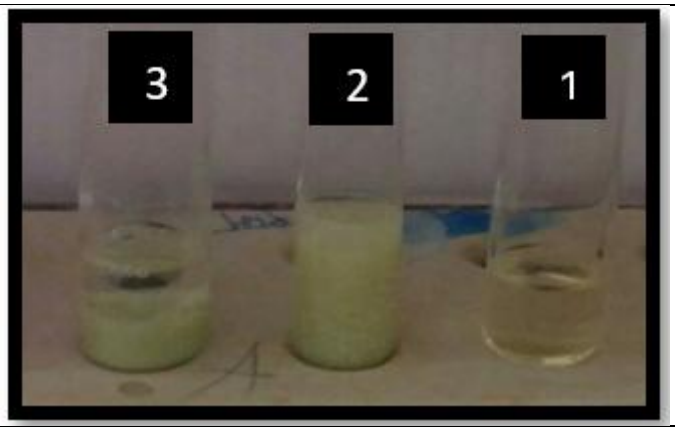

Figure 5.1. Arnow's Assay (Catecholate siderophore) 1. Control, 2.*Pseudomonas spp. A1113* (Negative), 3. *Achromobacter spp. 93* (Negative)

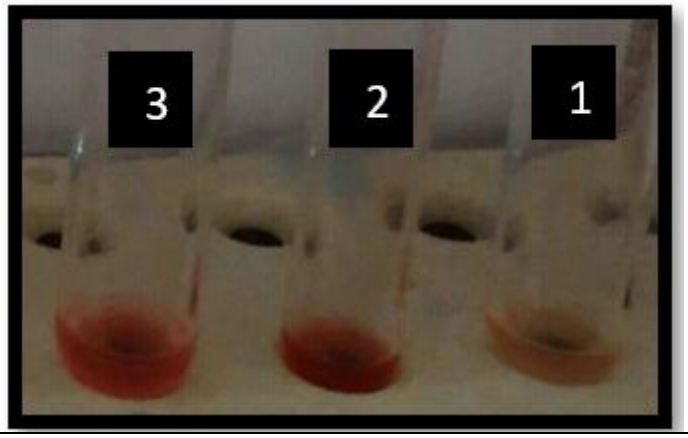

Figure 5.2. Tetrazolium Salt Test (Hydroxamate Siderophore) 1. Control, 2. *Pseudomonas spp. A1113* (Positive), 3. *Achromobacter spp. 93* (Positive)

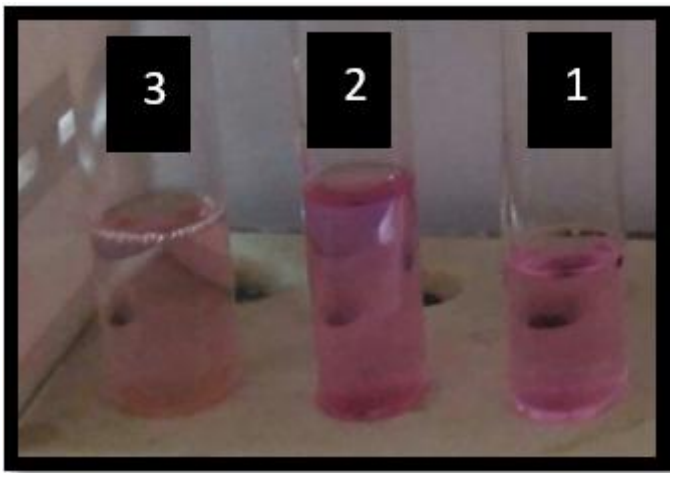

Figure 5.3. Vogel's Chemical Test (Carboxylate siderophore) 1. Control, 2.*Pseudomonas spp. A1113 (Positive),* 3. *Achromobacter spp. 93* (Negative)

CONCLUSION

Primarily screened dimethoate degrading bacterial isolates *P. fluorescens* and *A. pulmonis* found to be promising strains as they can degrade dimethoate to a metabolite (AM1/ AM2) which is non-toxic, non-mutagenic in nature. Besides that, isolates can produce number of plant growth promoting compounds makes them all round performer to improve soil condition for better plant growth and for eco-system management around rhizosphere.

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