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ISOLATION AND PHYSICOCHEMICAL CHARACTERIZATION OF FENVALERATE DEGRADING BACTERIA FROM FENVALERATE CONTAMINATED AGRICULTURAL SOIL

NEELAM KAUSHIK*	DEPARTMENT OF BIOTECHNOLOGY, HIMACHAL PRADESH UNIVERSITY, SUMMER HILL, SHIMLA, INDIA-171005.
SANGEETA KUMARI	DEPARTMENT OF BIOTECHNOLOGY, HIMACHAL PRADESH UNIVERSITY, SUMMER HILL, SHIMLA, INDIA-171005.
SAUMYA THOLAT	DEPARTMENT OF BIOTECHNOLOGY, HIMACHAL PRADESH UNIVERSITY, SUMMER HILL, SHIMLA, INDIA-171005.

ABSTRACT:

In this research, fenvalerate degrading bacteria were isolated from agricultural soil samples collected from Kaccholi, Gujarat, India. A total of 9 bacterial isolates were isolated and screened for fenvalerate degradation. Initial screening was done by growing the bacterial colonies on minimal salt media encompassing fenvalerate as the sole source of carbon. A biomass assay was conducted to identify the most efficient bacterial isolates capable of degrading fenvalerate. Out of 9 isolates that showed good biomass assay, one isolate (FDB-3) showing the best fenvalerate degrading potential was preferred for further study. Based on morphological and biochemical tests, isolate FDB-3 was recognized as *Pseudomonas* sp. The growth parameters were further refined under varying physicochemical conditions. The results showed that *Pseudomonas* sp. FDB-3 had maximum growth up to 72 hours with 94% of fenvalerate degradation. FTIR analysis of residual fenvalerate after 72 hours of incubation showed that FDB-3 was able to degrade fenvalerate. Therefore, the initial results obtained after optimization of culture conditions for biomass production and fenvalerate degradation indicated the possible utility of bacterial isolate FDB-3 for degradation of fenvalerate which can be utilized in the bioremediation of fenvalerate polluted soil.

KEYWORDS:

FENVALERATE, CONTAMINATION, BIOREMEDIATION, BACTERIAL ISOLATE, FTIR ANALYSIS.

INTRODUCTION

Pesticides are substances, either natural or synthetic, designed to eliminate unwanted plant or animal pests. Various pesticides are commonly utilized in agriculture to control pest infestations, safeguard crops, and prevent expected crop reductions and declines in product quality (Damalas, 2009). However, the extensive use and over-exploitation of such insecticides have several harmful effects which lead to microbial imbalance, environmental problems, and different health hazards, especially for farm workers (Triebskorn et al., 2013; Suman and Tanuja, 2021; Guerrero Ramírez et al., 2023). There are numerous physical and chemical strategies recommended to deal with pesticide contamination. While physical methods like incineration release toxic gases, chemical methods often result in the breakdown into simpler components that turn out to be equally harmful as the parent component and are hence ineffective (Andreotti et al, 2009). Further, the physicochemical treatment methods of pesticide-contami nated sites are expensive and also contaminate the enviro -nment. In this regard, bioremediation is observed as a more dependable, cost-effective, and efficient alternative to conventional decontamination methods for environmen tal clean-up and pollution control (Zhao et al., 2013; Cyco´n et al., 2013; Akbar et al., 2015). In this process, the microorganisms employ these complexes as their carbon, phosphorus, nitrogen, and energy source. *Bacillus*, *Sphingomonas*, *Pseudomonas*, *Arthrobacter*, *Trichoderma*, *Sphingobium, Aspergillus*, and *Candida* can be used as effective biocatalysts for pesticide bioremediation (Gangola et al., 2022).

Fenvalerate, a synthetic pyrethroid pesticide, is commonly utilized in agriculture and forestry worldwide to manage livestock and pest diseases. It is known for its short half-life and comparatively low harmfulness. Its chemical name is α-cyano-3-phenoxyphenyl 2-(4-chlorophenyl)-3-methylbutyrate (Giri et al., 2002). However, fenvalerate can negatively impact non-target species, including beetles (Desneux et al., 2007), bees (Decourtye et al., 2005), zebrafish (Yao et al., 2022), fish and aquatic insects (Antwi and Reddy, 2015; Mahmoud et al., 2020) and microbes (Das et al., 2016). Additionally, extensive exposure to fenvalerate has been linked to chronic disorders (Kolaczinski and Curtis, 2004) and accumulation of residues in soil can cause serious ecological contamination and environmental damage (Ye et al., 2016; Nesser et al., 2016). Therefore, it is essential to grow a quick and effective degradation progression to eradicate or decrease fenvalerate deposits in the environment. Different microorganisms have been described to eliminate artificial pyrethroid from contaminated soil (Cycon and

Piotrowska-Seget, 2016; Gangola et al. 2022). However limited reports are available regarding biodegradation of fenvalerate. It has been reported in the past that different bacteriological species have the potential to reduce fenvalerate (Gill et al., 2015; Fulekar, 2009; Tang et al., 2018). Yu et al. (2013) found that Sphingomonas sp. F-7 could fully degrade 101 mg/L of fenvalerate within 70-72 hours, with 3-phenoxybenzoic acid (3-PBA) identified as the primary metabolite. Tang et al., (2018) stated that *Bacillus licheniformis* strain CY-012 could degrade around 80.07% of fenvalerate within 59-60 h of incubation. Thus, the present study was carried out to isolate and identify bacteria from fenvalerate-contaminated agricultural soil with a high potential to degrade the fenvalerate present in the contaminated soil.

MATERIALS AND METHODS

Chemicals and reagents: All the chemicals/reagents and media components utilized in the present research were of high-purity analytic grade obtained from HiMedia and Sigma Aldrich, USA. Agricultural grade fenvalerate 20% EC was purchased from TATA Agrochemicals.

SOIL SAMPLE COLLECTION

Soil samples were collected from agricultural fields of mango, sapota (chiku), and papaya in Kachholi, Gujarat (20°50'26.5"N 72°58'59.7" E) in April 2022 at an elevation of 49.21 meters above sea level for the isolation of microbes capable of degrading fenvalerate. The location for collection of samples was chosen keeping in mind the fact that it had been exposed to the fenvalerate pesticide for a long extent of period. The soil samples were taken by scooping from the top 10 cm of the soil and stored in sterile polyethylene bags. The collected soil samples were transported to the Biotechnology Department, Himachal Pradesh University, and were kept at 4°C until further processing.

ISOLATION AND IDENTIFICATION OF FENVALERATE DEGRADING BACTERIA

Ten grams of soil sample were added to 50 ml of minimal salt media and enriched with 400 mg/l fenvalerate 20% EC powder. Samples were incubated on rotary shaker (150 rpm) at 30°C for 5 days. After a 5-day incubation period, 1 ml of the enriched culture sample was consecutively diluted from 10^{-1} to 10^{-9}. From every dilution, a 0.1 ml portion of the sample was spread onto an MSM agar plate containing fenvalerate and evenly spread using a sterilized L-shaped spreader. The plates were subsequently kept at 30°C for 24 hours under aerobic conditions for incubation. Colonies were observed on the plates and repeatedly streaked on fresh media until a single bacterial colony was obtained. Bacterial colonies able to grow on fenvalerate MSM agar were identified positive for fenvalerate utilization as fenvalerate was provided as the sole source of carbon.

QUANTITATIVE ESTIMATION OF FENVALERATE DEGRADING EFFICIENCY BY BACTERIAL ISOLATES

For direct assays of fenvalerate degradation efficiency, a simple spectrophotometric method proposed for the determination of pyrethroid pesticides such as cypermethrin and fenvalerate was used (Tamrakara et al., 2012). The method is based on the detection of cyanide which is released on hydrolysis of fenvalerate. The released cyanide undergoes a reaction with bromine and pyridine, resulting in the formation of glutaconic aldehyde. This compound then combines with sulphanilic acid in an acidic medium, producing an orange-red dye with a maximum absorbance at 480 nm.

To determine fenvalerate degradation efficiency, an aliquot containing 400 mg/ ml of fenvalerate was placed in a sequence of 250 ml standard flasks. To this, 1.0 ml of culture was added and incubated for 2-3 days at 30°C and 150 rpm for complete hydrolysis. After incubation, this was centrifuged for 5 minutes at 10000 rpm to separate cells, and the supernatant was taken in another flask. 3.0 ml of bromine water was introduced into the flask and allowed to stand for 3 minutes to ensure complete bromination. Additional bromine was removed by gradually adding 0.7 mL of 5% sulfosalicylic acid drop by drop. 0.4 ml of pyridine solution was added to it and then placed in a boiling water bath for 15 minutes. It was allowed to cool to room temperature, and then 0.5 ml of 5 M NaOH solution and 2-3 ml of 2% sulphanilic acid were introduced. The prepared solution was allowed to stand for 5 minutes to ensure full-color development. The resultant orange-red dye was analyzed at 480 nm using a blank reagent as a reference.

CHARACTERIZATION OF BACTERIAL ISOLATES

A pure isolate exhibiting the highest efficiency in fenvalerate degradation was chosen for further investigation and after characterization was designated as *Pseudomonas* sp. FDB-3. Cell morphology was determined by Gram staining and for further documentation, a sequence of biochemical tests like sugar utilization test, catalase test, urease test, Vogues Proskauer test, methyl red test, indole production test, citrate utilization test, gelatinase test, lipid hydrolysis was performed as per the second edition of the Bergey's Manual of Systematic Bacteriology (Brenner et al. 2005).

OPTIMIZATION OF GROWTH CONDITIONS OF *PSEUDOMONAS* SP. FDB-3

Pseudomonas sp. FDB-3 with the potential of fenvalerate degradation was subjected to diverse physicochemical conditions like nitrogen, pH, temperature, and carbon sources. The one-variable-at-a-time (OVAT) approach was utilized to examine the impact of cultural conditions influencing bacterial growth such as carbon source (glucose, fructose, maltose, cellulose, sucrose, starch, and lactose), nitrogen source (inorganic sources NH_4Cl, $(NH_4)_2SO_4$, NH_4NO_3, $(NH_4)_2C_2O_4$ and organic sources beef extract, casein, meat extract, peptone), incubation temperature (30-50^{o}C), initial medium pH (pH 5-9) was used. The control flask was also maintained without inoculating the bacterial culture. All cultures were incubated in a shaker (150 rpm) at 30°C for 2-3 days. The biomass concentration was determined through absorbance measurement at 600 nm using UV-visible spectrophotometry every other day, and turbidity was measured until the growth becomes constant.

OPTIMIZATION OF FENVALERATE DEGRADATION CONDITIONS FOR *PSEUDOMONAS* SP. FDB-3

Since apart from the culture conditions during the growth of the organism in the medium, the reaction conditions also affected the degradation and were subjected to varied degradation conditions of incubation time (72 hrs), temperature (20^{o}C, 25^{o}C, 30^{o}C, 35^{o}C, 40^{o}C, 45^{0}C), fenvalerate concentration (200 mg/ml to 450 mg/ml) to find out the optimum reaction conditions and results were recorded in each case after performing the spectro photometric assay as mentioned earlier (Tamrakara et al., 2012).

ANALYSES OF FENVALERATE DEGRADATION BY FTIR

FTIR is a widely used technique for analyzing a mixture of compounds to analyze the bonds present within the mixture and to understand its type and properties. It can also be used to identify certain compounds which show "fingerprint" peaks. For FTIR analysis, one control and one test medium were prepared. The test media used was fenvalerate MSM broth (400 mg/ml). Seed culture prepared using optimized conditions was inoculated in test media and allowed to incubate at 35^{o}C for 72 hours. The cells were isolated by centrifuging at 10000 rpm for 5 min. The control was made by dissolving an equivalent concentration of fenvalerate in sterile distilled water. FTIR (Fourier transform infrared spectroscopy) analysis was performed at the Directorate of Forensic Services, State FSL, Himachal Pradesh.

RESULTS AND DISCUSSION

ISOLATION AND CHARACTERIZATION OF BACTERIA CAPABLE OF DEGRADING FENVALERATE

The soil samples for isolating fenvalerate degrading bacteria were obtained from agronomic fields with a prolonged history of fenvalerate exposure. Fenvalerate degrading bacteria were isolated by enrichment method and 9 bacterial isolates were isolated based on the growth on minimal salt media containing fenvalerate as the only source of carbon. The selected pure colonies were used further for analysis of fenvalerate degradation by spectrophotometric method. Out of 9 isolates that showed good biomass assay, one isolate (FDB-3) showed 83% fenvalerate degradation potential after 48hours of incubation and was selected for morphological and physicochemical characterization.

The bacterial isolate (FDB-3) was further analyzed morphologically as well as microscopically. Based on morphological analysis, isolate FDB-3 was creamish with colonies of moderate size, opaque, slightly elevated, smooth surfaced, and margin irregularity and was found to be rod-shaped motile (unipolar), gram-negative, single arrangement bacteria (Table 1)

Further, the bacterial isolate FDB-3 was positive for catalase, citrate, methyl-red, lipid utilization, and gelatin hydrolysis and harmful for indole, urease, VP, and gas production. The isolate was also able to ferment glucose, fructose, sucrose, xylose, galactose, and maltose and was negative for lactose (Table 2). Based on the morphological and biochemical features, the genus of the isolate FDB-3 was inferred to be *Pseudomonas* sp. In the present study, 83% of fenvalerate degradation was reported after 48 hr of incubation while in a similar study, Yu et al., (2013) observed 80% degradation after 8 days of incubation using *B. Licheniformis*. The region for longer duration for fenvalerate degradation in this study may be the conc. of fenvalerate in the pesticide. In another study, Tang et al., (2018) reported, around 80.06% of fenvalerate degradation within an incubation period of 60 hours.

OPTIMIZATION OF GROWTH CONDITIONS FOR *PSEUDOMONAS* SP. FDB-3

Pseudomonas sp. FDB-3 with the possibility of fenvalerate degradation was subjected to different physicochemical conditions like nitrogen, temperature, pH, and carbon sources. The one-variable-at-a time (OVAT) approach was used to observe the outcome of diverse culture conditions on bacterial growth.

EFFECT OF TEMPERATURE AND PH

The general growth and production of metabolites by microbes is influenced by environmental conditions like pH and temperature. *Pseudomonas* sp. FDB-3 was subjected to different temperature (20^{o}C, 25^{o}C, 30^{o}C, 35^{o}C, 40^{o}C, 45^{o}C, 50^{o}C, 55^{o}C) and pH (5-9), as shown in Fig. 1 and 2. At pH 7.5, the bacterial isolate FDB-3 produced the maximum biomass in the current study, Tang et al., (2018) reported a pH of 7.4 as the most suitable pH for fenvalerate degradation. The production of biomass was reduced when the pH was below or above its optimal level. This might be caused by the inactivation of a metabolic or regulatory pathway of an enzyme. Another important factor in the production of biomass is temperature. *Pseudomonas* sp. FDB-3 produced the maximum biomass at a temperature of 35°C, and with an increase or decrease of optimum temperature, the amount of biomass produced gradually dropped, the results of the present study were quite similar to the findings of Tang et al., (2018).

EFFECT OF CARBON AND NITROGEN SOURCE

To optimize the growth conditions of the selected *Pseudomonas* sp. FDB-3, numerous carbon and nitrogen sources were tested, and the absorbance was measured at 12-hour intervals. The biomass yield was highest in a medium containing fructose (Fig 3). After fructose, the most preferred carbon source appeared to be glucose, and no biomass was produced in a medium containing lactose. Further, the maximum cell mass was obtained when media was supplemented with a 1.5% concentration of fructose. Like carbon, nitrogen is also an essential element for the growth of microbes. To determine the optimal nitrogen source for biomass production, various organic and inorganic sources were used. All selected nitrogen sources positively affected the biomass formation with maximum absorbance/ biomass production in the case of casein. Further, casein at conc. of 2% resulted in maximum biomass production. Besides this, some inorganic sources also enhanced biomass production with maximum biomass in the case of ammonium sulfate and least in the case of ammonium oxalate (Fig. 4).

OPTIMIZATION OF FENVALERATE DEGRADATION CONDITIONS BY *PSEUDOMONAS* SP. FDB-3

EFFECT OF TEMPERATURE ON FENVALERATE DEGRADATION BY *PSEUDOMONAS* SP. FDB-3

Degradation of fenvalerate was carried out at different temperatures (20-45°C). The degradation of fenvalerate was measured spectrophotometrically. *Pseudomonas* sp. FDB-3 showed a continuous increase in fenvalerate degradation from 25-35°C but thereafter, the degradation decreases with a further increase in temperature. Maximum fenvalerate degradation (87%) was recorded at 35°C followed by degradation (83%) at 30^{0}C (Fig. 5**)**. The degradation percentage was almost equal at temperatures 25 and 45°C.

OPTIMIZATION OF INCUBATION TIME ON FENVALERATE DEGRADATION BY *PSEUDOMONAS* SP. FDB-3

The effect of the incubation time on degradation of fenvalerate by *Pseudomonas* sp. FDB-3 was determined by performing the degradation assay at 35°C for a range of time periods (12, 24, 36, 48, 60, 72, 84 hr). The strain FDB-3 showed 94% degradation of fenvalerate after 72 hr of incubation after no further increase in degradation was observed. The least degradation was after 12 hr of incubation at 35°C (Fig. 6**)**. Our findings are consistent with Tang et al., (2020) who reported around 88% of fenvalerate degradation within 72 h of incubation.

OPTIMIZATION OF FENVALERATE CONCENTRATION FOR DEGRADATION BY *PSEUDOMONAS* SP. FDB-3

To study the effect of varying concentrations of fenvalerate on degradation by *Pseudomonas* sp. FDB-3, its concentration in the degradation mixture was varied from 200-450 mg/ml. The reaction mixture was kept for incubation at 35°C for 72 hr, thereafter degradation was recorded in each case. It was observed that the degradation of fenvalerate was highest (94%) when the medium contained 400 mg/ml followed by 350 mg/ml concentration (92%) of fenvalerate. The least degradation was observed with 200 mg/ml of fenvalerate thereafter degradation increased up to 400 mg/ml. After 400 mg/ml concentration, degradation activity decreases (Fig. 7). This might be because, with an increase in several substrate molecules beyond optimum value, complete saturation leads to a decrease in the degradation potential. Tang et al., (2020) reported 77 mg/L conc. of fenvalerate for degradation by CD-9 within 72 h of incubation.

FTIR ANALYSIS OF FENVALERATE DEGRADATION BY *PSEUDOMONAS* SP. FDB-3

FTIR was used to analyze the changes in the functional group of the untreated and treated samples to study the rate of degradation. Different authors have used gas chromatography-mass spectroscopy (GC-MS), thin-layer chromatography (TLC), and high-performance liquid chromatography (HPLC) techniques to identify metabolites of fenvalerate degradation (Tang et al., 2018; Tang et al., 2020; Selvam et al., 2013; Mulla et al., 2017). This is the first report in which the degradation of fenvalerate was analyzed by comparing the FTIR peaks of the degraded fenvalerate sample and control sample.

FTIR analysis was conducted to assess bond stretching during the degradation of fenvalerate in media inoculated with the active culture of strain FDB-3. Non-inoculated flasks with fenvalerate served as the control. Bond stretching was analyzed at a wavelength of 400-4000 cm^{-1}. FTIR analysis for fenvalerate degradation revealed that fenvalerate breaks down into two by-products one of which contains a C-N triple bond i.e. cyanide group. The Cyanide group shows distinctive peaks in the range of 2000 to 2400 cm-1, which can be used to infer that fenvalerate was successfully degraded (Smith, 2019). The various peaks in the control indicated the presence of different chemical bonds within the fenvalerate structure. Peaks were observed in the control in between 3500 to 3000 cm^{-1} which corresponded to stretching in the C-N and C-H bonds of fenvalerate. The peak length in this region decreases in *Pseudomonas* sp. FDB-3 treated sample revealing the degradation of fenvalerate as shown in Fig. 8. Further, extra peaks between 2000 to 1000 cm^{-1} were observed in the case of *Pseudomonas* sp. FDB-3 treated sample which corresponds to stretching in C-C, C-N, (C=O)-O- bonds confirmed the formation of by-products formed by degradation or breakage of bonds of fenvalerate. The findings confirmed that the degradation of fenvalerate by the Pseudomonas sp. FDB-3 isolate follows a systematic process, involving the breakdown and transformation of aromatic rings into intermediate compounds.

CONCLUSION

In this study, bacterial strain FDB-3 was isolated from agricultural soil samples collected from Kaccholi, Gujarat, India which was identified as *Pseudomonas* sp. based on morphological, microscopical, and biochemical tests. It was shown that *Pseudomonas* sp. FDB-3 had maximum growth up to 72 hours with 94% of fenvalerate degradation. FTIR analysis of residual fenvalerate after 72 hours of incubation showed that FDB-3 was able to degrade fenvalerate. Therefore, the initial results obtained after optimization of culture conditions for biomass production and fenvalerate degradation indicated the possible utility of *Pseudomonas* sp. FDB-3 for degradation of fenvalerate which can be utilized in the bioremediation of fenvalerate-contaminated soil.

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TABLE 1: MORPHOLOGICAL CHARACTERISTICS OF FDB-3

Sr. No.	Characteristics	Observation
1	Appearance	Creamish
2.	Margin	Irregular
3.	Elevation	Slightly elevated
4.	Surface	Smooth, Mucoid
5.	Shape of colony	Round
6.	Size	Moderate
7.	Opacity	Opaque
8.	Gram character	Negative
9.	Shape	Rods
10.	Arrangement	Single
11.	Motility	Motile (unipolar)

TABLE 2: BIOCHEMICAL CHARACTERISTICS OF FDB-3

Sr. No.	Biochemical tests	Result	Sugar Utilization tests	Result
1	Indole	Negative	Glucose	Positive
2.	Catalase	Positive	Sucrose	Positive
3.	Lipid utilization	Positive	Fructose	Positive
4.	Urease	Negative	Lactose	Negative
5.	Citrate and Methyl red test	Positive	Maltose	Positive
6.	Voges - Proskauer test and Gas production	Negative	Xylose	Positive
7.	Gelatine hydrolysis	Positive	Galactose	Positive

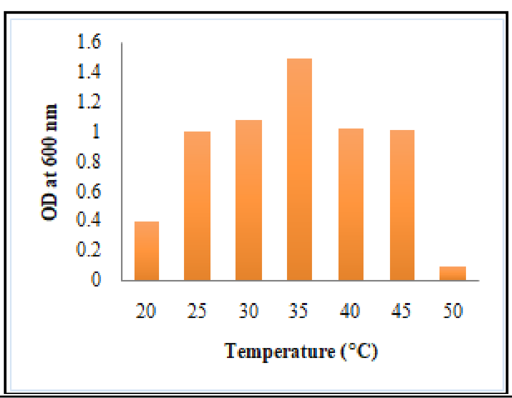

FIG. 1: EFFECT OF TEMPERATURE ON GROWTH OF *PSEUDOMONAS* SP. FDB-3

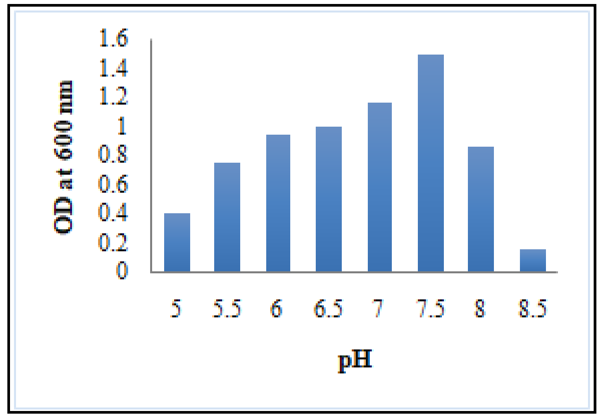

FIG. 2: EFFECT OF PH ON GROWTH OF *PSEUDOMONAS* SP. FDB-3

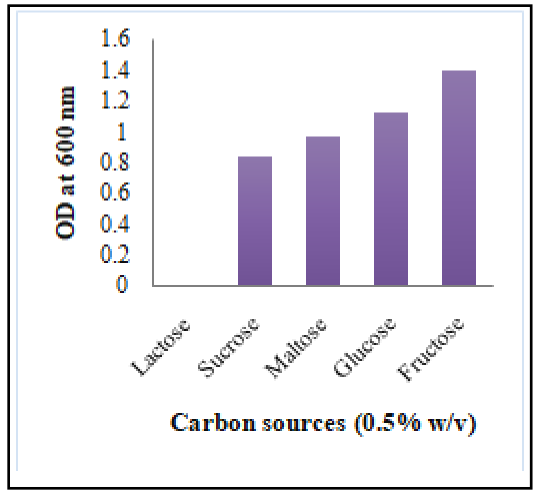

FIG. 3: EFFECT OF DIFFERENT CARBON SOURCES ON GROWTH OF *PSEUDOMONAS* SP. FDB-3

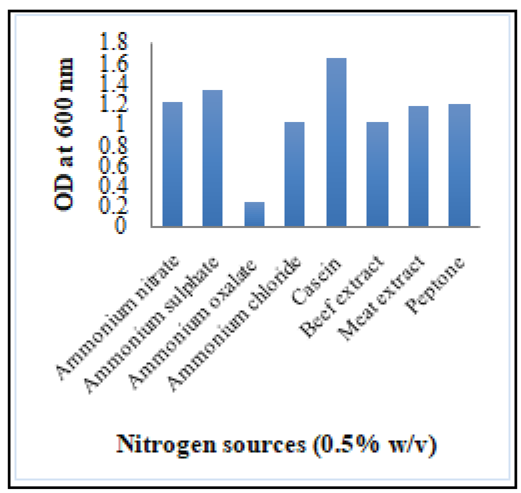

FIG. 4: EFFECT OF DIFFERENT NITROGEN SOURCES ON GROWTH OF *PSEUDOMONAS* SP. FDB-3

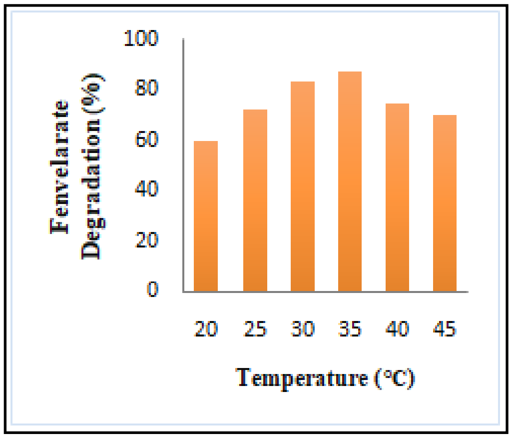

FIG. 5: EFFECT OF TEMPERATURE ON FENVALERATE DEGRADATION BY *PSEUDOMONAS* SP. FDB-3

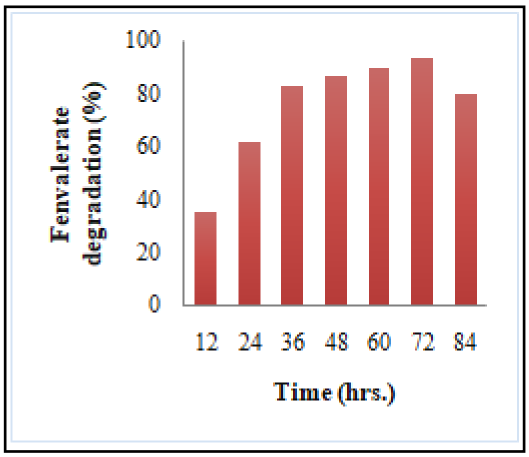

FIG. 6: EFFECT OF TIME OF INCUBATION ON FENVALERATE DEGRADATION BY *PSEUDOMONAS* SP. FDB-3

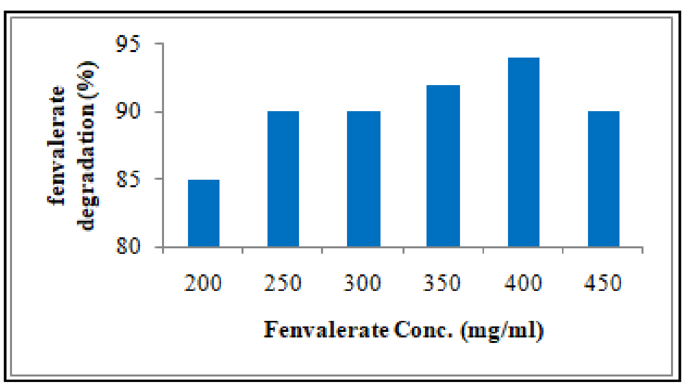

FIG.7: EFFECT OF FENVALERATE CONC. ON FENVELARATE DEGRADATION BY *PSEUDOMONAS* SP. FDB-3

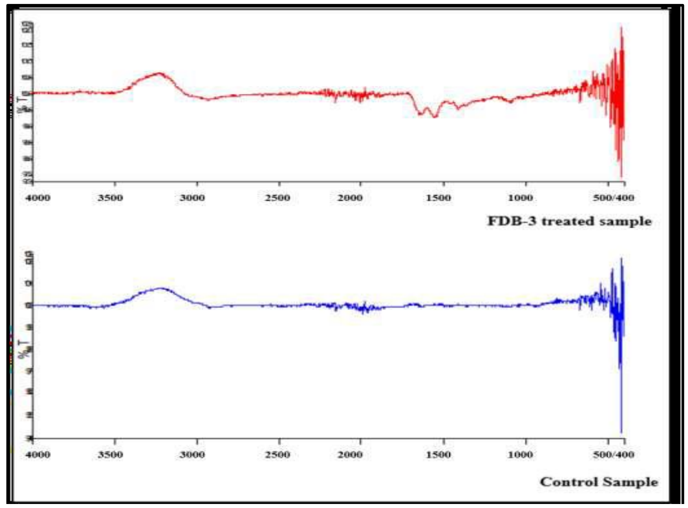

FIG 8: FTIR SPECTRA OF PSEUDOMONAS SP. FDB-3 TREATED AND UNTREATED SAMPLE OF FENVALERATE

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