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Displaying *in-vitro* and *ex-situ* microbial degradation of pesticide-thiram by *Pseudomonas aeruginosa* TD-9 strain deploying method using novel high performance thin layer chromatography (HPTLC)

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ABSTRACT
The carbamate group of pesticides is being widely used in the agriculture field for improving crops. The dithiocarbamate group of non-systemic fungicides known as thiram (tetramethylthiuramdisulfde) is used to treat seeds, control crop pests, repel animals, etc. It has been used as a sunscreen, bactericide-infused soap, and a therapy for human scabies. It's also effectively used in mice in vitro against Tricophyton and control fungal diseases on safflower (Pythium spp., Fusarium spp.) and damping-off diseases (Phytophthora and Pythium spp.) of maize, ornamentals, and vegetables. Advancing to prevent such toxic effects and prevention of soil fertility from thiram and thiram-like chemicals is indispensable There are many microbes which are helpful in this process, but some modification in the microbes can lead to pesticide degradation at an enhanced speed. For many years, the analytical HPTLC (High-Performance Thin-Layer Chromatography) method was proposed and confirmed for the detection and quantification of various compounds. The analytical High-Performance Thin-Layer Chromatography (HPTLC) is a simple, quick and a reliable method was proposed for the detection and quantification of thiram. The identification and quantification of thiram between 100 and 700 ng/spot on a TLC plate are revealed in this HPTLC procedure. The same concentration was then utilized to determine the percentage microbial degradation of thiram from the culture broth and soil sample. The isolated bacterial strains can efficiently degrade thiram and it's a very excellent candidate for application of pollution remedy. The cultivated strains TD-9 was displaying in-vitro and ex-situ microbial degradation of thiram pesticide using novel HPTLC.

Keywords *Thiram, in-vitro and ex-situ microbial degradation and HPTLC*

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INTRODUCTION

Pesticides are still being used extensively in crop care practices in the agricultural sector, which makes it extremely dangerous for them to leak into the soil in either significant or trace amounts. The microbiological and nutritional content of the fertile soil may also be impacted by the widespread application of pesticides. Such mishaps are especially frequent in agricultural settings where pesticides are frequently used. Since plants serve as animals' primary source of nutrition, crops cultivated with pesticides can easily enter the food chain; this phenomenon is referred to as "biomagnification." [1]. The herbicides,

fungicides, insecticides, nematicides, and rodenticides are typical terms for pesticides that are categorized according to the pests they are intended to control, which can include bugs, mites, worms, fungus, nematodes, and rodents, among others [2]. The carbamate group of pesticides is being widely used in the agriculture field for improving crops by protecting against fungal diseases. The application of pesticides causes serious concern about food safety and environmental pollution. Thiram is one of the most widely used fungicides for protection against crop damage [1]. Its careful application in accordance with National Institute of Safety and Health (NIOSH) regulation is used to stop the deterioration of harvested crops during storage and transportation [3]. It is also utilized to prevent cereals, seeds, vegetables, fruits and turf crops from variety of fungal diseases and at high dose, it is reported to work as an animal repeller [4]. According to Gupta et al. [5], thiram's metabolites are more hazardous than their parent molecule. Thiram degradation suggests that degradation of pesticides from the soil is crucial to restore the soil nutrient presence, hence, to maintain a healthy crop quality and yield productivity. Dimethyldithiocarbamic acid (DMDTC) and carbon disulphide (CS2) are two of them that are more hazardous than their parent compound, endangering soil fertility and even causing diseases like diabetes.

In modern years, various microbial strains were reported for their biodegradation of organophosphate pesticides [6]. Some soil bacterial communities included *Pseudomonas*, *Agrobacterium* and *Bacillus* sp. has been reported for their capabilities of chlorpyrifos degradation [7]. The *Pseudomonas aeruginosa Is-6* can break down methyl parathion, dimethoate, acephate, methamidophos, and malathion [8], *Serratia sp. SPL-2* can degrade methidathion [9]. A previous study [10], reported that several microorganisms including Gliocladium, Trichoderma, Penicillium, Pseudomonas, Flavobacterium, Alcaligenes and Rhodococcus, use pesticides as a carbon source. In the same manner, thiram (TMTD) breakdown has been reported by several researchers. According to Richardson 95% of thiram is completely breaking down in its metabolites after 55 days of incubation by soil microbial communities. Sometimes, this decomposition of pesticide also leads to the formation of less toxic organic compounds, called partial biodegradation products. The pesticides degradation or transformation through the microorganisms can be used as a carbon, nitrogen source, or other it can be used as a source of minerals [11] and ultimately help in enrich the soil's nutrient reserves. To study the microbial degradation of pesticides, it is first important to understand the pesticide metabolism within the microorganism, and then accordingly develop appropriate strategies for bioremediation of contaminated sites [10]. According to Shirkot (1983), *pseudomonas aeruginosa* uses thiram as a significant source of carbon and energy [12]. The nature of the medium and ambient condition have an impact on microbial degradation and its metabolites. The degradation of thiram is dependent on several environmental factors, including pH, temperature, and field conditions **[5]**.

Gas chromatography (GC), gas chromatography coupled with mass spectrometry (GC/MS) and high-performance liquid chromatography (HPLC) are a few of the several analytical techniques that are frequently employed for identifying and quantifying pesticide residues. When using the HPTLC technique, very small quantity of mobile phase is used to compare the other methods. The development of HPTLC has been carried out to develop for accuracy and simplicity technique to identification and quantification of thiram in comparison to the traditional TLC [13]. When compared to other methods, such as GC and HPLC methods, a very tiny amount of mobile phase is utilized when utilizing the HPTLC approach, and it also provides speedy an accurate detection of a variety of pesticides samples.

We have drawn out this work based on the main advantage of HPTLC, it will improve the lack of information about the detection, quantification and percentage degradation of thiram pesticide by microbes. To assess the effectiveness of thiram degradation in both *in-vitro and ex-situ* microbial degradation of *Pseudomonas aeurogenasa* TD-9 bacterial strain, we used a unique HPTLC approach.

MATERIAL AND METHODS

Apparatus

The experiment was carried out in HPTLC system which was purchased from CAMAG (Muttenz, Germany). The HPTLC system consisted of Linomate 5 auto sprayer which is equipped with a Hamilton syringe (100 µl) for samples loading on TLC silica gel 60F254 plates (Merck, Germany) and a Scanner 3 which was equipped with winCATS software (version 2.5.18262.1) for densitometry evaluation of HPTLC plates.

Materials and reagents

The carbamate group of fungicides thiram (TMTD) was purchased from commercially market of Gujarat. All other reagents and solvent acetone, ethyl acetate, chloroform, hexane Sisco Research Lab. Pvt. Ltd.) and methanol (Molychem, India) were purchased of analytical grade. The aluminium backed TLC silica gel 60F254 plates (0.25-mm thickness) were purchased from Merck (Darmstadt, Germany).

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Stock and standard solution

The 10 mg/ml stock solution of thiram was prepared in analytical grade chloroform by weighing 1.0 g of the analyte (thiram) into 100 ml volumetric flask and stored in dark under refrigeration for their use. The appropriate concentration of working standard solution was prepared through diluting this stock standard solution. The 0.05 mg/ml standard solution of thiram was prepared in chloroform.

Soil sampling

The soil sample were collected from thiram pesticides contaminated agricultural field that was in the satlasana (Gujarat). The soil has been exposed to continuous applications of fungicides thiram for last 4-5 years. The soil samples were air dried and sieved through a 2 mm mesh and stored in sterile glass bottles at 4 °C. the soil was sandy and loamy with 6.5 pH.

Qualitative screenings thiram degrading microbes by enrichment culture

The enrichment culture method was used for isolation of thiram degrading microorganism. About 1 gm of soil sample was added in 10 ml of sterile distilled water and soil suspension was shaken vigorously on vortex at room temperature and prepared serial dilutions (10^{-2} to 10^{-5}). After serial dilution, 0.1 ml aliquots were inoculated in petri dish containing M9 agar and only agar medium with thiram pesticide for isolation of pesticides tolerating microorganism. Plates were incubated at 30°C for up to 7 days. After screening, colonies were purified by nutrient agar medium. After purification colony were tested on different concentration of thiram pesticide and colony morphology was studies according to the Bergey's manual of Determinative Bacteriology [14], further than cultures were incubated on 100 ml liquid medium for identified degradation capabilities. The culture flasks were incubated for 4 and 8 days of incubation period for *in-vitro* studies and 10 days of incubation period for *ex-situ* studies. The outline of experimentation is shown in **Figure 1**.

HPTLC performance

Sample application

In sample application system, standard and extracted samples were loaded on 10×20 cm sized TLC plate using a Linomat 5 applicator (CAMAG, Muttenz, Germany) which contained 100 µl syringe and there was application rate 150 nl/s, table speed was 10 mm/s, band length was 6 mm and band distance was set automatically and the distance from plate edge to origin on the X-axis and Y-axis was 14 mm and 10 mm.

Calibration curves

For calibration curves, thiram standard solution (50 µg/ml) was prepared in chloroform and these concentrations of standards solution were loaded separately in the range of 2 to 14 µl on 10×10 cm sized TLC plate. The different volume of standard solution 7 bands was loaded on TLC plate. The volumes of standard thiram was loaded on TLC plate from track 1 to track 7 which were follow in the steps of 2 µl, 4 µl, 6 µl 8 µl, 10 µl, 12 µl and 14 µl per band on TLC plate for standard calibration. During the standard calibration, calibration curve was expanded with the increasing the concentration range (100 to 700 ng per band).

After loading the standard sample, TLC plate was developed in presaturated twin trough chamber with mobile phase hexane and acetone [7.5: 2.5 (v/v)], where saturation time was 20 min and developed solvent front (distance) was 7.5 cm (75 mm) on TLC plate. For spot visualities, plate was air dried for 10 min and then spots were visualized under UV chamber with 256 nm wavelength. TLC plate image and their densitograms are reported in the supplementary material.

Densitometric analysis of chromatogram

After TLC plate development, quantitative densitometric analysis was operated and peaks area of the developed spots of relative samples were quantified through linear scanning using at 256 nm Camag TLC Scanner 3 with deuterium source at 20 mm/s scanning speed, operated by winCATS software, data resolution was 100 µM/step using filter factor, slit dimension setting was 5-mm length×0.45-mm width, and lowest slope was set for baseline correction and display scaling was set to automatic. The limit of detection (LOD) and limit of quantitation (LOQ) were determined using X–Y scatter graph for the peak area of the developed spot versus ng of standard loaded per band. The sample detection and lowest concentration detection of samples were determined based on LOD and LOQ. The calculation of LOD and LOQ were performed with the formula for LOD=3.3 (SD/ slope) and for LOQ=10 (SD/slope). According to the formulas , standard deviation and slope were calculated from standard curve of ng of sample (X-axis) against peak area (Y-axis) using LINEST function in MS EXCEL 2010 [15].

Validation of method

The spot of thiram in the bacterial extract sample and control broth/soil was assured by comparing Rf values of spot with that of standard and control thiram.

Linearity

HPTLC method linearity was assessed interpretatively calibration curve at different levels of standard solution. The calibration curve was over planned range 100 -700 ng/spot. The linearity was evaluated by plotting the standard curve of ng of sample (X-axis) against peak area (Y-axis).

Precision and robustness

The precision was determined as a recovery study by standard addition method. The accuracy of HPTLC method was determined by the application of the analysis measurements for sample solution recovery studies. The precision of method was determined by adding the three different concentration of 450 ng, 700 ng & 1000 ng of standard in separate tracks of TLC plate. The experiments were performed in triplicate. The concentration of the thiram standard was evaluated using densitometric analysis by comparing the peak area of the spots with previously obtained standard curve. Percentage recovery was measured and considering the loaded amount of standard and control as 100%.

Specificity

The specificity of the developed method was determined by analysis of standard and test samples. Thiram samples were validated by comparing Rf values and UV spectrum that of the standard and spot.

System precision-repeatability

The system precision was determined by spotting thiram samples which was covering similar range of calibration curve seven times and analyzing them once.

Detection and analysis of thiram residual from bacterial cell free supernatant using the *in-vitro* and *ex-situ* microbial degradation

***In-vitro* microbial detection for check the degradation studies**

Following the *in-vitro* degradation extraction procedure, thiram's purity was checked on a thin layer chromatography (TLC) plate, and it was subsequently quantified using the HPTLC method. The method of HPTLC, thiram standard, control and bacterial extracts from each bacterial strain were loaded on TLC plate 20×10 cm sized. On TLC plate (20×10 cm sized) of *in-vitro* degradation method, total 18 bands were loaded with different volume of standard (7 µl, 14 µl and 21 µl), 3 bands loaded with different volume of control (2 µl, 4 µl and 6 µl), 3 bands loaded with 4th day extracted each bacterial samples in different volumes (15 µl, 30 µl, 45 µl) and 3 bands loaded with 8th day extracted each bacterial samples with volumes (15 µl, 30 µl and 45 µl), respectively. The TLC plate was prepared using a mobile phase of hexane: acetone [7.5: 2.5 (v/v)] in a pre-saturated twin trough chamber. The solvent front distance that was traveled 75 mm. Following the completion of the plate development, the plate was allowed to air dry before the spots were detected using the Scanner-3's absorbance-mode at 256 nm. Through the densitometric analysis, the residual thiram of presented bacterial strains were identified, and the percentage degradation was computed. The amount of thiram degraded was determined using a 100% recovery rate for thiram from control broths (broths without bacteria inoculation). Similar to this, the HPTLC chromatographic approach was used to accomplish the thiram *ex-situ* degradation extraction process, quality detection, and quantification on TLC plates.

***Ex-situ* microbial detection for check the degradation studies**

A similar method was used to detect thiram in *ex-situ* microbial decomposition. The 10 ml of bacterial active culture were prepared for ex-situ studies. After completing the incubation period, the broth was centrifuged to separate the bacterial cell pellets. The bacterial cell pellets were inoculated in 100 gm soil containing beaker with 100 ppm thiram pesticide and same as control uninoculated soil. Culture beakers were inoculated in room temperature for 10 days of incubation period. In two days of interval, sterile distill water was sprayed on soil beaker to maintain the survival condition of bacterial strain. After completing the incubation period, an extraction process was carried out for detection and quantification of thiram or thiram residues.

Following the incubation period , 100 gm of soil beaker sample was added to 200 µl of 1 N HCL to acidify the culture beaker to a pH 2.5 and the sample was extracted using three times the amount of ethyl acetate (300 ml). For the collection of ethyl acetate fraction, which concentrate the residues content up to 2.0 ml final volume, vacuum evaporator was used. The concentrates residues contents were taken for analytical HPTLC method. After the completion of extraction process, sample extracts were checked on TLC plate to confirm the quality detection of residues. After completed the quality detection on TLC plate, the work is extended for HPTLC technique. The thiram standard, control and bacterial extracts of soil from bacterial strain, were loaded on TLC plate 20×10 cm sized. On TLC plate, total 12 bands were loaded with different volume of standard (7 µl, 14 µl and 21 µl), 3 bands loaded with different volume of control (2 µl, 3.5 µl and 5 µl), 3-3 bands loaded with 10th day extracted of bacterial samples in different volumes (2 µl, 3.5 µl and 5 µl) respectively. Following sample loading, a TLC plate was developed using a mobile phase of hexane: acetone [7.5: 2.5 (v/v)] in pre saturated twin through chamber and the frontal distance to the moving

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solvent was 75 mm. After completing the plate development, plate was air dried, and spots were scanned using Scanner-3 absorbance–mode at 256 nm. To assess the recovery of thiram (residual thiram after microbial degradation) from samples, densitometric analysis was used. The amount of thiram degraded was calculated using a 100% recovery rate for thiram from control soil sample (soil without bacteria inoculated).

RESULT AND DISCUSSION

16s rRNA gene sequence analysis

Preliminary qualitative screening experiments were performed to obtain thiram resistant strains. A qualitative screen consisted of assessing the growth of isolates on plate containing M9 ager and only agar inoculated medium with thiram different concentration. A total of 18 microorganism strains were isolated from pesticides contaminated soil samples that was characterized by repeated application of thiram. The pure culture of strains was obtained five to six rounds of subculturing in enrichment culture media. A quantitative screen consisted of assessing the degradation of 100 ppm thiram in liquid medium after 4th to 8th days by strains showing good growth at the highest concentration of thiram tested in the previous screen. A concentration of 100 ppm thiram was chosen because thiram resistant strains showed higher growth and higher colony development compared to solid media containing 200 ppm thiram. Microbial degradability was calculated from this experiment.

In the present studies, TD-9 bacterial strain was preliminarily identified by their morphological, biochemical, and molecular characteristics. The presented strain was selected based on 100 ppm pesticides tolerance ability. Selected strains were gram-negative and short rod-shaped on microscopic morphology. The nucleotide sequence of TD-9 was deposited at the NCBI GenBank under Accession No. OP646604. By using an online alignment search engine, the bacterial strains TD-9 were compared to the other 10 strains of the pseudomonas genera in the GenBank database. Then, using MEGA X software, a phylogenic tree was built using the 16s rRNA gene sequences. The 16s rRNA gene sequence of the TD-9 bacterial strain shows 100% identical similarities to the 16s rRNA gene of *Pseudomonas* sp. (GenBank accession no. FJ972538) (**Figure 2**). In these experiments, isolation of bacterial strain was achieved through enrichment culture method and colony characteristics and morphological assessment highlighted the presence of *Pseudomonas* which was further affirmed from 16 s rRNA gene sequencing of the bacterial isolates. The isolate was recognized as a *Pseudomonas aeruginosa* TD-9 strain. Even at a greater thiram concentration of 200 ppm, the isolates were able to survive and may have used thiram or its byproducts as an energy source. After the extraction and identification procedure, the broth of the isolates was concentrated to one tenth of the total volume for an adequate application during TLC and HPTLC. TLC was used before HPTLC because it provides accurate information about the purity of the compounds and the best eluent and stationary phase combination to use when separating the compounds. The solvent system was first optimized using a conventional, straightforward TLC technique before being carried forward for HPTLC. These details are necessary to carry out a high-resolution HPTLC analysis [21]. The process of sample preparation in the HPLC technique is laborious because HPLC requires a sample that is extremely pure and takes a longer time to detect and standardize [22]. Therefore, to overcome such limitations, we hypothesized a HPTLC-based protocol to accurately detect and quantify thiram.

In these studies, find out the *in-vitro* and *ex-situ* microbial degradation in broth and pesticides treated soil samples and percentage degradation was quantified by HPTLC analytical method. The TD-9 bacterial strain is capable of consuming and breaking down the thiram pesticides.

Calibration curves

The composition of the mobile phase was based on polarity during the development of the HPTLC method for thiram analysis. For the confirmation of pesticide residues, different mobile phase was tried on TLC plate for thiram analysis such as ethyl acetate:cyclohexane, acetone:chloroform and hexane:acetone in a different volume. However, only the mobile phase hexane:acetone [7.5: 2.5 (v/v)] would produce compact and dense spots with significantly and appropriately different Rf values. For 10 minutes, a standard thiram loaded TLC plate was developed in pre saturated twin through chamber using mobile phase hexane:acetone [7.5: 2.5 (v/v)]. After development, the plate was air dried and analyzed for retention factor (Rf) values under 256 nm, where appeared thiram Rf value 0.33. For creating the calibration curve **(Figure 3)**, standard thiram was loaded separately in the range of 100 ng to 700 ng/spots. Due to densitometric analysis, it was determined that there was strong linear correlation between the loaded standard concentration and the peak area of the relevant spot, which ranged from 200 to 700 ng/spot. The calibration curve revealed that the limit of detection (LOD) and limit of quantification (LOQ) of thiram were found to be 354.44 ng and 1013.77 ng per/spot, respectively.

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Accuracy of method

By randomly applying three different concentrations of standard thiram and reanalyzing them using the developed HPTLC method, the accuracy **(Table 1)** of the HPTLC method was assessed. This was done to ensure that the developed method quantified the same concentration of thiram that was applied on TLC. To do this 450 ng, 700 ng and 1000 ng standard thiram were loaded onto a TLC plate and run in a twin trough chamber with mobile phase hexane: acetone [7.5:2.5 (v/v)].

During the development of solvent front in 75 mm height, the plate was scanned at 256 nm to determine the peak area of developed spot. The standard concentration in the respective spot was determined by comparing its respective peak area of developed spot. Since the loaded amount was assumed to be 100% for each standard's measure quantity in **(Table 1),** the obtain value was shown as a percentage recovery value. Taking recovery values into account showed that the approach was accurate within the specified range.

Quantitative determination of pesticide residues in extracted samples

Check the degradation studies by *in-vitro* technique

The thiram degrading isolate, which was isolated from agricultural soil that had been treated with thiram pesticide, was chosen for the investigation using the selective enrichment culture method. The bacterial strain was determined based on their growth capacity on minimum concentration of pesticides on M9 and MSM medium. Out of many bacterial strains, presented bacterial strain was able to consume thiram up to 200 ppm of thiram pesticides. The TD-9 bacterial strain was grown on 100 ppm thiram concentration on Minimal media with 4 and 8 days of incubation periods. In this study, microorganisms with the potential to break down thiram were isolated from agricultural soil with a history of thiram treatments. A level of thiram tolerance was present in around 80% of the isolated bacteria in broth and soil extract. For the treatment of contaminated agricultural land and landfills in the modern day, it is crucial to create bioremediation technologies and allow microbial degradation of pesticide [16]**.** Natural microorganisms that degrade thiram might be rare in the environment, so it's important to isolate and identify strains of bacteria that can do this.

Presented, bacterial strain TD-9 are used for degradation studies and their extraction process of thiram residues is represented a form of a flowchart in **Figure 1.** During the precise calibration, the analytical technique HPTLC was used to identify and quantify the thiram pesticide in bacterial extract. Mobile phase hexane and acetone [7.5:2.5 (v/v)] produces a dense and compact spot with good resolution and Rf value of 0.33 during the development of a TLC plate. **Figure 3a** displays a TLC picture following the emergence of the solvent front and **Figure 3b** displays the calibration curve for the thiram standard.

In a concentration range of 100 to 700 ng per spot about the peak region, the analytical approach provided the linearity. Any analytical method's strength is its linearity, which allows it to immediately determine the test result within a specified range or to arrive at some mathematical transformation that is proportional to the concentration of an analyte. In **Figure 3** regression data showing good linearity which are suitable for analysis.

Tracks 1 to 3 were loaded with 7 µl, 14 µl and 21 µl of thiram standard as a reference on the TLC picture in **Figure 4**, while Tracks 4 to 6 were filled with 2 µl, 4 µl and 6 µl of uninoculated extracted sample as a control are presented in **Figure 5** along with its 2D densitogram.

Thiram degradation result are shown in **table 2**. The 15 µl, 30 µl and 45 µl of 4-day-grown broth (set I) sample extract for *Pseudomonas aeruginosa* TD-9 strain were put on three distinct bands on tracks 7 to 9 **(Figure 4).** A total of 10 compounds were found during TLC development **(Figure 4 (track 8))**. Thiram was found in tracks 7 through 9 at concentrations of 622.22 ng, 255.55 ng, and 288.88 ng respectively. Extracts from tracks 4 to 6 from controls (uninoculated broth) found thiram at concentrations of 532.22 ng, 1061.11 ng, and 1451.11 ng, respectively **(Table 2)**. Thiram concentration in the uninoculated broth was determined to be 5.15±0.27 µg/ml after back-calculating the densitometric TLC data, while *Pseudomonas aeruginosa* TD-9 strain 4-day grown culture extract revealed presence of just 0.37±0.11 µg/ml of thiram. As a result, 92.70% of thiram was degraded when it was exposed to TD-9 for 4 days in culture broth **(Table 2).**

Similarly, 8 days grown culture broth (set II) extract sample of *Pseudomonas aeruginosa* TD-9 strain was put on three different bands on track 10 to 12. The 8 compounds were observed during TLC development **(Figure 4 (track 11))**. Thiram was found in tracks 10 through 12 at concentrations of 44.44 ng, 33.33 ng and 411.11 ng, respectively **(Figure 4)**. On tracks 4 to 6 thiram were detected with concentrations 532.22 ng, 1061.11 ng and 1451.11 ng, respectively **(Table 2).** Thiram concentration in the uninoculated broth was determined to be 5.15±0.27 µg/ml after back-calculating the densitometric TLC data, while *Pseudomonas aeruginosa* TD-9 strain 8-day grown culture extract revealed presence of just

0.08±0.00 μg/ml of thiram. As a result, 98.29% of thiram was degraded when it was exposed to TD-9 for 8 days in culture broth **(Table 2).**
The central concentrations of (i) standard thiram; (ii) control uninoculated broth; (iii) 4-day *Pseudomonas aeruginosa* TD-9 strain-treated set I broth extract; (iv) 8-day *Pseudomonas aeruginosa* TD-9 strain-treated set II broth extract which are track 2, track 5, track 8, track and 11 respectively, are shown in **Figure 5**; along with its densitograms which are show other nonspecific compounds besides thiram and their relative abundance. In addition to providing simultaneous detection and quantification of thiram, HPTLC analytical method produces a capability to verify the absorbance of UV light by analyte or compound present in the spot created on the TLC plate.
The bacterial strains are capable of destroying pesticides up to 200 ppm, although in the present study, only 100 ppm of pesticide was used. Thiram can be broken down by the *Pseudomonas aeruginosa* TD-9 strain up to 92% and 98% in broths that have been treated for 4 and 8 days, respectively. A comparison of the percentage degradation data to the typical thiram peak region revealed that the sample containing the thiram has a lower height. The outcome demonstrated that thiram degradation existed. The outcome demonstrated that there were significant differences in thiram degradation at various points during the incubation period **(Table 2).** According to a literary analysis by Elhussein et al. [12], the rate of thiram breakdown in soil is inversely correlated with its concentration. Elhussein et al. [12] found that after 55 days of incubation, soil microorganisms entirely transformed thiram to its metabolites. In the presented thiram degradation experiment, bacterial strain of *Pseudomonas* species showed the greatest degradation of thiram. Various technique can be used to analyze the pesticides but presented HPTLC technique is the most suitable for analyzing most types of pesticides because it is easy to use and effective [16]**.** Pillai et al. [18] assert that the chromatographic method's accuracy is extremely high and that it indicates values that are close to their true values. For the analytical technique of HPTLC, accuracy of the method was evaluated by performing a decomposition study on the samples. Numerous bacterial and fungal species, including *Aeromonas, Azospirillum, Alternaria, Bacillus, Brevibacillus, Nocardioides, Pseudomonas, Ralstonia, Rhodococcus, Sphingomonas, Streptomyces* and *Trichoderma* have also been implicated in previous studies as biodegrades or detoxification agents of the fungicide carbendazim (CBZ) in both soil and aquatic ecosystems [18].
Pseudomonas species has shown high metabolic adaptability to many toxic pollutants such as mancozeb [19], methyl parathion, endosulfan, dimethoate and malathion [20]. The purpose of the current study is to assess the potential as thiram degraders of *Pseudomonas aeruginosa* TD-9 strain. The use of microbes to clean up polluted soil fields could be a more progressive alternative than using expensive and unreliable methods of incineration or pyrolysis for creating a pollution-free environment.
The current manuscript claims for the first time an easy and quick technique to separate and identify thiram or thiram residue in bacterial extract sample. Before HPTLC, when spot was observed in TLC, it is further checked and quantified by HPTLC. During the *in-vitro* degradation studies, the experimental set used for the TD-9 bacterial strain's degradation study was 100 ml of broth containing a total of 5.15 μg/ml of thiram pesticide. Thiram degradation rate was 0.37 μg/ml and 0.08 μg/ml, respectively, after the incubation periods of 4 days and 8 days. The sample that had been treated for 8 days showed the greatest degradation. The findings show that the degradation was higher in the sample incubated for 8 days as opposed to 4 days, and this was true for both strains under study.
Check the degradation studies by *ex-situ* technique
Similar, presented bacterial strains *Pseudomonas aeruginosa* TD-9 are used for *ex-situ* degradation studies and their extraction process of thiram residues is represented in the form of a flowchart in **Figure 1**. During the precise calibration, the analytical technique HPTLC was used to identify and quantify the thiram pesticide in bacterial extract through the *ex-situ* degradation studies. For *ex-situ* studies, the same mobile phase hexane and acetone [7.5:2.5 (v/v)] was used for good resolution and compact spot. Calibration curve for thiram standard is presented in **Figure 3b**.
For *ex-situ* studies, TLC tracks were loaded with different concentration of thiram standard, control and thiram residues. Tracks 1 to 3 were loaded with 6 μl, 10 μl and 14 μl of thiram standard as a reference on the TLC picture in **Figure 6,** while Tracks 4 to 6 were filled with 2.0 μl, 3.5 μl and 5 μl of uninoculated extracted sample as a control are presented in **Figure 7** along with its 2D densitogram.
Thiram *ex-situ* degradation result is shown in **table 3**. The 10 days grown culture of soil sample extract sample of *Pseudomonas aeruginosa* TD-9 strain was put on three different bands on track 7 to 9. The 4 compounds were observed during TLC development **(Figure 6 (track 8))**. Thiram was found in tracks 7 through 9 at concentrations of 23.33 ng, 17.77 ng, and 101.11 ng respectively **.** On tracks 4 to 6 thiram were detected with concentrations 218.88 ng, 888.88 ng, and 988.88 ng, respectively **(Table 2).** Thiram concentration in the uninoculated broth was determined to be 3.7333 μg/ml after back-calculating the

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densitometric TLC data, while *Pseudomonas aeruginosa* TD-9 strain 10 days grown culture extract of soil sample (set Ш) revealed presence of just 0.2366 µg/ml of thiram. As a result, 94.02% of thiram was degraded when it was exposed to 10 days in culture soil sample **(Table 3)**.
The central concentrations of (i) standard thiram; (ii) control uninoculated soil; (iii) 10-day *Pseudomonas aeruginosa* TD-9 strain-treated set Ш soil sample extract which are track 2, track 5, track 8 respectively, are shown in **Figure 7** along with its densitograms which are show other nonspecific compounds besides thiram and their relative abundance. HPTLC analytical method produces a capability to verify the absorbance of UV light by analyte or compound present in the spot created on the TLC plate.
Thiram can be broken down by the *Pseudomonas aeruginosa* TD-9 strain up to 94% in soil treated beaker that have been treated for 10 days, respectively. The TD-9 strain are also capable of degrading thiram in soil at room temperature. A comparison of the percentage degradation data to the typical thiram peak region revealed that the sample containing the thiram has a lower height. The outcome demonstrated that thiram degradation existed. The outcome demonstrated that there were significant differences in thiram *in-vitro* and *ex-situ* degradation at presented strain **(Table 2 & 3)**. During the *ex-situ* degradation studies, the experimental set used for the TD-9 bacterial strain's degradation study was 100 gm of soil sample (untreated) containing a total of 3.74 µg/ml of thiram pesticide. Thiram degradation rate was 0.11 µg/ml and 0.24 µg/ml, respectively, after the incubation periods of 10- days. When the soil sample that had been treated for 10 days showed the degradation. The present results showed that the *Pseudomonas aeruginosa* TD-9 strain have great thiram degraders in broth as well as soil samples. The outcome also demonstrated that thiram degradation is closely correlated with incubation time; as incubation time increases, greater degradation is seen. All the thiram residue chemicals are removed from the broth and soil sample and then separated on a TLC plate as part of the sample extraction process for TLC analysis. In this experiment, we found that the percentage degradation increased together with the incubation period. This study will help commercialize this bacterial isolate for the removal of thiram pesticide from contaminated fields.

Table 1 Determination of accuracy for thiram standard.

Sample loaded (ul)	Loaded concentration of Thiram (ng)	Calculated concentration of Thiram (ng)	% Recovery
7	450	464.444	103.2098765
14	700	717.778	102.5396825
21	1000	1037.778	103.7777778
Thiram Concentration of Stock solution = 0.05 mg/ml			

Table 2 Quantification of thiram from bacterial extracted sample broth through the *in-vitro* technique

	µl of sample loaded	Area under the curve	ng of thiram per spot	Per ml of extract (µg)	Thiram per ml of broth (µg)	Average with standard error (µg)	% Thiram degradation
Control							
Uninoculated broth (control)	2	0.00689	532.22	266.11	5.32	5.15±0.27	NA
	4	0.01165	1061.11	265.27	5.30		
	6	0.01516	1451.11	241.85	4.83		
***Pseudomonas aeurogenasa* TD-9**							
4 days of incubation	15	0.0077	622.22	41.48	0.82	0.37±0.110	92.70%
	30	0.0044	255.55	8.51	0.17		
	45	0.0047	288.88	6.41	0.12		
8 days of incubation	15	0.0025	44.44	2.96	0.05	0.088±0.008	98.29%
	30	0.0024	33.33	1.11	0.022		
	45	0.0058	411.11	9.13	0.81		

Table 3 Quantification of thiram from bacterial extracted soil sample through the *ex-situ* technique

	µl of sample loaded	Area under the curve	ng of thiram per spot	Per ml of extract (µg)	Thiram per ml of broth (µg)	Average with standard error (µg)	% Thiram degradation
Control							
Uninoculated broth(control)	2	0.00407	218.88	109.44	2.18	3.73±1.45	100
	3.5	0.0101	888.88	253.96	5.07		
	5	0.011	988.88	197.77	3.95		
***Pseudomonas aeurogenasa* TD-9**							
	2	0.00231	23.33	11.66	0.26		
	3.5	0.00226	17.77	5.07	0.37		
	5	0.00301	101.11	20.22	0.08	0.23±0.14	94.02

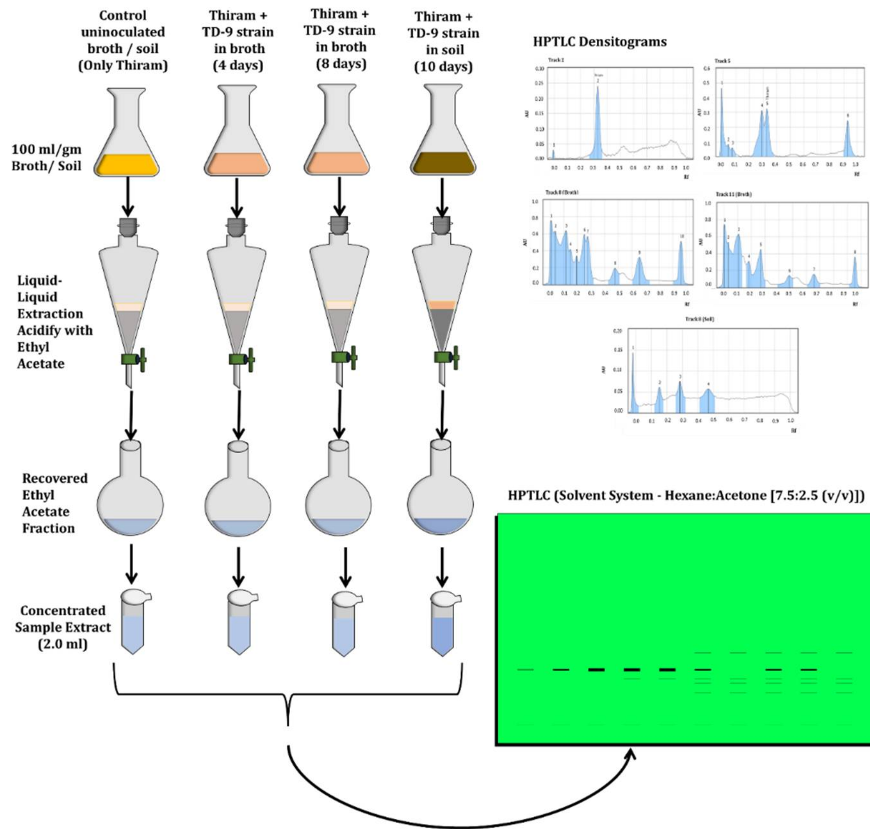

Figure 1 Workflow of extraction of residual thiram from microbial culture broth and soil sample and their sample preparation for HPTLC assessment for studying microbial degradation of thiram

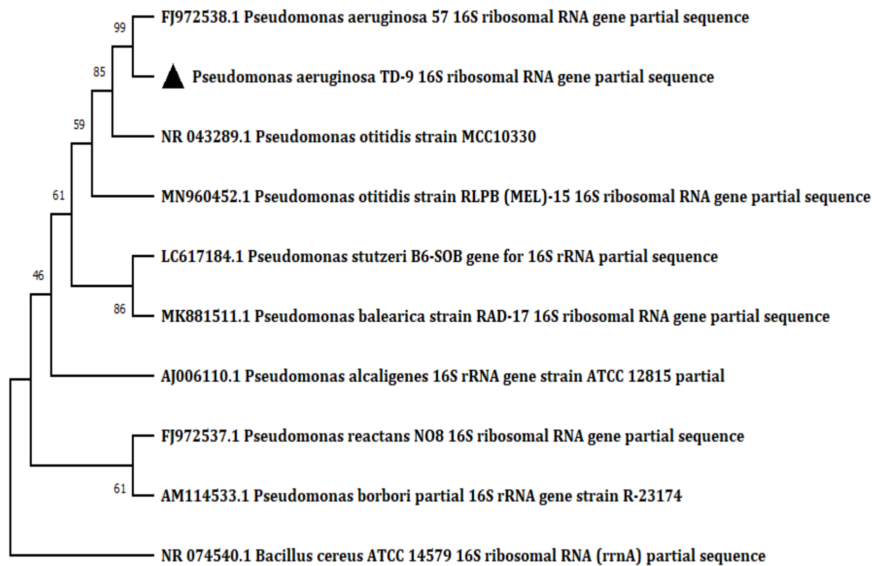

Figure 2 Phylogenetic tree showing the affiliation of isolated thiram-degrading bacteria in relation to representative bacteria with ability to degrade pesticides. Asterisk indicates sequences from bacteria reported as thiram-degraders. Phylogenetic tree derived from analysis of 16s rRNA gene sequence of strain TD-9. Phylogenetic analysis based on 16s rRNA gene sequences available from NCBI library constructed after multiple alignments of data ClustalW with neighbour-joining method using MEGA 11 software.

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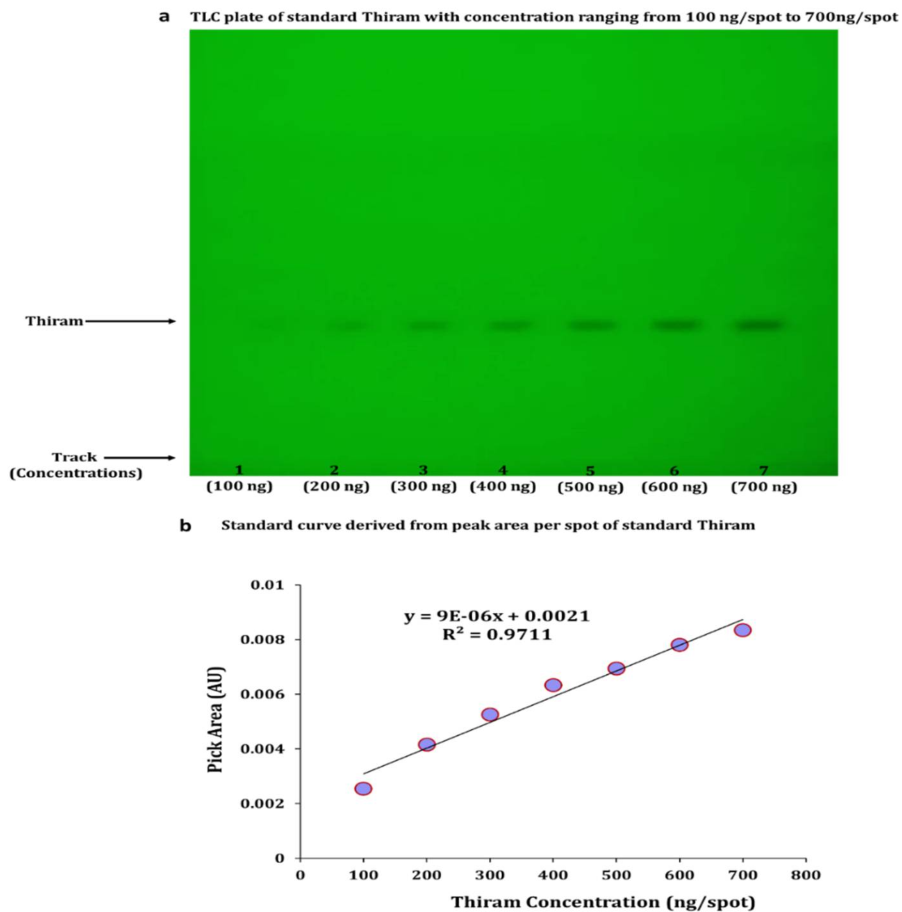

Figure 3 (a) The image of thiram standard on TLC plate under 256 nm UV light, **(b)** standard curve of peak area versus thiram concentration. It can be noticed that as the concentration of standard increases, the area under the curve also increases linearly

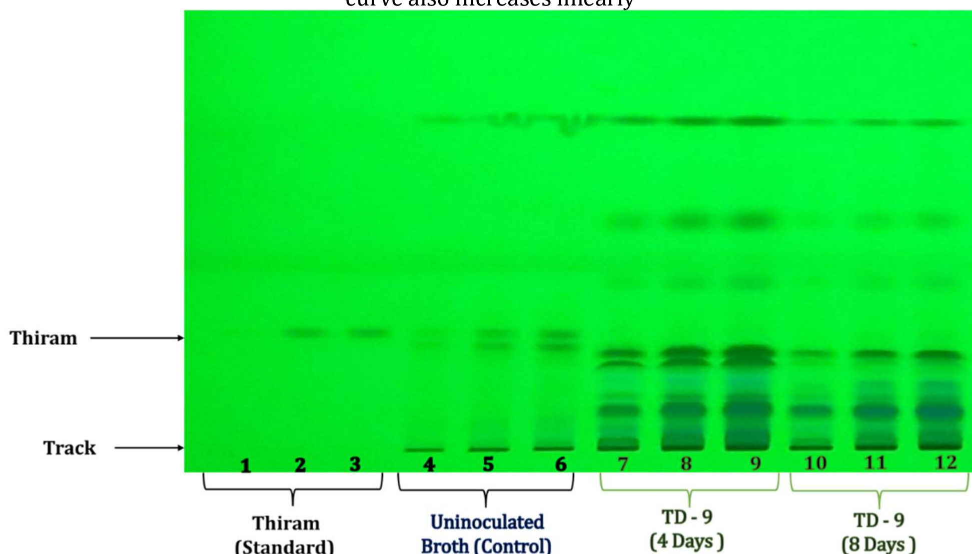

Figure 4 Image of TLC plate under 256 nm UV light where tracks 1 to 3 were loaded with 450 ng, 700 ng and 1000 ng of thiram, respectively; tracks 4 to 6 were loaded with uninoculated broth extracts as a control (2 µl, 4 µl and 6 µl, respectively); tracks 7 to 9 were loaded with 4-day treated sample from *Pseudomonas aeruginosa* TD-9 strain (5 µl, 10 µl and 15 µl, respectively); tracks 10 to 12 were loaded with 8-day treated extract sample from *Pseudomonas aeruginosa* TD-9 strain (15 µl, 30 µl and 45 µl, respectively)

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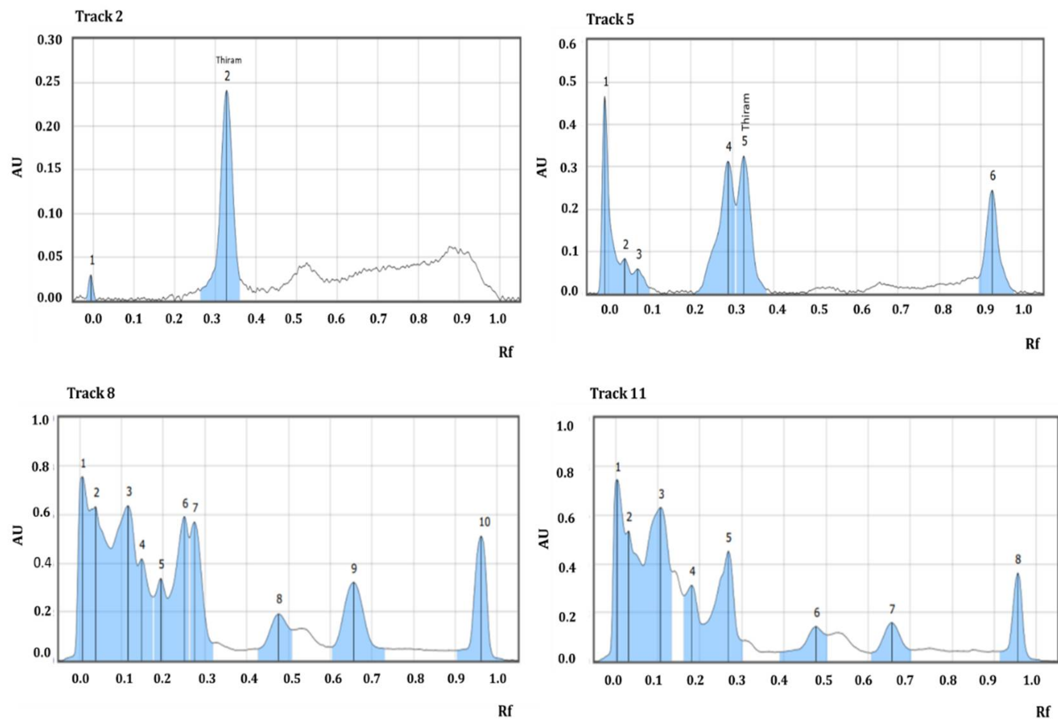
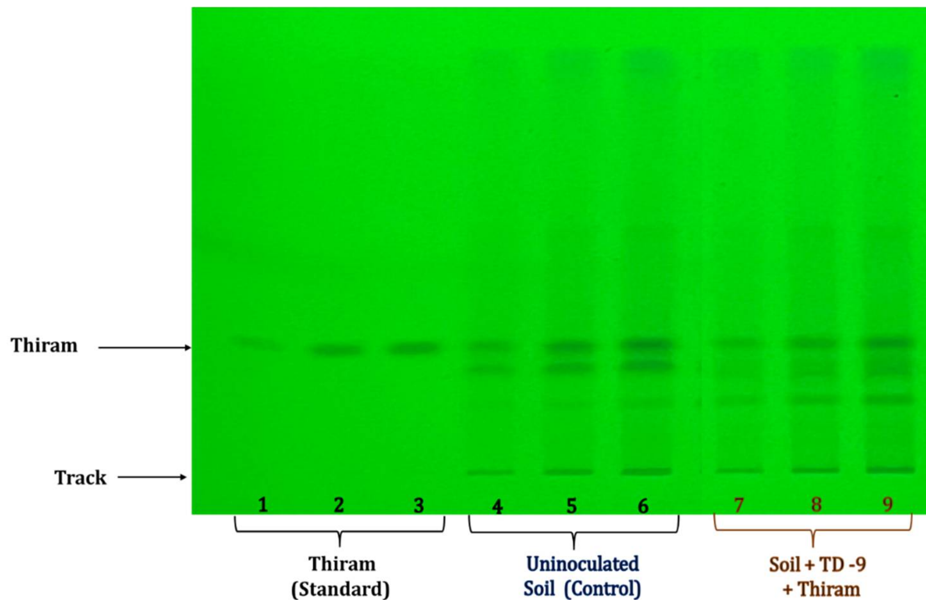


Figure 5 2D densitograms of bacterial extracts thiram and thiram residue in a sample in track 2 loaded with thiram standard (track 2 of the TLC image shown in Figure 4), track 5 loaded with uninoculated broth extract control (track 5 of the TLC image shown in Figure 4), tracks 8 and 11 loaded with 4-day and 8-day treated sample extract of *Pseudomonas aeruginosa* TD-9 strain (tracks 8 and 11 of the TLC image shown in Figure 4)



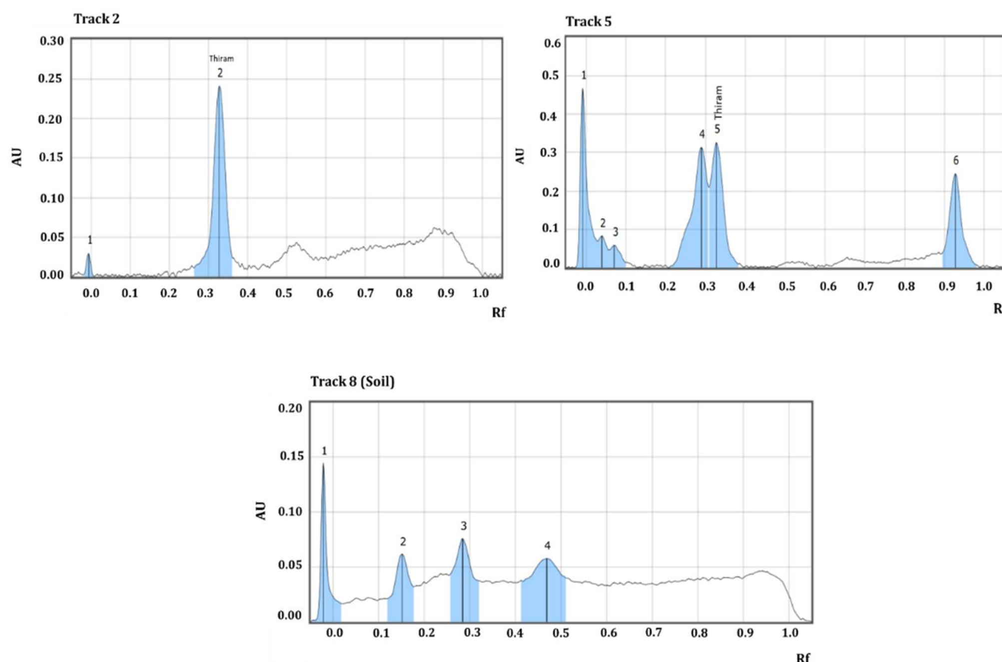


Figure 6 Image of TLC plate under 256 nm UV light where tracks 1 to 3 were loaded with 450 ng, 700 ng and 1000 ng of thiram, respectively; tracks 4 to 6 were loaded with uninoculated soil extracts as a control (2.0 µl, 3.5 µl and 5 µl respectively); tracks 7 to 9 were loaded with 10-day treated sample extract from *Pseudomonas aeruginosa* TD-9 strain (2.0 µl, 3.5 µl and 5 µl respectively)

CONCLUSION

Due to the high demand for pesticides in agriculture, this study aims to reduce pesticides contamination with the help of pesticides degrading microbes. A bacterial strain was isolated and measured their ability to degrade in culture medium and soil experiment. When the thiram containing culture medium was incubated with the bacterial culture for 4 days, the degradation was less when compared to bacterial treatment for 8 days. The result obtained in this study will help us understand the persistence of pesticide toxicity, efficacy, and bacterial degradation capacity with the help of developed HPTLC analytical method. The HPTLC method is simple, easy to use, precise, accurate and cost-effective. It can be used to evaluate microbial degradation as well as the quantitative determination of pesticide concentrations. Throughout the work, this paper successfully developed a HTPLC method to detect, quantify and measure thiram or thiram pesticides residues.

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