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**Dinesh Rajaram Hegde**
PhD Scholar, Agricultural Entomology, Department of Agricultural Entomology, Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India.

**T Manoharan**
Professor, Department of Agricultural Entomology Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India.

**R Sridar**
Professor, Department of Microbiology, Tamil Nadu Agricultural University, Coimbatore Tamil Nadu

**Correspondence**
**Dinesh Rajaram Hegde**
PhD Scholar, Agricultural Entomology, Department of Agricultural Entomology, Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India

# Identification and characterization of soil bacterial isolates and their role in the degradation of flubendiamide insecticide in the artificial medium

**Dinesh Rajaram Hegde, T Manoharan and R Sridar**

**Abstract**
Soil bacterial colonies capable of degrading flubendiamide (FBD) were isolated from three different ecosystems such as cotton, cabbage and paddy fields, Coimbatore, Tamil Nadu followed by enrichment technique. Totally, six colonies were isolated and were morphologically and biochemically tested. Among six bacterial isolates, three isolates named as FBD-1, FBD-2 and FBD-6 were found to be recorded maximum growth in the medium supplemented with flubendiamide as sole carbon source and hence taken for further dissipation studies. Highest dissipation of flubendiamide was recorded in the mineral salt medium inoculated with FBD-2 (92.14%) followed by FBD- 1 (88.13%) compared to 65.57 percent in the control after 15 days of inoculation. The bacterial isolates *viz.*, FBD-1, FBD-2 and FBD-6 were later identified as *Micrococcus sp.*, *Corynebacterium sp.* and *Pseudoxanthomonas sp.*, respectively through molecular characterization by 16S rRNA gene sequencing. These results highlight the potential of bacterial cultures to be used in the cleanup of pesticide contaminated sites in the environment.

**Keywords:** Enrichment, Dissipation, 16S rRNA, *Micrococcus* sp., *Corynebacterium* sp. *and Pseudoxanthomonas* sp

## 1. Introduction
In modern days, an intensive crop protection measure has led use of indiscriminate use of pesticides in order to harness maximum yield. Although the use of such synthetic chemicals has led to increased production of food and fiber, their use also has been associated with several concerns including risks to human health and alteration of local environment [1]. Among several abiotic (temperature, moisture, soil pH, etc.) and biotic factors (microbial community or plant species) determining the metabolic fate of pesticides in the soil, enzymatic transformation which is mainly the result of biotic processes mediated by plants and microorganisms is by far the major route of detoxification [2]. Microorganisms are considered as prominent driving force behind many soil processes such as transformation of organic matter, nutrient release and degradation of xenobiotics. Unrivaled enzymatic and nutritional versatility made them as potent recyclers of xenobiotic compounds operating in soil ecosystem apart from their small size, ubiquitous distribution, high specificity and rapid growth [3]. They have been effectively utilized in cleanup (via detoxification, degradation and removal processes) of contaminated sites in the environment. Flubendiamide is a novel insecticide belonging to phthalic acid diamide group. It is a potent insecticide with ryanodine receptor modulator type of biochemical action and been proved effective against vast range of lepidopterous pests including resistant strains in rice, cotton, corn, grapes, other fruits and vegetables [4, 5]. The reports indicated that the insecticide is hydro stable in nature and it degrades in field condition very slowly [6]. Australian Pesticides and Veterinary Medicines Authority (2009) reported that photolytic breakdown of flubendiamide on soil surface gave calculated half-life of 11.4 days. There is least information available on the role of natural communities of bacteria responsible for degradation of flubendiamide in the soil. Hence, in concern with the stability of the compound in the environment, a laboratory study was attempted to explore diverse group of soil bacteria which are capable of degrading flubendiamide in the artificial medium with an idea that they may be further exploited for bioremediation studies in the future.

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## 2. Materials and methods

### 2.1. Collection of soil samples and enrichment technique

The study was conducted in Tamil Nadu Agricultural University, Coimbatore during the month of January, 2014. Initially, soil samples of 2.5 kg were collected randomly from crop fields such as cabbage, cotton and paddy in Coimbatore, Tamil Nadu with prehistory of application of flubendiamide. Physico-chemical properties were tested and later the samples were subjected to enrichment with flubendiamide @ 50 µg/g five times at an interval of seven days [7]. In case of broth enrichment technique [8], 10 g of sieved samples were suspended in a flask containing 50 ml of modified nutrient broth supplemented with flubendiamide (50 mg/l) as sole source of carbon.

### 2.2. Isolation and purification of the isolates from the enrichment cultures

Serial dilution and plating technique was used to isolate bacterial colonies from both soil and broth enrichment cultures which were plated on mineral salt medium (MSM) supplemented with flubendiamide (50 µg/ml).

### 2.3. Morphological and biochemical characterization of the isolates and the estimation of the growth of the isolates in the medium

The purified colonies were morphologically identified for their shape, size, gram staining ability etc., and biochemical tests *viz.*, utilization of carbon sources, extracellular enzymatic activities, IMViC tests etc., were conducted [9, 10]. Growth of the isolates in the MSM was estimated by measuring the increase in turbidity of the medium using UV-VIS spectrometer at 600 nm.

### 2.4. Testing the degradation potential of the isolates in the MSM and estimation of residue using HPLC

The isolates were inoculated into flasks containing 50 ml of the MSM broth supplemented with flubendiamide besides maintaining a control (without bacterial inoculums). Flubendiamide residues were extracted from the media using the liquid-liquid extraction method by making slight modification [11]. A quantity of 50 ml of dichloromethane and 10 ml of 10 per cent sodium chloride solution was added to the sampled culture medium in a separating funnel. The mixture was vigorously shaken for two minutes and pressure was released intermittently. The solvent mixtures were left to stand until phase separation took place. The dichloromethane layer (bottom layer) was collected in a rotary flask by passing through anhydrous sodium sulphate and then the aqueous sample was reextracted twice with dichloromethane (25 ml x 2). The organic layer (dichloromethane) was combined and concentrated to near dryness in a rotary vacuum evaporator (Roteva # 8763RD, M/s Media Instrument, Mfg., Co., Mumbai) at 40°C. The residue was redissolved in 5 ml of HPLC grade acetonitrile and diluted to 50 times (200 µl of the sample to 10 ml with acetonitrile) before injecting into HPLC (High Performance Liquid Chromatography, Shimadzu 20 AT). The quantification of residue was carried out by HPLC using Photo Diode Array (PDA) detector. The recovery studies were conducted in triplicate at three different concentration level (0.1, 0.5 and 1.0 ppm) to evaluate extraction efficiency of the analytical method.

### 2.5. Molecular Characterization of screened isolates

Genomic DNA was extracted, PCR was done with 16S rRNA gene specific primers FL1 (5= - CCGAATTCGTCGACAACAGAGTTTGATCCTGGCTCA G- 3=) and RL1 (5= - CCCGGGATCCAAGCTTACGGCTACCTTGTTAC GACTT- 3=) [12, 13]. and later PCR products were sequenced (Rajeev Gandhi Centre for Biotechnology, Trivendrum). BLAST software (nucleotide-nucleotide BLAST [http: //www.blast.ncbi.nlm.nih.gov/Blast.cgi]) was used to identify related sequences in GenBank.

### 2.6. Statistical analysis

Statistical parameters like confidence intervals for intercept (a), slope of regression line (b) and coefficient of determination ($r^2$) were determined [14]. The different functions were worked out for the residue data to fit the dissipation curves.

## 3. Results

Altogether, six bacterial colonies were isolated and purified *viz.*, FBD-1 to FBD-6. Among six isolates, three isolates were selected further, studied for their flubendiamide degrading ability based on their growth response in the MSM containing flubendiamide (50µg/ml) as sole source of carbon. FBD-2 recorded maximum growth with an optical density of 1.323 at 600 nm followed by followed by FBD-1 (1.212) and FBD-6 (1.189).

Initial studies pertaining to morphological characteristics and biochemical test analysis results are represented in Table 1. The enzymatic activity of the isolates FBD-1, FBD-2 and FBD-6 had shown positive results for casein hydrolysis and

that of FBD-1 and FBD-2 had shown positivity for starch hydrolysis.

### 3.1. Degradation of flubendiamide in the MSM medium

The mean recovery of flubendiamide in the artificial medium was 81.12, 86.34 and 98.13 per cent for the three different fortification levels of 0.1, 0.5 and 1.0 ppm, respectively. The method was found to be satisfied with more than 80 percent recovery (Fig, 1). The dissipation of flubendiamide inoculated with the isolates was found to be farthest from the control. There was a greater reduction in half-life of the compound in the MSM medium from 10.38 days in control to 4.21 days with FBD-2 isolate. The half-life of flubendiamide in other two isolate was 4.71 days and 6.03 days with FBD-1 and FBD-6, respectively (Table 2). The half-life was calculated in 1st order kinetics which was observed to be best fit model based on regression coefficient ($R^2$) of determination.

### 3.2. 16S rRNA analysis for identification

NCBI sequence data bank by BLAST analysis revealed that the isolates FBD-1, FBD-2 and FBD-6 were *Micrococcus sp.*, *Corynebacterium sp. and Pseudoxanthomonas sp*., respectively. Gel electrophoresis of 16S rRNA gene of these isolates is shown in (Figure 2).

**Table 1:** Morphological and biochemical characteristics of flubendiamide degrading bacterial isolates

| Characteristics | Bacterial isolates | | |
|---|---|---|---|
| | FBD-1 | FBD-2 | FBD-6 |
| **A. Morphology** | | | |
| 1. Grams Stain reaction and shape of the cell | +ve, small cocci | -ve and tiny cocci | -ve and short rods |
| 2. Size of the cell (µm) | 1.0-1.5 | 0.7-1.0 | 1.0-1.2 |
| **B. Biochemical tests** | | | |
| 1. Glucose utilization | - | + | - |
| 2. Sucrose utilization | + | - | + |
| 3. $H_2S$ production | + | + | + |
| 4. Indole production | + | + | - |
| 5. MR Reaction | - | - | - |
| 6. VP reaction | + | + | + |
| 7. Citrate utilization | - | + | - |
| 8. Catalase activity | + | + | - |
| 9. Gelatin Liquefaction | + | - | - |
| 10. Starch Hydrolysis | + | + | - |
| 11. Lipid hydrolysis | - | - | - |
| 12. Casein hydrolysis | + | + | + |
| 13. Growth on Mackonkey Agar | + | - | + |

(+) Positive, (-) Negative, MR – Methyl Red, VP – Voges Proskauer,

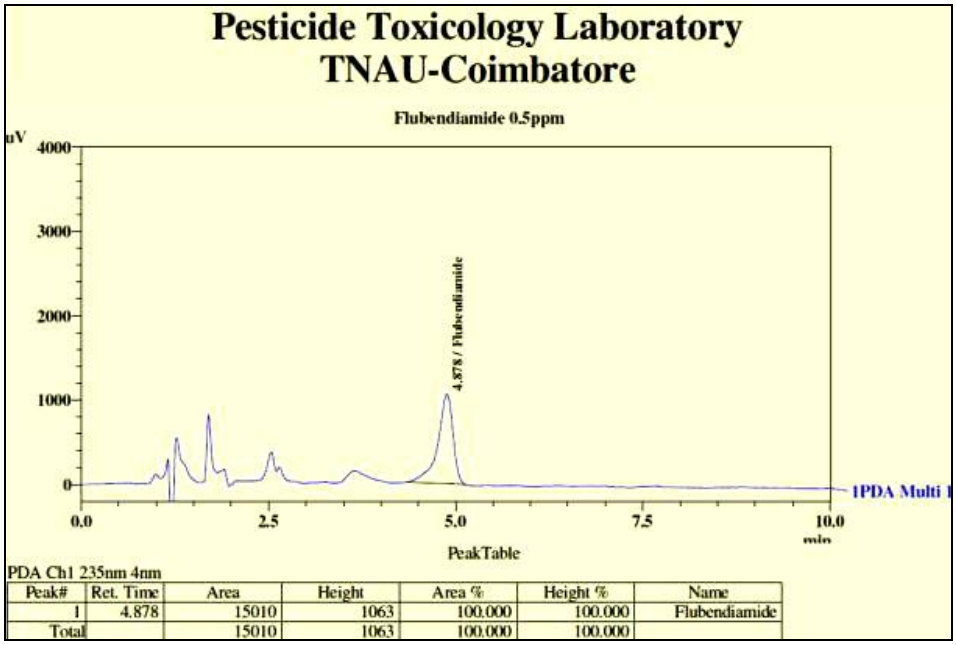
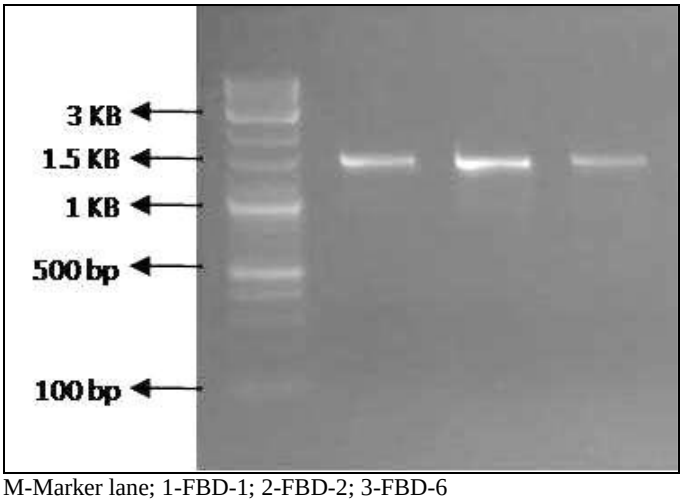



**Fig 1:** Standard chromatogram of flubendiamide at 0.5 ppm concentration

**Table 2:** Persistence and dissipation behaviour of flubendiamide in the MSM medium

| Days after inoculation | Control | | Bacterial isolates | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | FBD-1 | | FBD-2 | | FBD-6 | |
| | Residues (µg/ml) | Dissipation (%) | Residues (µg/ml) | Dissipation (%) | Residues (µg/ml) | Dissipation (%) | Residues (µg/ml) | Dissipation (%) |
| 0 (2 h) | 47.23 | - | 44.17 | - | 46.62 | - | 48.7 | - |
| 1 | 43.57 | 7.74 | 41.41 | 6.2 | 36.33 | 22.07 | 42.33 | 13.08 |
| 4 | 38.21 | 19.09 | 35.11 | 20.51 | 26.83 | 42.44 | 32.61 | 33.03 |
| 7 | 33.9 | 28.22 | 20.10 | 54.95 | 18.11 | 61.54 | 23.14 | 52.48 |
| 10 | 27.57 | 41.62 | 11.25 | 74.53 | 9.71 | 79.17 | 16.51 | 66.09 |
| 15 | 16.26 | 65.57 | 5.24 | 88.13 | 3.66 | 92.14 | 8.42 | 82.71 |
| $R^2$ | 0.9568 | | 0.9763 | | 0.9856 | | 0.9959 | |
| $T_{1/2}$ (days) | 10.38 | | 4.71 | | 4.21 | | 6.03 | |




M-Marker lane; 1-FBD-1; 2-FBD-2; 3-FBD-6

**Fig 2:** Gel electrophoresis of amplified 16S rRNA gene of flubendiamide degrading bacterial isolates through PCR

## 4. Discussion

Microbes have capacity to withstand higher concentration of pesticides in the soil and rapidly utilizing them as energy source [15]. Enrichment culturing employed in the current experiment enabled to select those bacterial genera which have got higher adaptation capability against flubendiamide residues in soil. Enzymatic hydrolysis is the primary source of microbial degradation of pesticides. In the present study, the purified bacterial isolates developed clear zone on solid synthetic medium provided with flubendiamide as the sole source of carbon. These clear zones can be explained by the liberation of extracellular enzymes produced by the microbial cells [16]. This phenomenon indicates the degradative ability of the isolated strains in the present study. As far as dissipation of flubendiamide is concerned, the present study notably showed that percentage of dissipation of flubendiamide in the MSM medium varied from 82.71 to 92.14 per cent after fifteen days of incubation whereas in control it is around 65.57 percent. Other studies pertaining to persistence of flubendiamide in the soil have shown greater residual nature of this insecticide. Flubendiamide degrades to des-iodo flubendiamide under field soil photolysis with a half-life estimated as 11.56 days. Shaon Kumar and Mukharjee, 2011 [17] reported that persistence of flubendiamide is more in dry soil with half-life of 206-215 days compared to field capacity (177-181 days) and submerged condition (150-158 days). Hence, the study proves that bacteria cultures are quite effective in reducing the insecticide (flubendiamide) load in the environment. This study in in parallel with the report that soil enrichment culture rapidly degraded 96 percent of 200 mg/L thiamethoxam in mineral salt medium broth within 30 days[18]. Identified isolates have also shown to degrade other contaminants in the environment as indicated by previous studies. For example, *Micrococcus* sp. named in the study as FBD-1 was able to utilize nitrobenzene as a sole source of carbon, nitrogen and energy under aerobic condition [19]. Similarly, *Corynebacterium* sp. (FBD-2) and *Pseudoxanthomonas* sp. (FBD-6) were found to degrade dichlorobenzoic acids and DDT respectively [20, 21].

## 5. Conclusion

Thus, we can conclude that the bacterial isolates such as *Micrococcus* sp. *Corynebacterium* sp. and *Pseudoxanthomonas* sp. are involved in the degradation of flubendiamide both in the medium as well as in soil. Hence, the study signifies their usage and focuses on further research concentrating upon the enzyme involved in the degradation is of great importance to formulate an effective consortium for remediation of affected soils.

## 6. Acknowledgements

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