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# Studies of organophosphate pesticide degrading microorganism isolated from Vaigai River, Madurai

**[1] KP Karuppaiah, [2] Ramesh U**

[1] Research and Development Centre, Department of Microbiology, Bharathiar University, Coimbatore, Tamil Nadu, India

[2] Department of Molecular Biology, School of Biological Sciences, Madurai Kamaraj University, Madurai, Tamil Nadu, India

**Abstract**
Biodegradation progressions which are based on the breakdown of pesticide by Pesticide tolerance microorganism. Vaigai, the major river of Madurai (course of 258 km) originates at Varusa Nadu Hills flowing through the districts of Theni, Dindigul, Madurai, Sivagangai and Ramanadhapuram. Due to the anthropogenic activities and wide usage of different pesticides in agricultural lands (in the form of spray drift, aerial spray), atmospheric fallout, soil erosion and runoff from agricultural areas directly enter the stream of river. Samples were collected from various areas in Tamil Nadu and analyzed for its Monocrotophos SL 36% degradation. Water samples were cultured on minimal media containing 100ppm/L Monocrotophos pesticide concentration. The isolates were identification by using various techniques like Staining, Biochemical Analysis, Antibiotic Sensitivity and pesticide Sensitivity Tests and molecular characterization. Determination of concentration of pesticide on the bacterial growth OD value UV spectroscope was carried out to confirm degradation. Using HPLC technique, Monocrotophos SL 36% was confirmed.

**Keywords:** Vaigai River, pesticides, microbial degradation, bioremediation, organ phosphorus pesticide, monocrotophos SL

## 1. Introduction
Allow agricultural producers to improve the quality, quantity, and diversity of our food supply Used in timber, turf, horticulture, aquatic, and structural pest control industries, When pesticides are applied the goal is that they will remain in the target area long enough to control a specific pest and then degrade into harmless compounds without contaminating the environment, Once applied, many pesticides are mobile in the environment (air, soil, water). This movement can be beneficial (moving pesticide to target area, such as roots) but can also reduce the effect on the target pest and injure non target plants and animals Bioremediation processes is an effective methods that stimulate the biodegradation in contaminated soils (McLaughlin, 2001; Swannell *et al*.,1996). Biodegradation and bioremediation are matching process to an extent that both of these are based on the conversion or metabolism of pesticide by microorganisms. The difference between these two is that the biodegradation is the natural process where as bioremediation is the technology. Temperature, pH, water potential, nutrients and the amount of pesticides or metabolites in soil may also act as limiting factor for pesticide degrading microorganism which require further exploration in relation to total microbial population and their biochemical activities (Fogarty and Tuovinen, 1990) Monocrotophos (MCP), an organophophorous insecticide used in agricultural operations persists as soil residue and seeps into ground water. Natural degradation of MCP takes place over a period of 12-16 days and the process could be expedited through bioremediation. (Sam Manohar Das & Anitha 2007) The ability of actinomycetes to transfor organochlorine pesticides has not been widely investigated, despite studies demonstrating that actinomycetes, specifi-cally of the genus Streptomyces. Cultural characteristics of *Streptomyces* sp. was examined using the method of the International *Streptomyces* Project (ISP) that was described by Waksman (1961), Shirling and Gottlieb (1966) and Locci (1989). The Vaigai is 258 kilometers (160mi) long, with a drainage basin 7,031 square kilometers (2,715 sq mi) large. It falls within the co-ordinates of Latitude 7º21'00'' N and Longitude 79º00'00''E. Vaigai Dam is built across the Vaigai River near Anaipatty, in the Theni district of Tamil Nadu, Southern India. The Enrichment of nitrogen and phosphorous in effluent – receiving Rivers as a result of pond effluent has the potential to set off subsidy stress responses in these systems, based on which of these nutrients is limiting in that ecosystem (Odum 1979)

## Materials and Methods
### 1. Chemical
The organophosphorus pesticide, Monocrotophos SL 36% (MSL) was purchased from the local market. Sediment samples from Vaigai River were collected in sterile polythene bags, transported and processed immediately for microbial isolation.

### 2. Collection of soil samples
The soil samples were collected from the different sits from vaigai river sediment top layer 0-10 cm which had been exposed to Monocrotophos pesticide in vaigai river Madurai Tamil Nadu, India. Transported and processed immediately for microbial isolation.

### 3. Isolation and identification bacteria from river sediment sample
The collected soil samples were exposed to serial dilution and

spread plate method for the isolation of pesticide degrading bacteria with the support of Nutrient agar and LB agar plates after bacterial colonies were selected and additional purified by streaking. The isolated strains were continued on Nutrient agar and LB agar slants and stored at 4°C. Identification development for the three altered bacterial isolates were carried out by after the routine bacteriological methods. After isolated bacterial colonies were inoculated to 100 ml MSM and enriched with an addition of 25ppm pesticide. Samples were incubated on rotary shaker (150 rpm) at 30°C for 7 days The growth curve obtained for the three selected bacteria were optical density(OD) value using UV- spectrophotometer at various time intervals. After which one the cultures were frequently transferred every 3-4 days or until increased turbidity were evidenced. After 3-4 times of repeated sub-culturing, 0.1 ml culture broth was pipetted and spreader on Minimal salt medium (MSM) + pesticide agar. Single colonies were selected and streaked on Minimal medium (MSM) supplemented with 25, 50 and 100 ppm of the pesticide. Cultures were incubated at 30°C for 3 days. Pesticide degrading isolates were selected from isolates which developed clear zone surrounding their colonies when grown on NA supplemented with 25, 50 and 100 ppm of the pesticide.

**4. Cultural Characteristics**

The colonies were identified by gram staining and biochemical tests (IMViC, starch hydrolysis, casein hydrolysis, carbohydrate fermentation, hydrogen sulfide ($H_2S$) production, urease production, hydrolysis of gelatin) further culture maintaining to Bennett's **agar and** Carbohydrate utilization agar (ISP-9**)**

**4.1. Bennett's agar (g/l)**

Agar- Agar-15.0g,Glucose-10.0g,pancreatic digest of casein-2.0g,Yeast extract-1.0g,Beef extract-1.0 g, calcium carbonate-1.0g,pH-6.8-7.2.

**4.2. Carbohydrate utilization agar International Streptomyces Project (ISP-9)**

$K_2HPO_4.3H_2$ - 5.65 g, $(NH_4)_2SO_4$ - 2.64 g$KH_2PO_4$-2.38 g, MgSO4.7H2O-1.0 g

pH- 6.8-7.2

Carbohydrate solution

Carbohydrate-10.0g, Distilled water-100ml,Pridham and Gottlieb trace salts MnCl2.7H2O 0.79 g,CuSO4.5 H2O- 0.64 g,ZnSO4.7 H2O- 0.15 g,FeSO4.7 H2O-0.11 g

**5. Isolation of DNA**

DNA samples were analyzed by the method of Sambrook *et al*. (1989). 0.7% (w/v) agarose gel was prepared with TBE buffer, pH 8.3. The agarose was melted into a clear solution using a microwave oven. The molten agarose was cooled to about 50ºC and ethidium bromide (10 mg/ml stock solution) was added to a final concentration of 0.5 µg/ml. The molten agarose was poured in a preset template with well forming comb and left as such for 30-45 min for gel formation. The comb and sealing tapes were removed and the gel template was mounted in a horizontal electrophoresis tank (Biotech, Yercaud). The tank was filled with TBE buffer (pH 8.3) just to immerse the gel up to 1 mm. Each 10µl of *Streptomyces* DNA and λ DNA/*Hind III* plus digest were loaded in the wells and the electrophoresis was carried out at 30V. When bromophenol blue migrated more than 2/3rd of the gel length, electrophoresis was stopped and the DNA bands were visualized in an UV transilluminator and photographed to obtain a permanent record of the band pattern in the gel.

**6. Analysis of 16S rDNA**

The 16S rRNA gene of *Streptomyces sp, S-14* was amplified by PCR according to Messner *et al*. (1994). 25 µl of master mix contained 10 X Taq buffer, 2mM $MgCl_2$, and 0.4mM dNTP mix and 2U Taq polymerase. The primers used in the study were: 27f (5' AGT TTG ATC CTG GCT CAG 3') and 1492r (5' ACG GCT ACC TTG TTA CGA CTT 3'). The reaction components were mixed and placed into the thermocycler (Eppendorf, Mastercycler, Germany).The product of the PCR amplification was analyzed by agarose gel electrophoresis. Five µl of PCR product was mixed with 1 µl of gel loading buffer. The mixture was loaded on agarose gels (1% w/v) in TBE buffer (pH 8.3). The separation was carried out at 90V for 40 min in TBE buffer. The resulting DNA patterns were examined with UV light and photographed, then analyzed by gel documentation system.

**6.1. Sequencing of 16s rRNA and phylogenetic analysis**

The PCR product obtained was sequenced by an automated sequencer (Genetic Analyzer 3130, Applied Biosystems, and USA). The sequence was compared for similarity with the reference species of bacteria contained in genomic database banks, using the NCBI-BLAST available at http://www.ncbi-nlm-nih.gov/. The phylogenetic tree based upon the neighbor-joining (NJ) algorithm was generated by using MEGA software (Kumar *et al*., 2004). The resultant unrooted tree topologies were evaluated in light of a bootstrap analysis of the neighbor-joining method using the seqboot and consensus programs in the MEGA package.

**7. Sensitivity to antimicrobial agents**

Bennett's agar plate was uniformly swabbed with a light suspension of spores of *Streptomyces sp.S14*. Discs impregnated with ofloxacin (5 µg), sparfloxacin (5 µg), norfloxacin (10 µg), perfloxacin (5 µg), chloramphenicol (30 µg), neomycin (30 µg), penicillin G (2 U), erythromycin (15 µg) and nystatin (10 U) were placed over the inoculated plates and incubated at 28°C for 18 to 24 h. The zones of inhibition were then measured (S. T. WILLIAMS, J. gen. Microbiol, 1967).

**8. Results and Discussion**

**1. Colony morphology**

The isolates appeared as mucoid, irregular, rhizoid and some of the colonies initially appeared as mucoid and after 10 days of incubation shows rhizoid. Red, Gray, Yellow colored spores were observed.

**2. Gram's staining**

Gram's staining smear showed gram positive rods.

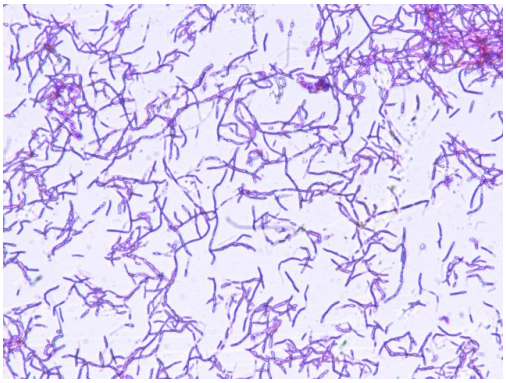

**Fig: 1** slide is bacterial *colony* observed *under 40 x magnifications Microscope* showed.

## 4. Biochemical and physiological characteristics of *Streptomyces griseus*

**Table 1**

| Type of Biochemical test | Results |
|---|---|
| Starch hydrolysis | +ve |
| Casein hydrolysis | + ve |
| Fermentation of carbohydrate (glucose fermentation) | +ve |
| Hydrogen sulfide (H2S) production | -ve |
| Urease production | -ve |
| Methyl-Red(MRVP) | +ve |
| Voges-Proskauer | -ve |
| Indole production | -ve |
| Citrate utilization | -ve |
| Hydrolysis of gelatin | -ve |
| Catalase test | +ve |

**Note:** +ve-Positive, -ve- Negative

**Table 2:** Sensitivity of *Streptomyces sp. S14* to different antibiotics

| Name of the Antibiotic | Zone of Diameter (mm) |
|---|---|
| Ofloxacin | 25 |
| Sparfloxacin | 23 |
| Norfloxacin | 10 |
| Perfloxacin | 18 |
| Chloramphenicol | 55 |
| Nystatin | 10 |
| Penicillin G | Nil |
| Erythromycin | Nil |

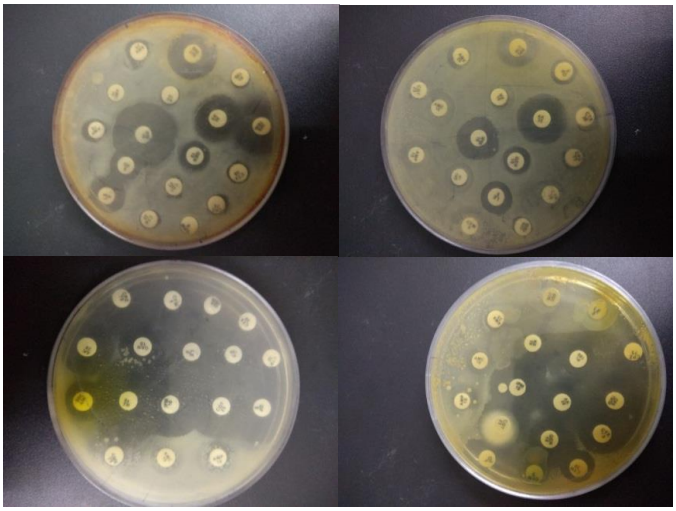

**Fig 2:** Sensitivity of Streptomyces sp. S14 to different antibiotics

**DNA Analysis and 16S rRNA Sequencing**

The molecular weight of the chromosomal DNA of *Streptomyces sp. S-14* was found to be 23,000 bp. For molecular identification of the isolated *Streptomyces sp. S-14*, amplification of isolated DNA with 27f (5' AGT TTG ATC CTG GCT CAG 3') and 1492r (5' ACG GCT ACC TTG TTA CGA CTT 3') primers for the 16S rRNA was proceeded by PCR. Agarose gel electrophoresis showed a single band of size 700 bp. A 675bp 16S rRNA gene sequence was determined for *Streptomyces sp. S-14*. The gene sequence obtained through 16srRNA analysis was published in National centre for Biotechnology information (NCBI) (GenBank ID: KU736843.1).

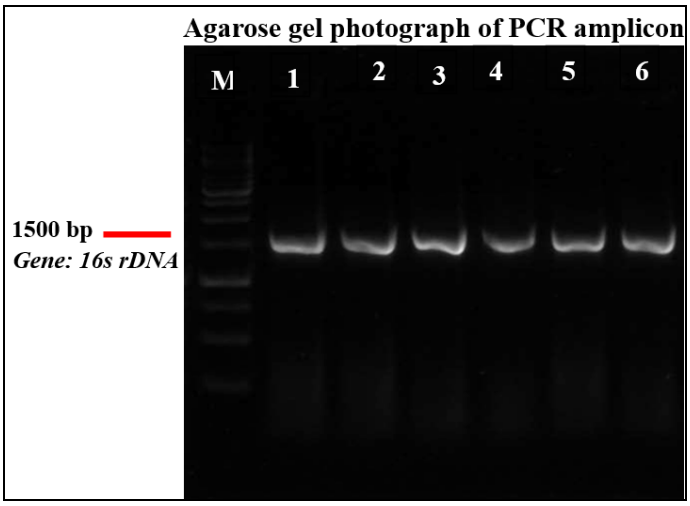


**Fig 3:** 16s rDNA Agarose gel photograph PCR application

The tree showed that there was a high degree of similarity (98%) to 16S rRNA gene sequence of *Streptomyces ghanaensis* NBRC 15414 (GenBank accession no. AB184662) and *Streptomyces espinosus* NRRL 5729 (GenBank accession no. X80826).

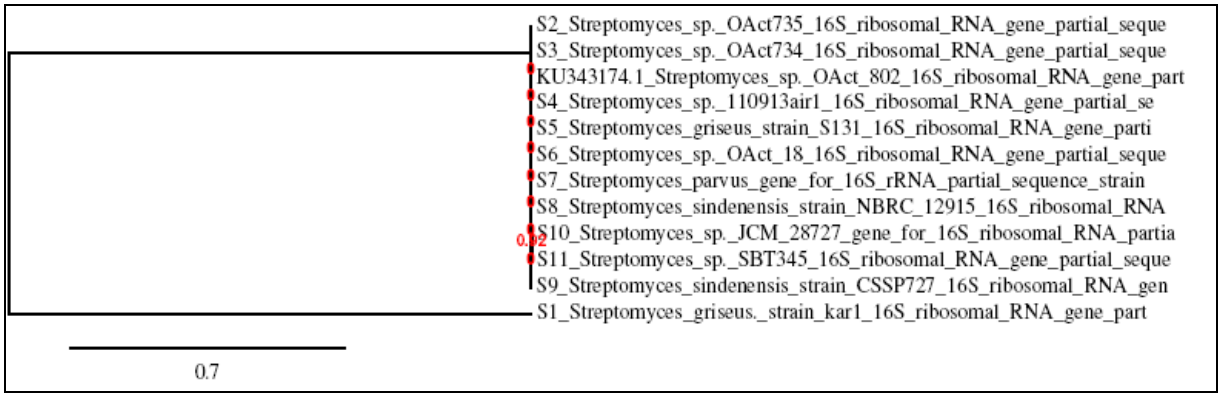


**Fig 4**

**Effect of pesticide concentration**

The effect of different concentrations of pesticide represented. The optical density was taken at 660 nm.

**Table 3**

| Hrs. | Control | 5ppm | 10ppm | 15ppm | 30ppm |
|---|---|---|---|---|---|
| | **CFU/ml** | **CFU/ml** | **CFU/ml** | **CFU/ml** | **CFU/ml** |
| 0 | 0.25 | 0.25 | 0.25 | 0.25 | 0.25 |
| 2 | 0.28 | 0.51 | 0.53 | 0.56 | 0.51 |
| 4 | 0.29 | 0.62 | 0.61 | 0.69 | 0.64 |
| 6 | 0.31 | 0.63 | 0.67 | 1.23 | 0.75 |
| 8 | 0.33 | 0.64 | 0.69 | 1.26 | 0.96 |
| 10 | 0.37 | 0.66 | 0.73 | 1.25 | 0.98 |
| 12 | 0.45 | 0.68 | 0.91 | 1.27 | 0.91 |
| 14 | 0.48 | 0.79 | 1.01 | 1.24 | 0.92 |

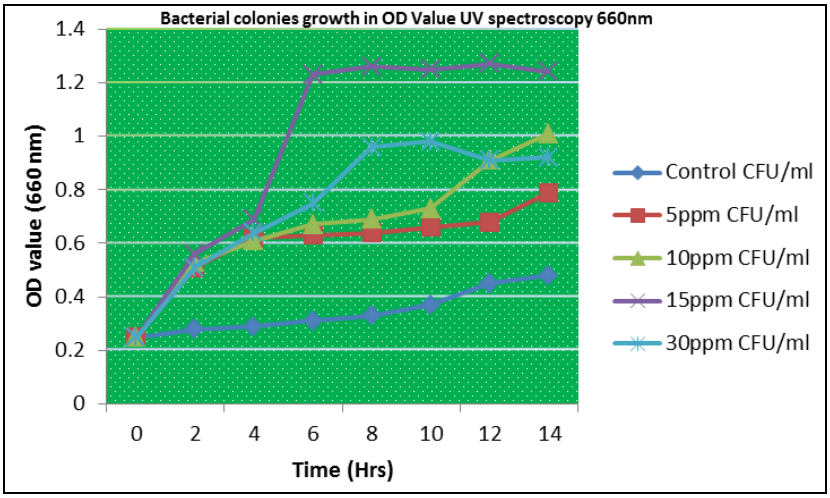


**Fig 5**

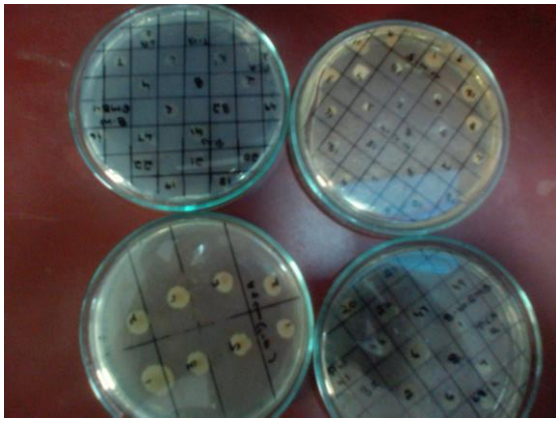

**Fig 6:** Pesticide Terence test MSM

**High Pressure Liquid Chromatography:**

Monocrotophos SL 36% was biodegraded at the retention time of 9.973 minutes, and for bacteria retention times is 10.372 (Figure7 & 8). Using self-determining enrichment step with Monocrotophos SL 36% as the sole carbon and energy, five Monocrotophos SL 36% -degrading bacteria were isolated out of which one organism were used. The ability of the isolates to utilize Monocrotophos SL 36% as carbon source was considered using HPLC over a period of 7 days with the reduction in the concentration of Monocrotophos SL 36% was determined against Monocrotophos SL 36% normal curve. The amount of biodegradation varied among the isolate Microorganisms. Growth of isolate was also examined against alteration in absorption of Monocrotophos SL 36%.

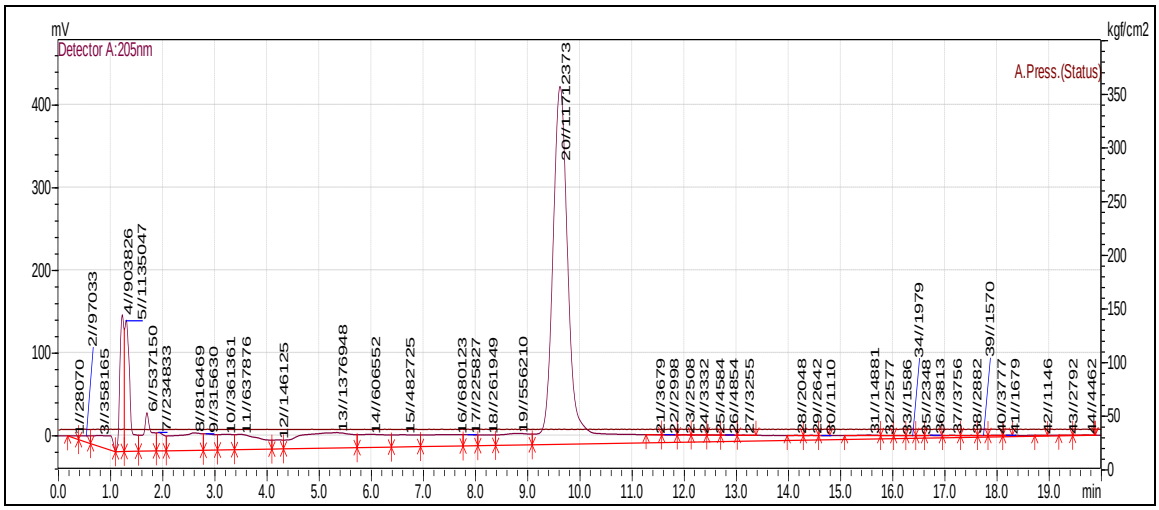


**Fig 7:** HPLC standard for Monocrotophos

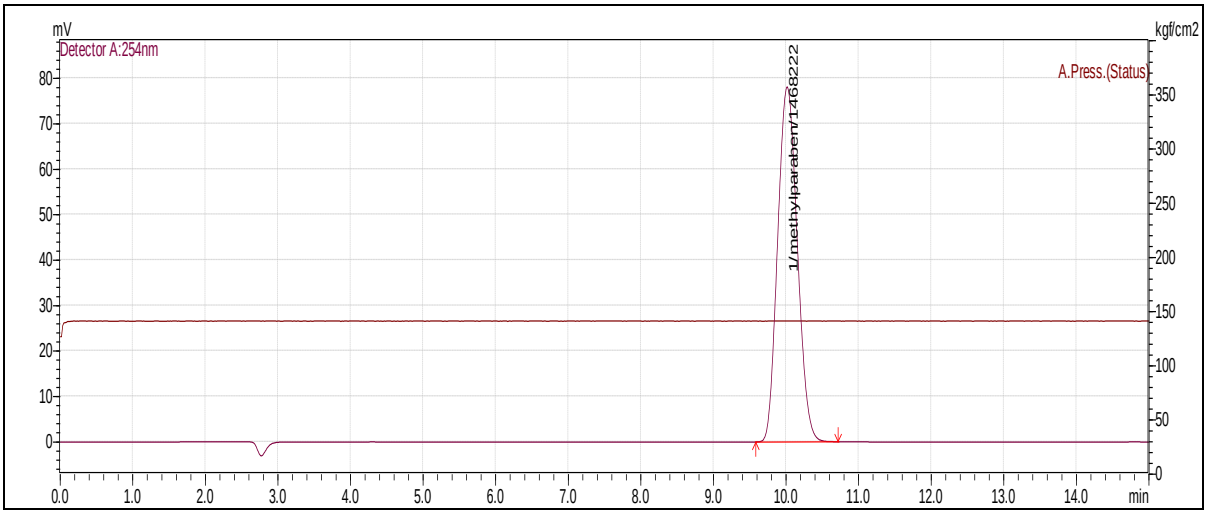


**Fig 8:** HPLC for *Streptomyces sp. s-14* degrading monocrotophos

## 9. Conclusion

Bacteria isolated from defective Vaigai river sediment soil with traditional enrichment techniques using ISO 11464 method (Soil quality- pre-treatment of samples for physicochemical and biological analysis)as an organ phosphorus pesticides model were able to grow on and to degrade Monocrotophos 36% SL in MSM liquid media. The highest pesticide biodegradation was obtained when the presence of certain metabolites such as biomass dry weight get result in Different volume in depending the Monocrotophos 36% SL ppm in MSM Medium. *KU736843Streptomyces griseous. Strain kar1*was found to be *Streptomyces griseus.* Biodegradation of these pesticide residues can ensue in soil, although at a measured amount. To improve degradation in situ several approaches have been future. Isolated strains from long period polluted soil are prospective tools for in situ remediation processes (bioaugmentation) at polluted sites.

## 10. Acknowledgement

Research and Development, Department Microbiology, Bharathiar University, Coimbatore.

Department of Molecular Biology, School of Biological Sciences, Madurai Kamaraj University, Madurai.

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