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Biodegradation of the Synthetic Pyrethroid, Fenvalerate by *Pseudomonas viridiflava*

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Abstract A current environmental concern is the contamination of aquatic ecosystems due to pesticide discharges from manufacturing plant, agricultural runoff, leaching, accidental spills and other sources. The degradation of synthetic pyrethroids in the environment is an important index in the evaluation of ecological risk of pesticides. Microorganisms play a significant role in detoxifying pesticides in the environment. There are few reports on the degradation of pyrethroid insecticides in soils. This study may provide basis for prevention and control of synthetic pyrethroid, fenvalerate pollution. The present study investigated the potential of the chosen bacterium, a natural isolate *Pseudomonas viridiflava* isolated from an agricultural field and its degradation efficiency was evaluated by the assessment of various parameters like pH, carbon dioxide, turbidity and esterase activity during the long term and short term degradation process. HPLC analysis revealed that the peaks with different retention time and disappearance of several peaks confirm the degradation of fenvalerate by *P. viridiflava.* These findings suggest that the utilization of fenvalerate by *P .viridiflava* may be a feasible treatment option for the removal of pesticides from soil environment.

Keywords: *pesticide, pyrethroid, fenvalerate, P.viridiflava, HPLC*

1. Introduction

A vast number of pollutants and waste materials including heavy metals are disposed into the environment per annum. Approximately 6 x 10^6 chemical compounds have been synthesized, with 1,000 new chemicals being synthesized annually. Almost 60,000 to 95,000 chemicals are in commercial use. According to Third World Network reports, more than one billion pounds (450 million kg) of toxins are released globally in air and water. The contaminants causing ecological problems leading to imbalance in nature is of global concern [1]. Among these chemicals, Pesticides are widely used in most areas of crop production to minimize infestations by pests and thus protect crops from potential yield losses and reduction of product quality [2]. However, the application of pesticides may cause adverse effects among the different forms of life and among the ecosystems; this will depend on the sensibility grade of the organisms and the pesticide [3].

Many soil-applied pesticides are also intentionally introduced into the soil environment for the control of soil borne pests and pathogens, which results in the accumulation of their residues and metabolites in soil at unacceptably high levels [4]. Pesticides are washed into aquatic ecosystems by water runoff and soil erosion. Pesticides also can drift during application and contaminate aquatic systems. Wild birds and mammals are damaged and destroyed by pesticides and these animals make excellent "indicator species". Pesticides easily find their way into soils, where they may be toxic to arthropods, earthworms, fungi, bacteria, and protozoa [5]. Besides, pesticide application generates social conflicts because of the elevated number of workers who are intoxicated by these products, with a high mortality rate, as well as for the suspicion of adverse effects on the health of surrounding communities, flora and fauna [6].

The damages caused to the environment and health, such as the existence of obsolete pesticides, make necessary the development of technologies that guarantee their elimination in a safe, efficient and economic way. Among the existent technologies there are those that apply physical treatments, such as adsorption and percolator filters; chemical treatments such as the advanced oxidation or inverse osmosis, and incineration, a treatment option usually not available in developing countries [7]. However, a treatment that promises to be efficient, economic and safe is the biological treatment, because several reactions catalyzed by enzymes of specific microorganisms take place. Biological processes have been used to give treatment to wastes and polluted sites with pesticides [8]. Biodegradation of these pesticides provides a cheap and efficient solution for their final disposal or for treatment of agricultural soils, contaminated water or polluted ecosystems.

With the decline of organochlorine pesticides in the late 1960s, organophosphorous, then carbamates and finally synthetic pyrethroids arrived. These so called soft pesticides though short lived than organochlorines and do not accumulate, have resulted in increased incidences of fish and wildlife kills [9]. Synthetic pyrethroids (SPs) are

synthetic analogs and derivatives of the original pyrethrins, the six active insecticidal compounds of pyrethrum, which is a natural extract of the flowers of *Chrysanthemum cinerariaefolium* and *Chrysanthemum cineum*, which possess insecticidal properties. SPs include a diverse group of over 1,000 powerful insecticides and their development involved extensive chemical modifications that make these compounds more toxic and less degradable in the environment. Pyrethroids are broad-spectrum insecticides, effective against a wide range of flying, crawling, chewing, and sucking insects of the orders Coleoptera, Diptera, Homoptera, Heteroptera, Hymenoptera, Lepidoptera, Orthoptera and Thysanoptera. To increase the insecticidal activity of pyrethroids, they are often formulated with compounds which may be potentially toxic and prevent some enzymes from breaking down the pyrethroids, thus increasing their toxicity.

Fenvalerate is a type II synthetic pyrethroid lacking a cyclopropane ring. Approximately 1000 tons of fenvalerate are used annually worldwide. It is used primarily in agriculture but also in homes and gardens for insect control, and on cattle, alone or in combination with other insecticides [10]. Metabolism of fenvalerate proceeds by way of oxidation and hydrolysis to produce metabolites considered pharmacologically inactive or inferior to the parent compound. Insects and fish are extremely susceptible to fenvalerate when compared to mammals and birds. It is considered supertoxic to aquatic species like algae, invertebrates, chordates and vertebrates like fish and it has high bioaccumulation potential and persists in sediments [11]. It reduces populations of beneficial non-target organisms including spiders, ground beetles and crickets [12].

Fenvalerate is one of the most persistent synthetic pyrethroids in soils [13]. Fenvalerate degradation rate in soil depends on soil type, moisture, temperature and microbial activity. Microbial degradation is most rapid under aerobic conditions and transformed products do not persist longer than the parent compound [14]. Microbes play significant roles in degrading and detoxifying pyrethroid residues in the environment, and many pyrethroid-degrading bacteria have been isolated and characterized [15].

Therefore, in the present study an attempt has been made to isolate a bacterial strain capable of degrading fenvalerate effectively.

2. Materials and Methods

2.1. Collection of Soil Sample

Soil sample was collected in sterile screw cap bottles from an agricultural field at Chekkanoorani near Madurai where fenvalerate was already applied.

2.2. Isolation of Fenvalerate-degrading Bacteria

The collected soil sample was serially diluted and 0.1ml from the 10^{-6} dilution was taken and spread plated on nutrient agar plates and incubated at 37℃ for 24 hours. The isolated bacteria were plated on minimal agar containing various concentrations of fenvalerate (100, 200,300, 400, 500, 750, 1000, 1500 and 2000ppm).

2.3. Identification of Fenvalerate-degrading Bacterial Strain

Gram staining was done and the isolates were grown in selective medium like Pseudomonas Agar. Biochemical tests like catalase, oxidase, MR-VP, Indole production, Citrate utilization and TSI agar tests were carried out for strain identification [16].

2.4. Degradation Efficiency

The isolate was inoculated onto minimal broth containing different concentrations of fenvalerate like 250, 500, 750 and 1000ppm and incubated at room temperature for a period of 16 days and the degradation was confirmed by analyzing various parameters such as pH, biomass, carbon dioxide production, esterase activity and the degradation products. A short term study was done by measuring the above parameters every 24 hours for 4 days.

2.5. PH

The pH of the sample was measured on the 4^{th} ,8^{th}, 12^{th} and 16^{th} day of incubation and another set of samples were analyzed on the 1^{st}, 2^{nd}, 3^{rd} and 4^{th} day of the treatment.

2.6. Carbon Dioxide Estimation

The release of carbon dioxide during the degradation of various concentrations of fenvalerate was estimated by the method proposed by Eaton *et al*. (1995) [17].

2.7. Esterase Activity

100 µl of cells or cell extract was incubated with 1.5ml of 0.42mM 1- naphthyl acetate, 0.5ml Na_2HPO_4 buffer (0.2M, pH 7.0) and 0.4ml glass distilled water at 39 ℃ for 10 minutes. 500 µl of 10% lauryl sulfate containing 2.5mg of Fast Garnet GBC was added to the mixture and incubated at room temperature for 15 minutes for color development. Absorbance was measured at 560nm and compared with the absorbance of 1-naphthol curve (linear from 0- 0.08 µM) [18].

2.8. Biomass Estimation

Turbidometric method was followed for estimating the biomass by measuring the turbidity at 600nm.

2.9. High Pressure Liquid Chromatography (HPLC)

The samples containing the minimal medium, isolate and 500ppm concentration of fenvalerate taken on the 0^{th}, 4^{th} and 8^{th} day were subjected to HPLC analysis.

3. Statistical Analysis

Two way ANOVA was performed for the parameters pH, carbon dioxide released, esterase activity and biomass using MS Excel. Variability was considered significant only when the statistic value was greater than the tabulated value at P is less than or equal to 0.05.

4. Results and Discussion

The details of the synthetic pyrethroid, fenvalerate is given in Table 1. The bacterial strain isolated from the soil was identified as Gram negative rod. Then the isolate was tentatively identified as *Pseudomonas viridiflava* on the basis of the results obtained in the biochemical tests (Table 2).

The isolate *P.viridiflava* was able to grow on minimal agar containing various concentrations of fenvalerate (Plate 1).

Table 1. Details of Fenvalerate

CAS number	51630-58-1
Chemical formula	$C_{25}H_{22}ClNO_3$
Physical state	viscous liquid
Color	yellow or brown
Odour	mild "chemical" odour
Relative molecular mass	419.9
Boiling point	300 ℃ at 4.93 kPa (37mmHg)
Water solubility	2 µg/Liter
Solubility in organic solvents	Soluble
Relative density (25 ℃)	1.175
Vapour pressure (25 ℃)	0.037 mPa

Table 2. Tests for the Identification of the Isolate, *P. viridiflava*

Test	Response of the organism
Gram staining	Gram –ve
Methyl Red	-
Vogus Proskauer	-
Simmons citrate Agar	+
Oxidase	-
Catalase	+
Indole	-
Triple Sugar Iron Agar	no change

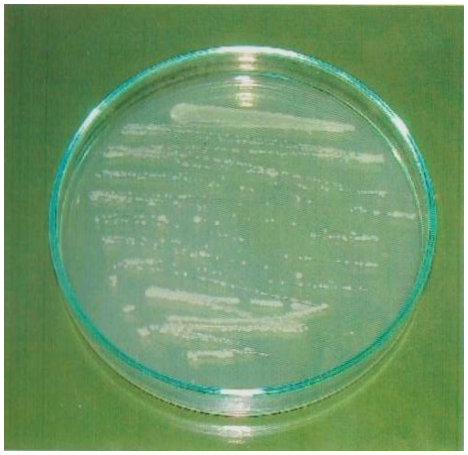

Plate 1. Growth of *P.viridiflava* in minimal medium containing 500 ppm fenvalerate

In Figure 1, the pH of the medium during the long term treatment of fenvalerate by *P.viridiflava* is observed to decline till the 8th day and increased after that. Figure 3 illustrates the changes in the pH recorded on the 1st, 2nd, 3rd and 4th days of treatment by *P.viridiflava*. During the short term treatment, the pH declined drastically indicating the degradation of fenvalerate. 3-phenoxy benzoic acid, an intermediate of synthetic pyrethroid degradation may be the reason for the decline in the pH of the medium during the degradation of fenvalerate. *Pseudomonas pseudoalcaligenes* POB310 and two modified strains of *Pseudomonas* were found to degrade 3- phenoxy benzoic acid efficiently [19]. Maloney *et al.* (1992) [20] reported the isolation of *Bacillus stearothermophilus* capable of degrading second and third generation of pyrethroids to non-insecticidal products. 3-Phenoxybenzoic acid and the respective halovinyl or haloacid moieties were detected as the major hydrolytic products of the pyrethroids.

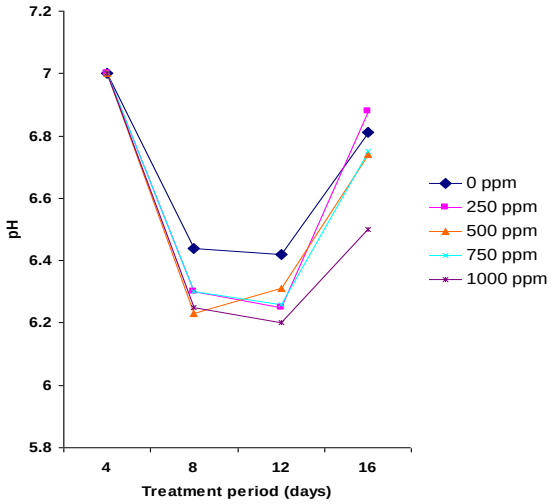

Figure 1. Changes in pH of the Medium during the degradation of Fenvalerate by *P. viridiflava* in the Long Term Treatment

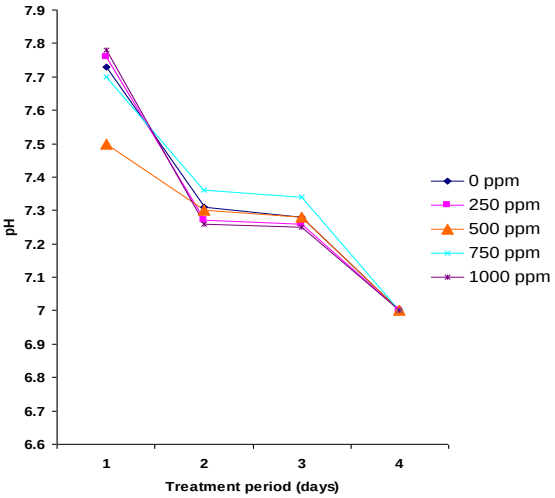

Figure 2. Changes in pH of the Medium during the Degradation of Fenvalerate by *P. viridiflava* in Short Term Treatment

The biodegradation of fenvalerate resulted in the production of carbon dioxide which was found to increase for the first 8 days and diminished after the 8th day. During the degradation of 500ppm of fenvalerate, more amount of carbon dioxide was released and in the case of 250ppm concentration, there was no release of carbon dioxide on the 16th day of treatment (Figure 3). The amount of carbon dioxide released during the first four days of degradation of fenvalerate by *P.viridiflava* is shown in Fig. 4. In both the cases, the amount of carbon dioxide release was maximum for 500ppm concentration of fenvalerate. The release of carbon dioxide as fenvalerate degrades is in line with the fact that fenvalerate is degraded into carbon dioxide by microbes in the soil. Motayama *et al*. [21] reported *that* fenvalerate was degraded in the diamond back moth by the cytochrome p450 monooxygenase system and the degradation products like the volatile metabolites and carbon dioxide were measured. Rodriguez-Cruz *et al.*

(2006) [22] showed that pesticides are mineralized to carbon dioxide.

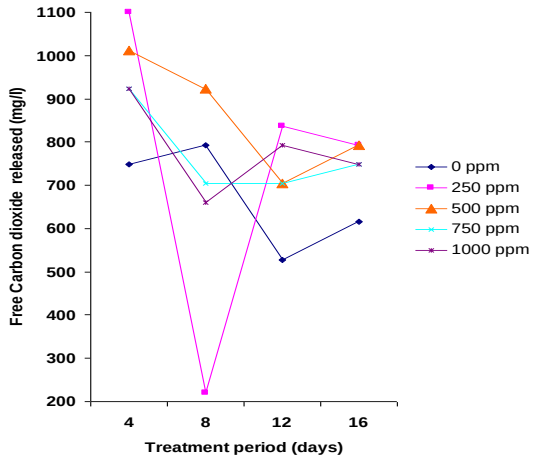

Figure 3. Carbon dioxide Released during the Degradation of Fenvalerate by *P. viridiflava* in the Long Term Treatment

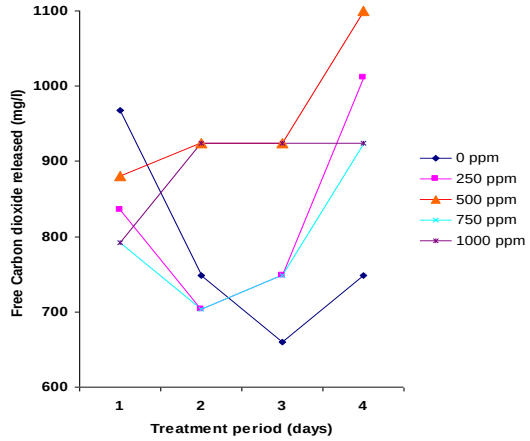

Figure 4. Carbon dioxide Released during the Degradation of Fenvalerate by *P. viridiflava* in the Short Term Treatment

Changes in the turbidity of the medium during the long term and short term treatment of fenvalerate by *P.viridiflava* are shown in the Figures 5 and 6. It seems to be fluctuating but there is a linear increase in the case of 500ppm concentration of fenvalerate indicating an increase in the growth of the organism during the long term treatment. Turbidity changes during the degradation of fenvalerate in the first four days by *P.viridiflava* were observed to be highly fluctuating.

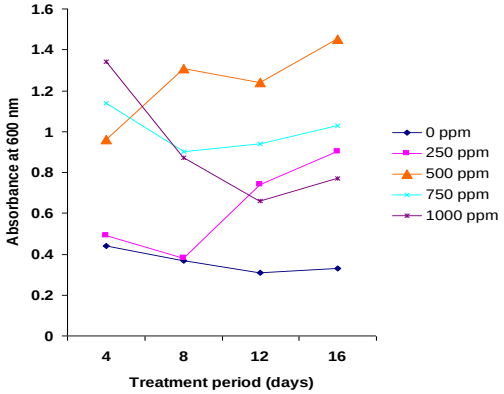

Figure 5. Changes in Turbidity during the Degradation of Fenvalerate by *P. viridiflava* in the Long Term Treatment

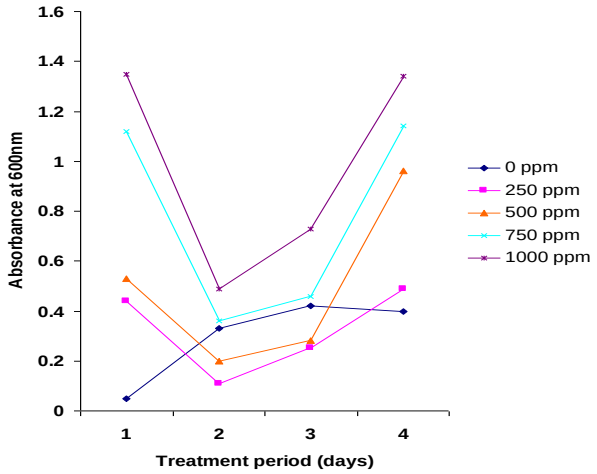

Figure 6. Changes in Turbidity during the Degradation of Fenvalerate by *P. viridiflava* in the Short Term Treatment

Esterase activity during the long term treatment of fenvalerate by *P.viridiflava* is shown in Figure 7. It was found to be gradually increased till the 8th day and decreased after the 12th day of treatment.

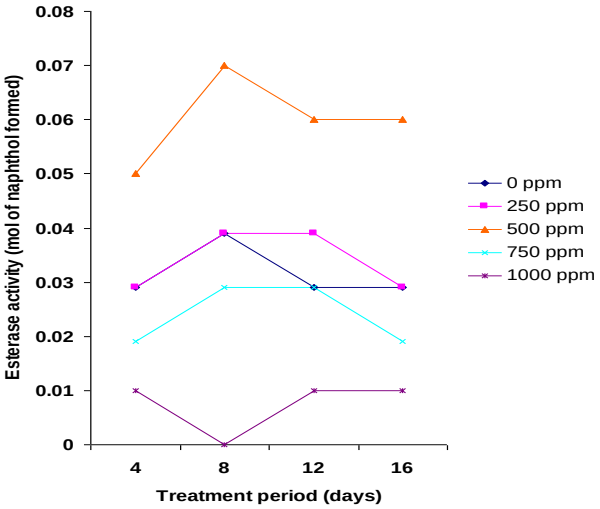

Figure 7. Esterase Activity during the Degradation of Fenvalerate by *P. viridiflava* in the Long Term Treatment

The highest activity was observed in 500ppm of fenvalerate. Esterase activity in the first four days of treatment by *P.viridflava* increased linearly (Figure 8) and the highest activity was recorded in the case of 500ppm of fenvalerate. Pesticide degradation is either by broad spectrum enzymes such as hydrolases, reductases and oxidases or by specific enzymes such as esterases commonly present in large numbers in the organisms. Increase in the esterase activity during the degradation of fenvalerate is justified by the fact that the carboxylesterases hydrolyze a large array of endogenous and exogenous esterase containing compounds, including synthetic pyrethroids [23].

Table 3 shows the HPLC analysis report for 500ppm fenvalerate which explores the retention time and heights of peak obtained during the treatment with *P.viridiflava*. Figure 9 divulges the HPLC analysis for 500ppm of fenvalerate on the 0th day of treatment. The peaks observed here were missing in the HPLC analysis of the same concentration of fenvalerate on the 4th day of degradation by *P.viridiflava* and there were few new

peaks with different retention times indicating the formation of intermediates (Figure 10). Figure 11 exhibits the degradation of 500ppm of fenvalerate on the 8th day of treatment by *P.viridiflava*. The peaks on the 8th day of treatment were different in their retention times and they were different from those on the 4th day of treatment indicating the formation of new intermediates.

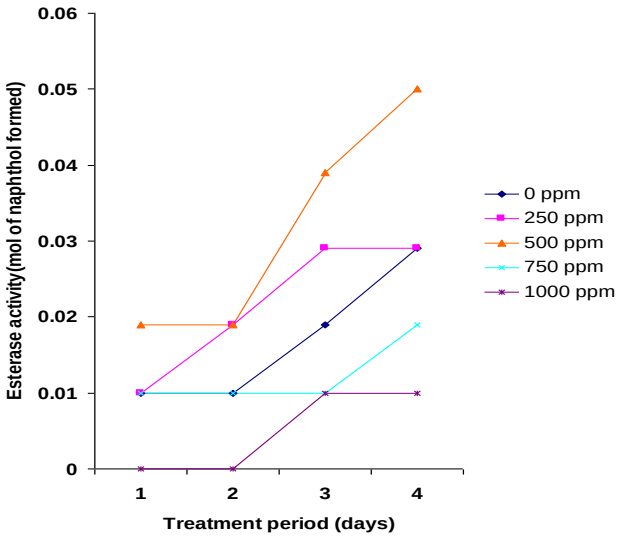

Figure 8. Esterase Activity during the Degradation of Fenvalerate by *P. viridiflava* in the Short Term Treatment

Table 4 and 5 exhibit the two way analysis of variance for the factors such as pH, carbon dioxide, turbidity and esterase activity with the variables, fenvalerate concentration and treatment period for *P. viridiflava* during the long term and short term treatment respectively. The variations due to the concentration and treatment period were statistically significant at 5% level.

From the present study, *P. viridiflava* isolated from the pyrethroid contaminated soil show the potential to be used in bioremediation application for the treatment of synthetic pyrethroid residues.

Table 3. HPLC Analysis Report for 500ppm Fenvalerate before and after Treatment with *P.viridiflava*

Sample	Retention Time [min]	Height [µV]
Before treatment	2.830	12.28
	3.003	14.10
	4.560	2.61
	5.163	9.54
	5.837	10.06
	6.540	15.15
	Total	63.74
After 4 days of treatment	2.02	0.50
	2.80	21.64
	2.97	22.19
	4.49	1.71
	4.85	1.91
	5.10	5.50
	Total	53.45
After 8 days of treatment	1.927	0.33
	2.803	8.86
	3.020	16.73
	4.553	1.35
	5.137	2.87
	6.463	0.34
	Total	30.48

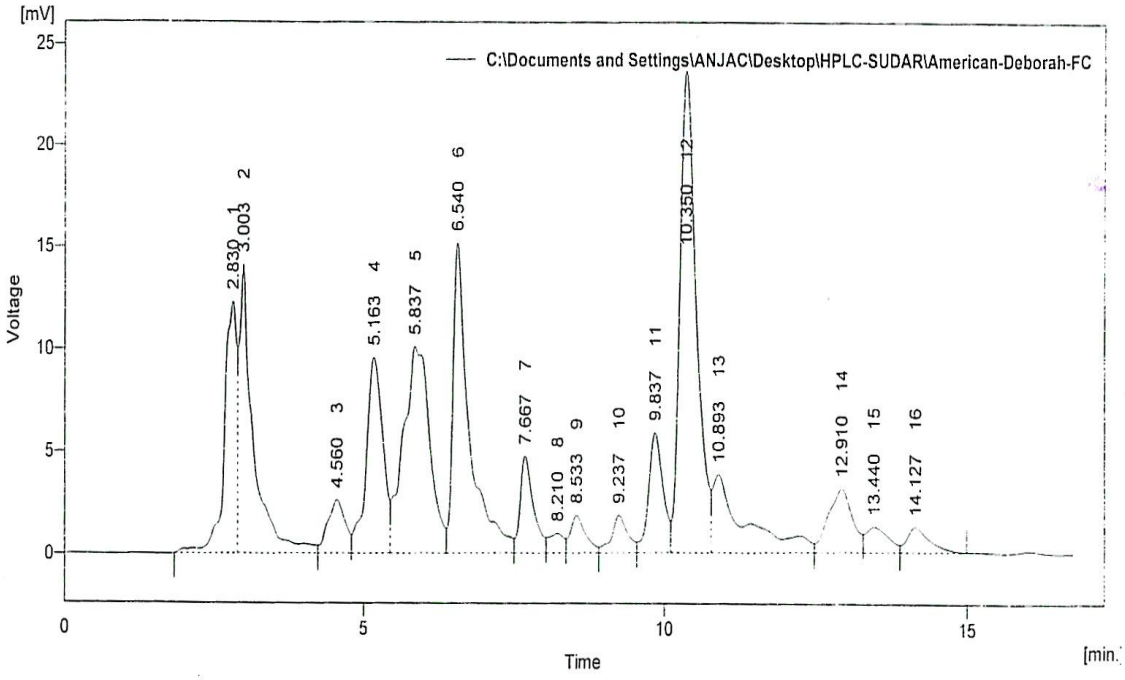

Figure 9. HPLC analysis for 500ppm of fenvalerate

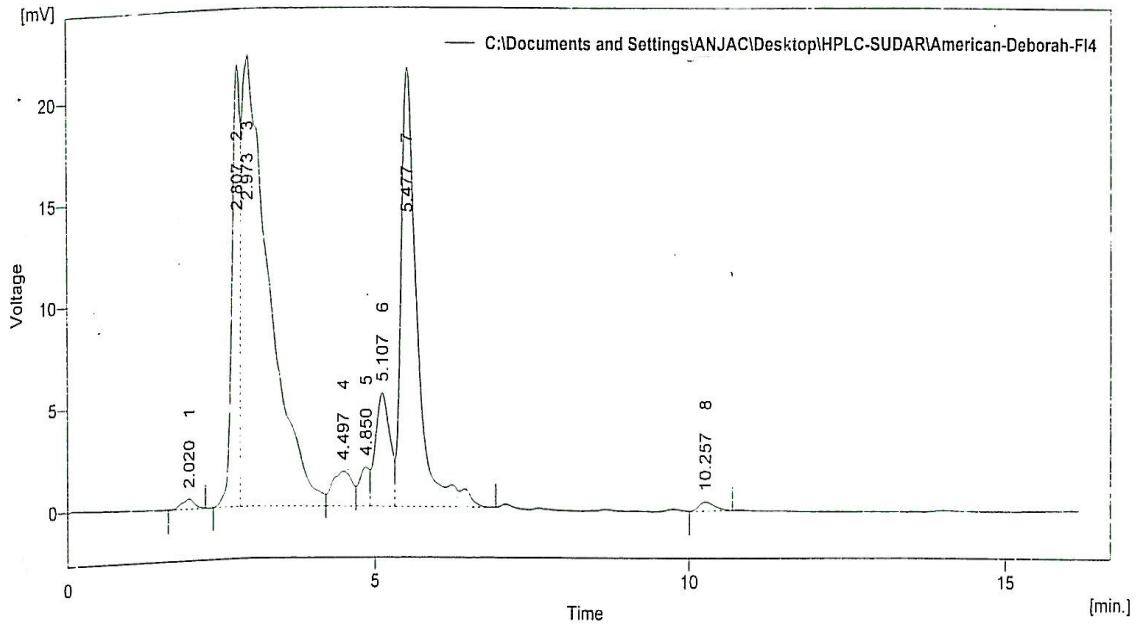

Figure 10. HPLC analysis for 500ppm of fenvalerate treated with *Pseudomonas viridiflava* (4th day)

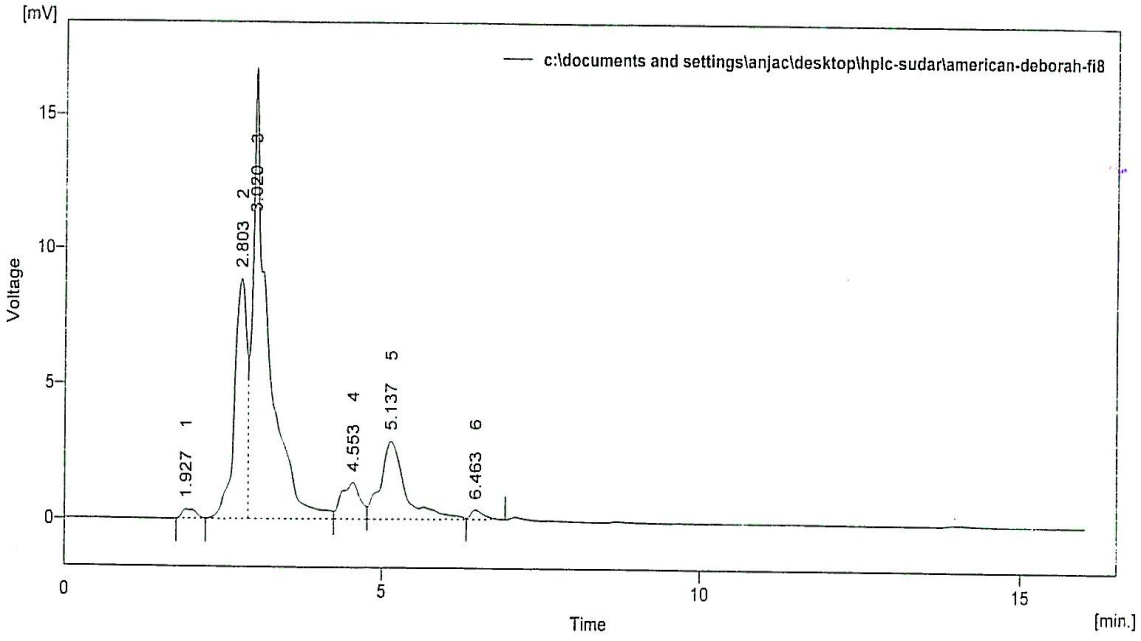

Figure 11. HPLC analysis for 500ppm of fenvalerate treated with *Pseudomonas viridiflava* (8th day)

Table 4. Two Way Analysis of Variance for the Factors with the Variables, Treatment Period and Fenvalerate Concentration for Pseudomonas viridiflava (Long Term Treatment)

Factor	Source of variation	df	MS	F value	Table value at 5% level	Level of Significance
pH	Concentration	4	0.017055	2.979039	0.063658	Significant
	Treatment period	3	0.6036	105.4323	6.88	Significant
Carbon dioxide	Concentration	4	18537.2	0.629589	0.118616	Significant
	Treatment period	3	75988	2.580822	0.349553	Significant
Turbidity	Concentration	4	0.463808	10.2762	0.000601	Significant
	Treatment period	3	0.21805	0.483115	0.700185	Insignificant
Esterase activity	Concentration	4	0.001451	49.73829	2.26	Significant
	Treatment period	3	6.67E-05	2.285714	0.13085	Significant

Table 5. Two Way Analysis of Variance for the Factors with the Variables, Treatment Period and Fenvalerate Concentration for *Pseudomonas viridiflava* (Short Term Treatment)

Factor	Source of variation	df	MS	F value	Table value at 5% level	Level of Significance
pH	Concentration	4	0.003507	0.881097	0.503855	Significant
	Treatment period	3	0.40686	102.2047	8.23	Significant
Carbon dioxide	Concentration	4	21876.8	2.387324	0.109101	Significant
	Treatment period	3	22070.4	2408451	0.117878	Significant
Turbidity	Concentration	4	0.346188	6.564767	0.004873	Significant
	Treatment period	3	0.330205	6.26169	0.008389	Significant
Esterase activity	Concentration	4	0.000405	14.65812	0.000144	Significant
	Treatment period	3	0.000346	12.49985	0.00053	Significant

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