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Fipronil degradation kinetics and resource recovery potential of *Bacillus* sp. strain FA4 isolated from a contaminated agricultural field in Uttarakhand, India

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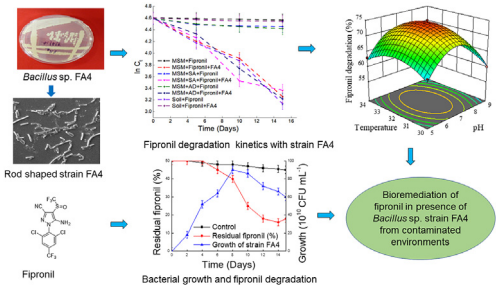
HIGHLIGHTS

- A *Bacillus* sp. FA4 capable of degrading fipronil was isolated and characterized.
- Fipronil degradation was observed with strain FA4 in mineral salt medium and soil.
- Immobilizing FA4 in sodium alginate and agar disc accelerated fipronil degradation.
- FA4 can be applied *in-situ* for remediating fipronil contaminated soil environments.

GRAPHICAL ABSTRACT



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ABSTRACT

This study investigates the potential role of *Bacillus* sp. FA4 for the bioremediation of fipronil in a contaminated environment and resource recovery from natural sites. The degradation parameters for fipronil were optimized using response surface methodology (RSM): pH - 7.0, temperature - 32 °C, inocula - 6.0 × 10^8 CFU mL^{-1}, and fipronil concentration - 50 mg L^{-1}. Degradation of fipronil was confirmed in the mineral salt medium (MSM), soil, immobilized agar discs, and sodium alginate beads. The significant reduction of the half-life of fipronil suggested that the strain FA4 could be used for the treatment of large-scale fipronil degradation from contaminated environments. The kinetic parameters, such as q_{max}, K_s, and K_i for fipronil degradation with strain FA4, were 0.698 day^{-1}, 12.08 mg L^{-1}, and 479.35 mg L^{-1}, respectively. Immobilized FA4 cells with sodium alginate and agar disc beads showed enhanced degradation with reductions in half-life at 7.83 and 7.34 days, respectively. The biodegradation in soil further confirmed the degradation potential of strain FA4 with a half-life of 7.40 days as compared to the sterilized soil control's 169.02 days. The application of the strain FA4 on fipronil degradation, under

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Resource recovery
Soil

different *in vitro* conditions, showed that the strain could be used for bioremediation and resource recovery of contaminated wastewater and soil in natural contaminated sites.

1. Introduction

Pesticides are used in huge quantities in agriculture and for home applications. The large-scale application of pesticides causes severe health and environmental impacts (Topping et al., 2020). Approximately 3 million human lives are affected due to pesticide application. In agricultural systems, pesticides contaminate the soil and water systems (Cycoń et al., 2017; Lin et al., 2020). Due to the biomagnification of the pesticides, they enter into the food chain and ultimately reach humans and animals (Birolli et al., 2018; Mishra et al., 2020; Pang et al., 2020). Fipronil is a commonly used, broad-spectrum insecticide used to control pests in crops, veterinary pests, and for public hygiene (Bhatt et al., 2021). It belongs to the phenylpyrazole group of chemicals. Fipronil was first discovered by a French company, Rhone-Poulenc, in 1987, and used to kill approximately 250 pests. The toxicity of fipronil was investigated in marine animals, lizards, zooplanktons, bees, termites, rabbits, and other mammals (Abraham and Gajendiran, 2019). It is a next-generation pesticide, and its mode of action is different from that of other groups (Bhatti et al., 2019). Fipronil and its metabolites have been reported in the soil and sediments of cultivated rice fields and adjacent rivers (Bhatt et al., 2021). The residues of fipronil have been reported from agricultural fields, water bodies, rivers, and indoor and outdoor dust (Bhatti et al., 2019). Fipronil and its metabolites are also reported in the matrixes of chicken eggs, muscle rat plasma, and foodstuffs (van der Merwe et al., 2019). In 2017, the European Union (EU) labeled it as a pollutant due to the presence in hen eggs. The United States Environmental Protection Agency (USEPA) classified fipronil as a group C carcinogen (Aparicio-Muriana et al., 2019). The concentration of fipronil in food products and environments has been detected in the range of 0.04–1.67 mg L^{-1} (Bhatti et al., 2019). In Florida, the canals and ponds are contaminated with fipronil at a concentration of 0.54–207.3 ng L^{-1}, whereas in urban surface water of California the concentration was reported in the range of 11–280 ng L^{-1} (Junior et al., 2016).

Fipronil is toxic to insects. It acts as an inhibitor for the gamma amino butyric acid (GABA) receptors. That causes hyperexcitation in chloride ion channels, which leads to paralysis and insect death (Bhatti et al., 2019). Besides its targeted specific action, fipronil can also affect the non-targeted organisms. Mortality of many non-target organisms has been reported, e.g., oysters, bees, fish, crustaceans, etc. (Bonmatin et al., 2015). Fipronil alters the growth and development of birds, fish, and amphibians, and showed moderate toxicity in rats (Slotkin et al., 2016). A previous report suggested that the human intestinal bacterium *Escherichia coli* (non-pathogenic) can degrade fipronil. However, higher concentration of fipronil can affect the membrane potential and its viability (Bhatti et al., 2019). It induced apoptosis using the mitochondrial caspase-dependent pathway in *Drosophila melanogaster* (Ajoud et al., 2008). The lethal effects of fipronil have been investigated in fish and the kidney cells of mice (Cole et al., 1993). Fipronil and fipronil sulfone, found commonly in meat products, are considered to be toxic metabolites (Nardeli et al., 2020). The toxicity of fipronil in living systems is a reason for its immediate removal from the environment.

Fipronil is degraded in the contaminated sites using physico-chemical and microbial-based methods (Qian et al., 2020; Bhatt et al., 2021). Microbial bioremediation of fipronil was found to be effective for the complete mineralization in soil and water environments. Indigenous microbial strains are able to mineralize fipronil from contaminated water and soil. The bacterial and fungal strains belonging to the genera *Streptomyces* (Abraham and Gajendiran, 2019), *Bacillus* (Bhatt et al., 2021), *Paracoccus* (Kumar et al., 2012), *Stenotrophomonas* (Uniyal et al., 2016), *Klebsiella pneumoniae* (Sayi et al., 2020), *Staphylococcus arlettae*, *Bacillus thuringiensis* (Keerthi et al., 2019), and *Aspergillus* (Gajendiran and Abraham, 2017) were found to be effective for the biodegradation of fipronil in various contaminated soil and water sites. The earthworm *Eisenia foetida* was also reported to be useful in the degradation of fipronil (Qu et al., 2014). The main metabolites of fipronil degradation were identified as sulfone, desulfinyl, amide and sulfide (Tan et al., 2008).

In this study, the role of *Bacillus* sp. FA4 was investigated for the bioremediation of fipronil, with the following objectives; (i) characterization of the bacterial strain FA4 for fipronil degradation, (ii) optimization of process parameters for fipronil degradation using response surface methodology, (iii) strain FA4 growth-linked degradation of fipronil, (iv) immobilization assay for the bioremediation of fipronil, and (v) analysis of the degradation metabolites of fipronil.

2. Materials and methods

2.1. Chemicals, media, and bacterial strain

Analytical grade chemicals were used for the fipronil bioremediation assay tests. The technical grade of 99% fipronil was used throughout the experiments. The stock solution of fipronil (100 mg L^{-1}) was prepared in acetonitrile. For the fipronil biodegradation study, the mineral salt medium (MSM) had the following composition: 2 g $(NH_4)_2SO_4$, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.01 g $CaCl_2 \cdot 2H_2O$, 0.001 g $FeSO_4 \cdot 7H_2O$, 1.5 g $Na_2HPO_4 \cdot 12H_2O$, and 1.5 g KH_2PO_4 per L of distilled water. The nutrient broth (NB) containing 1 g beef extract, 2 g yeast extract, 5 g peptone, and 5 g sodium chloride per L of distilled water was used for the preparation of the active bacterial culture. The pH of the MSM and NB was adjusted to ~ 7.0 and sterilized at 121 °C, for 20 min, by wet heat sterilization.

2.2. Isolation and characterization of the fipronil degrading strain FA4

Contaminated soil samples were collected from the agricultural fields of Pantnagar, Uttarakhand, India. The samples were stored at 4 °C in the laboratory for further screening of the fipronil degrading isolates. MSM flasks were prepared in sterile conditions with 50 mg L^{-1} of fipronil, and 5 g of soil was added into each of the flasks. The MSM flasks were incubated at 32 °C, pH 7.0, and an agitation speed of 110 rpm in an incubator shaker for up to 15 days. The 5 mL culture transferred to the new MSM flask contained 50 mg L^{-1} of fipronil. This step was repeated three times periodically to achieve enrichment, and isolate the potential fipronil

degrading strains. The process was also repeated with 100, 200, 300, 400, 500, 600, and 800 mg L^{-1} of fipronil in the MSM medium over the 15 days of experimentation (Bhatt et al., 2019a). The serial dilution method was used for isolation of the pure bacterial strains. The pure isolates were grown on MSM agar plates containing 50 mg L^{-1} of fipronil. The fipronil degrading bacterial isolate was able to grow in the MSM broth containing a fipronil concentration of up to 800 mg L^{-1} and on MSM agar plates. Potential fipronil-degrading bacterial isolates were purified in the nutrient agar plates, and stored at −20 °C in the form of glycerol stocks. The fipronil degradation potential of each of the isolates was confirmed using high-performance liquid chromatography (HPLC) (Dionex Corp., Sunnyvale, CA, USA). The bacterial strain considered to potentially degrade the fipronil was designated as FA4. The bacterial strain FA4 was further characterized on the basis of biochemical, physicochemical, and morphological methods. The morphology of the strain FA4 was determined using a scanning electron microscope. The Bergy's Manual of Determinative Bacteriology was used for the biochemical characterization of the strain FA4. Furthermore, to characterize the strain FA4 at the molecular level, the 16S rDNA technique was used for identification. For the molecular identification, genomic DNA of the bacterial strain FA4 was isolated. The genomic DNA (150 ng) was used as a template for the polymerase chain reaction (PCR) with a universal primer set (forward primer) 5′-AGAGTTTGATCCTGGCTCAG-3′ and (reverse primer) 5′-TACCTTGTTACGACTT-3'. The PCR conditions for the strain FA4 were initial denaturation at 95 °C, denaturation at 94 °C, annealing at 55 °C, and extension at 72 °C, followed by 35 cycles. The PCR amplified DNA bands were observed in the 1.0% agarose gel electrophoresis, and the bands were eluted. The purified amplicon of the strain FA4 was sequenced. The sequenced contig was used for the final deposition into the National Center for Biotechnology Information (NCBI) to get the GenBank accession number. The NCBI BLAST tool was used to retrieve similar sequences, and MEGA 5.0 was used for sequence alignment and phylogenetic analysis. The phylogenetic tree was constructed using the neighbor joining (NJ) method in MEGA software (Huang et al., 2020).

2.3. Box–Behnken design for optimization of fipronil degradation

Fipronil degradation tests were designed using response surface methodology (RSM). RSM is a statistical technique that has been used to optimize bioremediation processes where the response is affected by certain variables or process parameters (Swati et al., 2019). In the present work, four process parameters were used for optimizing fipronil degradation using strain FA4 (Chen et al., 2011). The Box–Behnken design was used to investigate the effect of the critical factors on fipronil bioremediation (Zhan et al., 2018). The pH (5.0, 7.0, 9.0), temperature (30 °C, 32 °C, 34 °C), inocula (OD~ 0.3, 0.6, 0.9), and fipronil concentration (25, 50, 75 mg L^{-1}) were used for the optimization process (Bhatt et al., 2020b, c). Fipronil degradation (%) was used as the dependent variable. The Box–Behnken design, with four variables, consisted of 29 experiments (in triplicate), wherein triplicate measurements were used for the generation of statistical analysis system (SAS) at the midpoint. A quadratic polynomial regression equation that relates the process variables/factors to the response was obtained (Equation (1)):

$$Y_i = b_0 + \sum b_i X_i + \sum b_{ij} X_i X_j + b_{ij} X_i^2 \quad (1)$$

where Y_i is the predicted response, X_i and X_j are variables, b_0 denotes the constant, b_i represents the linear coefficient, b_{ij} is the interaction coefficient, and b_{ii} represents the quadratic coefficient.

A substrate inhibition model was used to fit the specific degradation rate (q) (Equation (2)) using various initial concentrations of fipronil (Chen et al., 2011).

$$q = \frac{q_{max} S}{S + K_s + (S^2 / K_i)} \quad (2)$$

In Equation (2), q_{max} is the maximum specific degradation rate, K_s denotes the half-saturation point, S represents the inhibitor concentration, and K_i denotes the substrate inhibition constant. The semi-logarithmic plot of fipronil at different initial concentrations was used for the calculation of the q value. In the present work, the fipronil concentrations used for the experiments were 50, 100, 200, 300, 400, 500, 600, and 800 mg L^{-1} at 32 °C, and an agitation speed of 110 rpm with strain FA4. All the experiments were performed in triplicates and the fipronil residues were extracted and collected after 15 days and analyzed using HPLC (Bhatt et al., 2021).

2.4. Fipronil degradation associated with bacterial growth

The bacterial growth was monitored in the presence of fipronil in a MSM medium. 50 mL of the MSM was supplemented with 50 mg L^{-1} of fipronil, and the fresh bacterial culture was inoculated from the NB plates. Uninoculated flasks acted as controls for the comparison of fipronil degradation. The growth of strain FA4 was monitored using a UV−visible spectrophotometer (Ray-Leigh UV 2601), at a λ_{max} of 600 nm. The fipronil from each of the flasks was recovered and analyzed using high-performance liquid chromatography (HPLC) (Dionex Corp., Sunnyvale, CA, USA). The correlation between fipronil degradation and bacterial growth was monitored (Bhatt et al., 2020e).

2.5. Effect of immobilization on fipronil degradation

Immobilized cells of the FA4 were used for the enhanced degradation of fipronil in liquid media. Sodium alginate beads and agar discs were used for immobilizing the strain FA4. Sodium alginate beads were prepared by using 4% sodium alginate with homogenized FA4 cells. Beads of 2 mm size were prepared with pre-chilled 0.4 M calcium chloride and stored in a refrigerator at 4 °C (Bhatt et al., 2020d). For the formation of the agar discs, 2.5% sterilized agar were used. The 20 beads of sodium alginate and agar discs were used for the immobilization in MSM medium. The abiotic treatment (beads without FA4 cells), considered as the control, contained 50 mg L^{-1} of fipronil in the MSM. The residual fipronil was quantified using HPLC.

2.6. Fipronil degradation in the soil

Fipronil degradation was studied in soil with strain FA4. The soil was collected from the agricultural fields of Udham Singh Nagar, Uttarakhand, India. The top layer of soil (0−25 cm) was used for the biodegradation study. The collected soil does not have a previous history of fipronil or other pesticides. The collected soil was sieved (5 mm) and autoclaved at 121 °C for 25 min on three successive days. The experiment was performed in 250-mL flasks and it contained 50 g of the autoclaved soil in triplicate. 20 mL of the sterilized MSM and 50 mg L^{-1} of fipronil were added to each of the flasks. The active strain FA4 was inoculated into each of the flasks. The fipronil degradation was also monitored in an abiotic control. 1 mL of the soil slurry was used for the analysis at 0-, 5-, 10-, and 15-day intervals. Fipronil was extracted and quantified from each of the samples using HPLC.

2.7. Analytical methods

Fipronil and its degraded metabolites were extracted from the soil and liquid media. For the analysis, 5 mL of media was collected from each of the experiments and 10 mL of acetone was added to each sample and the contents were well mixed by agitation for 30 min. The mixture was filtered using a Buchner funnel and the residue was washed with 10 mL acetone and filtered. The filtered solution was collected in round-bottomed flasks. After that, the mixture was evaporated under vacuum, at 45 °C. Fipronil and its degradation metabolites were collected from each of the round-bottomed flasks after acetonitrile washing (Bhatt et al., 2021). The prepared samples were filtered through a bacterial filter with the help of a 5-mL syringe and collected in 2-mL vials. The HPLC conditions for the quantification of fipronil residue from the degraded samples were column - C_{18} reverse phase; detector - UV; solvent - acetonitrile: water (80:20); flow rate - 1 mL/min; and the retention time for the fipronil - 6 min.

A gas chromatography–mass spectrometry (GC–MS) system (Shimadzu QP-2010 Plus with Thermal Desorption System TD 20, Shimadzu, Kyoto, Japan) was used for the analysis of the fipronil degradation metabolites with strain FA4. The Column oven temperature of the GC was 70 °C and the injection temperature was 260 °C. The injection mode of GC was split, with flow control mode as linear velocity. The total flow rate was 16.3 mL min^{-1}, column flow 1.21 mL min^{-1}, and purge flow 3.0 mL min^{-1}. For analysis of fipronil and its intermediates, 1 μL of sample was injected in splitless mode. The degradation metabolites of fipronil were identified and compared with the library database of the National Institute of Standards and Technology (NIST), USA. The fipronil degradation metabolites were characterized based on their retention time (RT) and molecular weight (*m/z*) from the NIST library. The compounds showing higher similarity were considered for the identification of fipronil and its degradation intermediates with strain FA4.

The statistical analysis was performed using the IBM SPSS 25 software (IBM, NY, USA). Three replicates were used for the statistical analysis of fipronil degradation data. One-way analysis of variance (ANOVA) and averages were compared using the Duncan test. An estimation of the lowest significance differences (LSD) at $p < 0.005$ confirmed the statistical difference within different experiments during fipronil degradation.

3. Results and discussion

3.1. Characterization of the strain FA4 for the bioremediation of fipronil

The results from the SEM shows that, the strain FA4 appeared as a rod-shaped bacteria, and Gram's staining results confirmed that it is a Gram-positive bacterium. The results from 16S rDNA analysis confirmed that strain FA4 belongs to *Bacillus* sp., and the nucleotide sequence was deposited in the NCBI GenBank with an accession number KT186611 (Fig. 1). The results of biochemical, morphological, and molecular analysis confirmed that the isolate FA4 was *Bacillus* sp. The results indicated that strain FA4 was able to use fipronil as a sole carbon and energy source. Bacterial strains belonging to *Streptomyces rochei*, *Bacillus thuringiensis*, *Bacillus firmus*, *Bacillus* sp., *Paracoccus* sp., *Comamonas aquatica*, *Stenotrophomonas acidaminiphila* and *Burkholderia* have been reported for the degradation of fipronil in various water and soil environments (Mandal et al., 2013, 2014; Thirumalaiselvan et al., 2015; Bhatt et al., 2021). Higher fipronil degradation occurs in unsterilized soil due to the cumulative effects of the presence of other microbial populations (Bhatt et al., 2021; Masutti and Mermut, 2007). Resource recovery of water and soil environments is possible with reductions of fipronil in the presence of the bacterial strains (Bhatt et al., 2021; Gajendiran and Abraham, 2017). In a previous study using microbial fuel cell (MFC), the biofilm containing the genera *Chryseobacterium*, *Sphaerochaeta*, *Azospirillum*, *Azoarcus*, and *Pseudomonas* were responsible for the bioremediation of fipronil. These groups of microbes were divided into two groups: electrogenic and organic compound degraders. The organic compound-degrading bacteria can degrade fipronil, whereas electrogenic bacteria provided the electrons required for co-metabolic activities during degradation (Zhang et al., 2019). On the basis of the bioremediation potential, strain FA4 was used for further study in soil and a MSM.

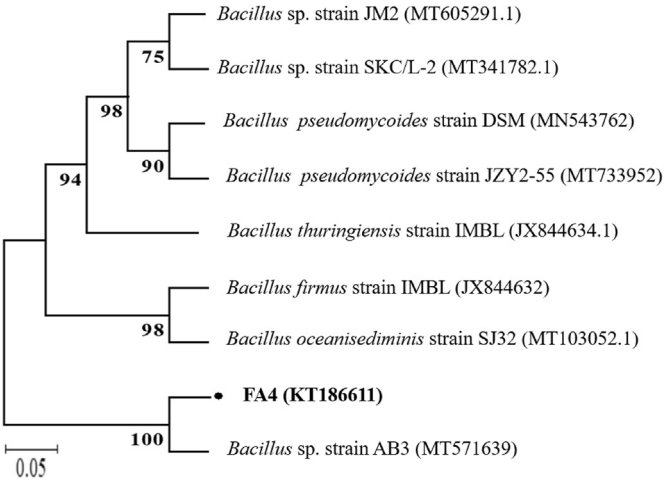


Fig. 1. Phylogenetic tree based on 16S rDNA sequences of strain FA4 and related bacterial strains. Numbers in parentheses represent the Genbank accession number. Numbers at the nodes indicate the bootstrap values. Bar represents the sequence divergence.

3.2. Response surface methodology for fipronil degradation using strain FA4

The results of the 29 experiments based on the Box–Behnken design showed a range of degradation patterns, varying from 59.9 to 75.0%. The important process factors namely, the pH, temperature, inocula size, and initial fipronil concentration were found to be significant in the quadratic model developed for fipronil degradation. Based on the degradation patterns, a 3D view of the optimized points are represented in Fig. 2a–f. Center points in the contour plot represent the maximum fipronil degradation with strain FA4 (Fig. 2g and h). The effects of each of the critical factors are represented in the contour and 3D plots. The determination coefficient (R^2) values indicated that the maximum responses were covered by the model, demonstrating that the predicted values of the model were in good agreement with the experimental values. The analysis of variance (ANOVA) of the quadratic model for fipronil degradation using strain FA4 showed a *F* value of 25.72, which implies that the model is significant (Table 1). The model for fipronil biodegradation was highly significant ($p < 0.0500$), indicating that the established quadratic model was adequate and reliable at representing the actual relationship between the response and the variables. A lack of fit *F* value of 2.04 implies that the lack of fit was not significant relative to the pure error. However, there is a 25.65% chance that the lack of fit value could have occured due to a noise. An insignificant lack of fit suggested that the model is suitable for fipronil degradation. The predicted R^2 of 0.810 was in

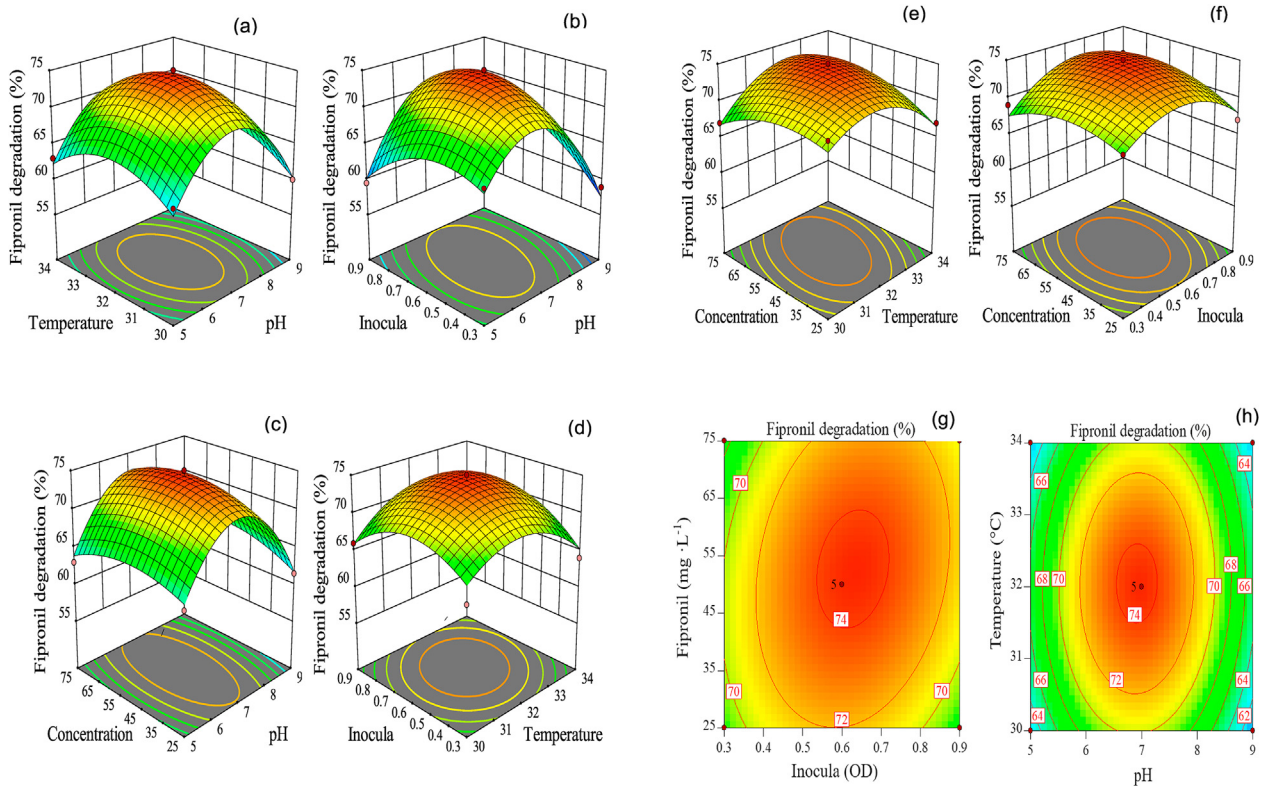


Fig. 2. Response surface 3D plots of fipronil degradation using strain FA4. (a) Effect of pH and temperature, (b) effect of inocula and pH, (c) effect of fipronil concentration and pH, (d) effect of inocula and temperature, (e) effect of fipronil concentration and temperature, (f) effect of fipronil concentration and inocula, (g) the contour plot representing the effect of fipronil concentration and inocula on fipronil biodegradation, (h) the contour plot representing the effect of temperature and pH on fipronil biodegradation.

reasonable agreement with the adjusted R^2 of 0.925, implying that the overall mean might be a better predictor of the fipronil degradation response. Adequate precision measures the signal to noise ratio; a ratio greater than 4 is desirable. This ratio for fipronil degradation was 17.693, denoting an adequate signal. The coefficient estimate represents the expected change per unit change in factor value when all the remaining factors are maintained at their constant values. The coded equation for fipronil bioremediation using strain FA4 is represented in Equation (3).

$$\begin{aligned}\textit{Biodegradation of fipronil}\,(\%) = {} & 74.28 - 0.7583 \times \textit{pH} + 0.2083 \times \textit{temperature} + 0.55 \times \textit{inocula} + 0.33 \times \textit{fipronil concentration} + \\ & 0.375 \times \textit{pH} \times \textit{temperature} + 3.15 \times \textit{pH} \times \textit{inocula} + 0.75 \times \textit{pH} \times \textit{fipronil concentration} + 1.25 \times \textit{temperature} \times \textit{inocula} + 1.75 \times \\ & \textit{temperature} \times \textit{fipronil concentration} + 1.0 \times \textit{inocula} \times \textit{fipronil concentration} - 8.73 \times \textit{pH}^2 - 3.91 \times \textit{temperature}^2 \times \\ & - 3.54 \times \textit{inocula}^2 - 1.97 \times \textit{fipronil concentration}\end{aligned} \quad (3)$$

The optimal temperature, pH, inocula and fipronil concentration were found to be 32 °C, pH 7.0, inocula (OD~0.6) 6.0×10^8 CFU mL^{-1} and 50 mg L^{-1}. The RSM is mainly useful to determine the dependent variables (Masutti and Mermut, 2007). The Box–Behnken design has been used previously for the optimization of process variables for the degradation of various pesticides and organic compounds (Bhatt et al., 2020a; Huang et al., 2020). Previous researchers have investigated the key growth-determining factors, such as agitation speed, temperature, pH, nutrient concentration, and inoculum size, for the degradation of insecticides, herbicides, and other organic pollutants (Zhan et al., 2018; Gangola et al., 2018; Yang et al., 2018). RSM is a popular optimization tool among the scientific community for studying the pesticide degradation process with indigenous microbial strains (Bhatt et al., 2020e; Huang et al., 2020). The RSM has been applied successfully for the degradation of pesticides by microbial strains belonging to the genera *Stenotrophomonas acidaminiphila* and *Bacillus* sp. FA3 (Uniyal et al., 2016). The optimized conditions showed effective degradation under all the tested laboratory conditions. The RSM technique has been used extensively for the carrying out optimization studies related to the degradation of dyes and aromatic compounds using pure and mixed consortia (Ridha et al., 2020; Fatima et al., 2020). In a previous work, *Bacillus badius* ABP6 was applied for the optimization of atrazine degradation using an RSM-based Box–Behnken design (Khatoon and Rai, 2020). RSM was also used for the degradation of fipronil with a combination of photocatalytic and microbial degradation (Junior et al., 2016; Bhatt et al., 2021). Chlorpyrifos degradation was optimized using RSM to maximize the oxidation using a mathematical model (Amiri et al., 2018). Pyraclostrobin bioremediation was also investigated using the central composite design of the RSM, and its removal efficiency found to be high using a bacterial consortium (Birolli et al., 2020).

Table 1
Analysis of variance (ANOVA) for the quadratic fipronil degradation model.

Source	Sum of squares	Degree of freedom	Mean square	*F*-value	*p*-value
Model	622.81	14	44.49	25.72	< 0.0001
A-pH	6.90	1	6.90	3.99	0.0656
B-Temp	0.5208	1	0.5208	0.3011	0.5918
C-Inocula	3.63	1	3.63	2.10	0.1694
D-concentration	1.33	1	1.33	0.7709	0.3948
AB	0.5625	1	0.5625	0.3252	0.5775
AC	39.69	1	39.69	22.95	0.0003
AD	2.25	1	2.25	1.30	0.2732
BC	6.25	1	6.25	3.61	0.0781
BD	12.25	1	12.25	7.08	0.0186
CD	4.00	1	4.00	2.31	0.1506
A^2	494.54	1	494.54	285.94	< 0.0001
B^2	99.00	1	99.00	57.24	< 0.0001
C^2	81.48	1	81.48	47.11	< 0.0001
D^2	25.15	1	25.15	14.54	0.0019
Residual	24.21	14	1.73		
Lack of Fit	20.25	10	2.02	2.04	0.2565
Pure Error	3.97	4	0.9920		
Cor Total	647.02	28			

3.3. Bacterial growth-linked biodegradation of fipronil

The growth of strain FA4 followed an established pattern of a long exponential phase, small stationary phase, and a decline stage. The growth of strain FA4 growth and the corresponding fipronil residue were well correlated; bacteria were able to use fipronil as a sole source for nutrition and energy. The growth of strain FA4 and degradation of fipronil are represented in Fig. 3. 75% of fipronil was degraded using strain FA4 in the MSM within 15 days of the experiment. The results of the growth-linked degradation showed a 0.005% level of significance. It was observed that the growth of strain FA4 was enhanced at 8–10 days with a significant reduction in fipronil residue. In the literature, indigenous bacterial strains have been used for the bioremediation of pesticides (Huang et al., 2021). These strains used pesticides as carbon, nitrogen, and phosphorous sources to fulfill their nutritional requirements (Zhang et al., 2019; Bhatt et al., 2019b). In this study, it was observed that the intermediate metabolites of fipronil degradation was not toxic to the strain FA4. Earlier researchers have also noted the lack of toxicity of fipronil intermediates to bacterial cell growth (Mandal et al., 2014). The strain FA4 was able to mineralize all the metabolites into an environmentally acceptable form. Previous researchers have investigated the growth-linked degradation of fipronil and other pesticides (Uniyal et al., 2016; Zhan et al., 2018). Microbes have the metabolic pathways for fipronil degradation (Bhatt et al., 2021). The metabolic pathways are helpful to mineralize fipronil completely from the contaminated sites (Mandal et al., 2014; Huang et al., 2019). Microbial strains are able to degrade fipronil in soil and water environments (Bhatti et al., 2019a, b, c). For example, the biodegradation of fipronil was investigated with *Comamonas* and *Bacillus* in a liquid medium (Thirumalaiselvan et al., 2015). The results of the present study are in line with the previous findings, which suggests that strain FA4 could efficiently degrade fipronil from contaminated sites.

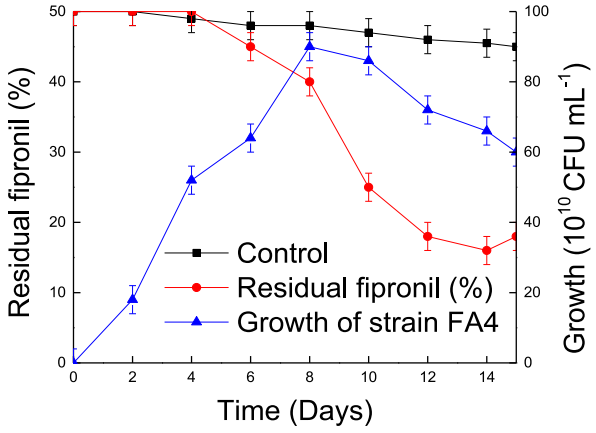


Fig. 3. Bacterial growth and fipronil degradation profiles.

3.4. Immobilization assay for fipronil degradation

The results of the agar disc and sodium alginate beads immobilized *Bacillus* sp. FA4 studies showed an increase in fipronil degradation. The fipronil degradation pattern with an agar disc was 24%, 58%, and 75% after 5, 10, and 15 days of incubation, respectively. The degradation with sodium alginate was 29%, 66%, and 71% after 5, 10, and 15 days of the experiment, respectively. The maximum fipronil bioremediation was observed with strain FA4 after 15 days of incubation (Fig. 4). The degradation of fipronil with an immobilized disc and beads followed the first-order reaction

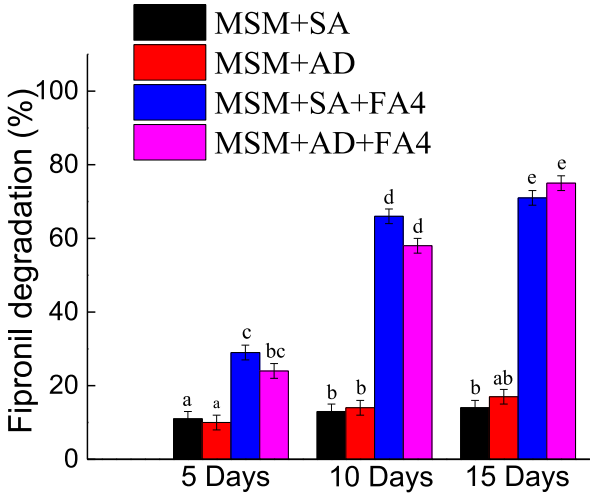


Fig. 4. Effect of immobilizing strain FA4 in sodium alginate and agar discs on fipronil degradation. SA = sodium alginate beads; AD = agar disc beads; MSM = mineral salt medium.

kinetics. The degradation kinetics calculated the half-life ($t_{1/2}$), degradation constant (k), and regression coefficient (R^2) for fipronil degradation with strain FA4. The $t_{1/2}$, k, and R^2 for the abiotic treatment with agar discs were calculated as 60.26 days, 0.0115 day^{-1}, and 0.944, respectively. The degradation parameters for FA4 immobilized with agar discs were determined as $t_{1/2}$ (24.48) days, k (0.0283) day^{-1}, and R^2 (0.857). The $t_{1/2}$ of fipronil in sodium alginate was 77.86 days and 7.83 days for the FA4 treatments. The results showed that the immobilized FA4 treatments are effective for the enhanced fipronil degradation in MSM medium. The k values for the sodium alginate immobilized beads were 0.0089 and 0.0884 day^{-1} for the FA4 incubated flasks. The enhancement in the degradation of fipronil in immobilized agar discs and sodium alginate could be due to the increased surface area for nutritional uptake by the bacterial strain. The immobilized beads are responsible for the fast and rapid degradation of fipronil in a liquid medium. Previous researchers have used whole bacterial cells for the degradation of the pesticides in a liquid medium (Bhatt et al., 2020a). Increased biodegradation of fipronil was reported with immobilized sodium alginate and agar discs in the MSM medium (Bhatt et al., 2021). The alginate-based beads were found to be effective for the degradation of organophosphate; besides, such beads can be used for wastewater treatment (Ha et al., 2009; Cruz et al., 2013; Doraiswamy et al., 2019). The immobilized microorganisms play an important role in the degradation of pesticides (Conde-Avila et al., 2019). In a recent study, biochar-based immobilization increased the degradation of tebuconazole using *Alcaligenes faecalis* WZ-2. The immobilized strain WZ-2 reduced the half-life from 40.8 to 18.7 days (Sun et al., 2020). In the literature, calcium alginate beads have been developed for the enhanced degradation of coumaphos and its hydrolysis products. It was reported that the hydrolysis of coumaphos was 2.5-fold faster in immobilized *Escherichia coli* (Ha et al., 2009). The sodium alginate and agar discs accelerated the cypermethrin and fipronil degradation in the presence of an indigenous bacterial strain (Bhatt et al., 2020d, 2021). The alginate beads increased the surface area, which could account for the enhanced reaction rate.

3.5. Fipronil degradation kinetics

The degradation kinetics results showed that strain FA4 was able to use fipronil at concentrations of 50 mg L^{-1} to 800 mg L^{-1}. The specific rate constant (q) was determined for fipronil degradation at different concentrations. Higher q (0.5 day^{-1}) values were found at lower concentrations, whereas lower q (0.2 day^{-1}) values were observed at higher fipronil concentrations, suggesting that higher concentrations are inhibitory for the growth of strain FA4. The kinetic parameters q_{max}, K_s, and K_i were estimated with strain FA4 at various fipronil concentrations. The values of the kinetic parameters were calculated as 0.698 day^{-1}, 12.08 mg L^{-1}, and 479.35 mg L^{-1} (Fig. 5). The degradation kinetics of fipronil followed the first-order reaction kinetics. The $t_{1/2}$, k, and R^2 were calculated for fipronil-treated samples and the abiotic control in MSM medium. The values of $t_{1/2}$, k, and R^2 for the abiotic control were calculated as 277.15 days, 0.0026 day^{-1}, and 0.999 (Fig. 5); in FA4-treated flasks $t_{1/2}$, k, and R^2 was found to be 8.04 days, 0.0861 day^{-1}, and 0.970, respectively (Table 2). Previously, *Bacillus* sp. strain FA3 was investigated for fipronil degradation from soil and water (Bhatt et al., 2021). *Bacillus thuringiensis* was implicated for fipronil degradation and was responsible for a significant reduction of fipronil in clay loam soil (Mandal et al., 2013, 2014). The results of the present study are in agreement with these previous findings (Huang et al., 2020). The half-life of the pesticides can be reduced by using microbial strains to clean the soil and water systems (Zhan et al., 2018; Gangola et al., 2018). In a recent study, the degradation

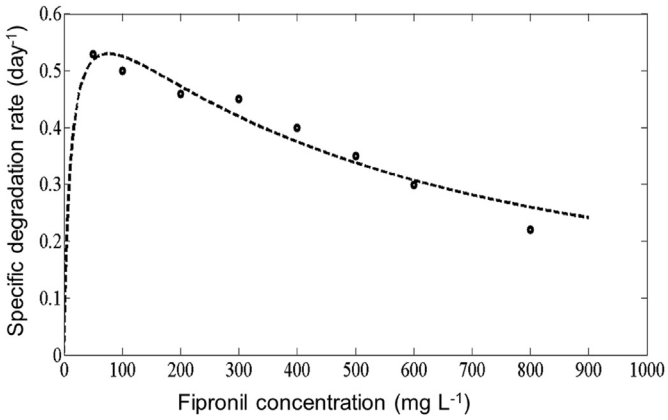


Fig. 5. Variation of specific degradation rate with initial fipronil concentration.

kinetics of phenylurea herbicides was investigated with *C. pyrenoidosa* and it proved to be effective for the remediation of different contaminants present in wastewater (Gao et al., 2020). *B. thuringiensis* and *B. arlettae* have also been used for the bioremediation of fipronil from soil and water environments (Keerthi et al., 2019). The degradation kinetics of fipronil in the presence of strain FA4 suggested an effective role in the reduction of half-life in various environments.

3.6. Fipronil bioremediation in soil

The degradation results of fipronil in soil with strain FA4 confirmed its bioremediation potential. The degradation of fipronil in soil was 35%, 52%, and 77% after 5, 10, and 15 days of incubation. Enhanced degradation of fipronil was observed in the soil-based experiments (Fig. 6). The kinetics for fipronil under FA4 treatment was investigated. The calculated kinetic parameters for fipronil degradation in soil were $t_{1/2} = 169.02$ days, $k = 0.955$ day^{-1} under abiotic treatment. In strain FA4 bioaugmented soil, $t_{1/2}$ and k values were calculated as 169.02 days and 0.955 day^{-1}, respectively. These results of soil-based bioremediation experiments confirmed that the strain FA4 not only degraded fipronil in a liquid medium but also had the potential to degrade fipronil in soil. In presvious studies, three bacterial strains, namely *Bacillus* sp. FA3, *Actinomycetes* strain AJAG7, and *Paracoccus* sp., were effectively used for the degradation of fipronil from contaminated soil (Kumar et al., 2012; Abraham and Gajendiran, 2019; Bhatt et al., 2021). On the basis of previous reports and findings, the present work encourages researchers to establish a mechanism for soil-based removal of fipronil. The potential of strain FA4 in fipronil degradation supports its large-scale application for fipronil degradation from contaminated agricultural fields. Biodegradation of fipronil was investigated in soil with *B. thuringiensis* and *S. arlettae*, which degraded 65.98% and 81.94% of fipronil, respectively (Keerthi et al., 2019). The indigenous bacterial strain was found to be suitable for the biodegradation of fipronil from the soil (Egbe et al., 2020). The microbial strains degraded fipronil in the top layer (0–25 cm) of soil, thereby reducing it in the ground water and food chain. Soil-based degradation of fipronil using *Bacillus* sp. strain FA4 showed that the strain can be used for the degradation of contaminated agricultural fields.

3.7. Metabolites of fipronil degradation with strain FA4

The results of GC–MS analysis identified the degradation products during FA4 metabolism. Fipronil and its degraded metabolites

Table 2
Degradation kinetics of fipronil using FA4 immobilized in sodium alginate (SA) and agar discs (AD).

Treatments	Regression equation	k (day^{-1})	R^2	$t_{1/2}$ (days)
MSM	$\ln (C_t/C_0) = -0.0026 + 4.605$	0.0026	0.999	277.15
MSM + FA4	$\ln (C_t/C_0) = -0.0861x + 4.638$	0.0861	0.970	8.04
MSM + SA	$\ln (C_t/C_0) = -0.0089x + 4.567$	0.0089	0.797	77.86
MSM + FA4+SA	$\ln (C_t/C_0) = -0.0884x + 4.600$	0.0884	0.946	7.83
MSM + AD	$\ln (C_t/C_0) = -0.0115x + 4.578$	0.0115	0.944	60.26
MSM + FA4+AD	$\ln (C_t/C_0) = -0.0943x + 4.6767$	0.0943	0.978	7.34
Soil + Fipronil	$\ln (C_t/C_0) = -0.0041x + 4.6002$	0.0041	0.955	169.02
Soil + Fipronil + FA4	$\ln (C_t/C_0) = -0.0936x + 4.6464$	0.0936	0.964	7.40

Note: MSM represents the mineral salt medium; $t_{1/2}$, k, and R^2 refer to the half-life, degradation constant, and determination coefficient, respectively.

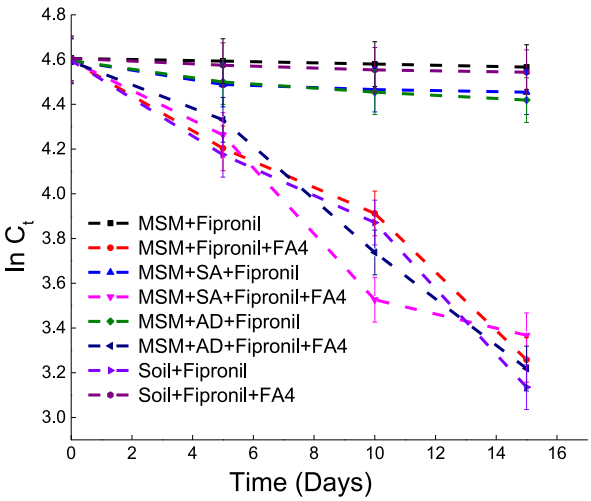
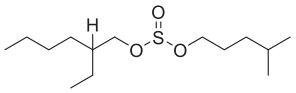
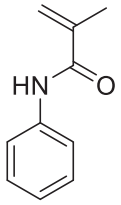
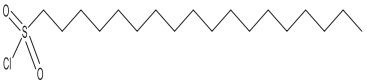
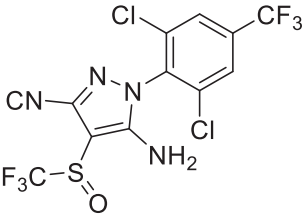
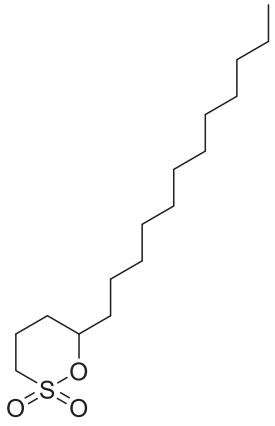
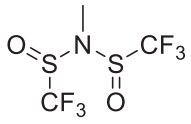


Fig. 6. (a) Degradation kinetics of fipronil (50 mg L^{-1}) with strain FA4; (b) biodegradation of fipronil with immobilized bacterial culture. SA = sodium alginate beads; AD = agar disc beads; MSM = mineral salt medium. All the values are presented as mean ± standard error of the mean (SEM) of the triplicate experiments, and different letters indicate the significant differences ($p < 0.05$) between the treatments.

were observed in the extracted samples. The analysis showed that the total retention time for fipronil degradation was up to 30 min. The identified retention time (RT), chemical structure, and mass spectra (m/z) were analyzed for each of the metabolites. The compound FP1 at RT, 11.19 min with m/z 278.46 was identified as sulfurous acid, 2-ethylhexyl isohexyl ester. Another metabolite, FP2, was detected at a RT of 14.00 min and m/z of 161.2 and it corresponded to *N*-phenylmethacrylamide. FP3, detected at a RT of 14.11 min and m/z of 353 was identified as octadecane suphonyl chloride. The concentration of the intermediate was found to be low compared to the parent compound. The fipronil peak area and concentrations were drastically reduced as the incubation time with strain FA4 increased. FP4 was characterized as fipronil at RT 19.02 min and m/z 437.1. The metabolites FP5 and FP6 were characterized as hexadecane-1-sulfonic acid, 4-hydroxy-, delta-sultone (m/z 304.5), and *N*-methyl bis (trifluoromethyl sulfin) amide (m/z 263.18) at RT of 18.98 min and 22.65, respectively (Table 3). In the abiotic (without FA4) control, a single parent compound was detected for fipronil under the same physicochemical conditions. To date, little information is available on the characterization of fipronil's intermediate metabolites in the presence of microbial strains. Sulfone and sulfide derivatives are formed during fipronil degradation via oxidation and reduction reactions. The concentration of these metabolites varied under different treatment conditions. Besides these two major metabolites, other metabolites have also been reported during fipronil degradation, such as amide derivatives (Masutti and Mermut, 2007). In a previous study, *Stenotrophomonas acidaminiphila* has been used for the degradation of fipronil and sulfone, sulfide, and amide intermediates were formed in the MSM medium (Uniyal et al., 2016). All major groups of fipronil intermediate metabolites were reported with strain FA4 treatments.

On the basis of the identified intermediate compounds of fipronil degradation using strain FA4, a metabolic degradation pathway has been proposed (Fig. 7). Fipronil can be transformed into fipronil sulfone via oxidation reaction. Fipronil sulfone is unstable and can be converted to other metabolites such as, *N*-methyl bis (trifluoromethyl sulfin) amide and 2-methyl *N*-phenyl-acrylamide. The intermediate fipronil sulfone can also be degraded into octadecanesulphonyl chloride. The intermediate octadecane-sulphonyl chloride can be transformed into sulfurous acid and 2-ethylhexyl isohexyl ester. Furthermore, the strain FA4 could degrade sulfurous acid and 2-ethylhexyl isohexyl ester into hexadecane-1-sulfonic acid and 4-hydroxy-delta-sultone. Previous researchers reported that fipronil is cleaved to sulfone-based metabolites via an oxidative reaction, and could be degraded to sulfide via a reduction mechanism. The amide intermediate can be formed by the oxidation, reduction and hydrolytic cleavage of fipronil (Mandal et al., 2013, 2014). The fungi *Aspergillus glaucus* strain AJAG1 degrades fipronil into the following main metabolites: bis [2-chloro-4-ethoxyphenyl] sulfone, sulfuric acid, 5,8,11-heptadecatrienyl methyl ester, asycarpidan-1-methanol, acetate ester, and isomethone (Gajendiran and Abraham, 2017). Fipronil sulfone was also identified with the white rot fungus *Trametes versicolor*. Fipronil is metabolized by the enzyme cytochrome P450 complex (Wolfand et al., 2016). The results of the present study are supported by previous research related to the intermediate metabolites and the proposed metabolic pathway of fipronil bioremediation (Mandal et al., 2014; Bhatt et al., 2021). Furthermore, discoveries related to the degradation of fipronil and its intermediate metabolites could be the beneficial to further explore enzymatic catalysis. In a previous study, the degradation and metabolic products of fipronil were investigated in a photo-Fenton/solar process located at a sewage treatment plant (Junior et al., 2020). Fipronil and its degradation metabolites were identified during photocatalytic degradation (Junior et al., 2016). The degradation products of fipronil were recovered and identified as desulfinyl fipronil and fipronil sulfide from indoor and outdoor dust using GC–MS (Mahler et al., 2009; Shi et al., 2020). Fipronil and fipronil sulfone were detected in food products and characterized using GC–MS (Nardeli et al., 2020). *C. pyrenoidosa* also has the potential to degrade the phenylurea herbicide flufiprole, and produce amides, sulfones, and sulfide metabolites via specific metabolic pathways (Gao et al., 2020). Bacteria, fungi, and algae have been found to be effective for the degradation of fipronil from different environments, at low concentrations, whereas higher concentrations have inhibitory effects on these organisms due to its inherent toxicity (Uniyal et al., 2016).

Table 3
Properties of the metabolites formed during fipronil degradation using strain FA4.

Compound code	Name	Retention time (min)	Molecular mass (gm·mol^{-1})	Chemical structural formula
FP1	Sulfurous acid, 2-ethylhexyl isohexyl ester	11.19	278.45	
FP2	*N*-Phenylmethacrylamide	14.00	161.2	
FP3	Octadecane suphonyl chloride	14.11	353	
FP4	Fipronil	19.02	437.1	
FP5	Hexadecane-1-sulfonic acid, 4-hydroxy-, delta-sultone	18.98	304.5	
FP6	*N*-Methyl bis (trifluoromethyl sulfin) amide	22.65	263.18	

3.8. Resource recovery potential of strain FA4

The results from fipronil degradation indicated that the strain FA4 was able to recover contaminated water and soil resources. Fipronil and its metabolites were successfully recovered using ethyl acetate and an acetone-based extraction method. Maximum degradation of fipronil indicated low recovery from the contaminated sites. After 5, 10, and 15 days of experiments, the fipronil residue recovered from the liquid media was 63.3%, 49.0%, and 26.0%, respectively, which indicated that the bacterial strain FA4 can play a direct role in the recovery of the natural environment. The residual recovery of fipronil from soil was 65.0%, 48.1%, and 23.0% with FA4 treatments after 5, 10, and 15 days of the experiments, respectively. It was observed that fipronil recovery in MSM was greater compared to the FA4-treated soil. The $t_{1/2}$ of fipronil in MSM and soil with FA4 treatment was 8.04 and 7.40 days, respectively. The smaller value of $t_{1/2}$ confirmed that the strain FA4 showed effectiveness in degradation as compared to the control. In the literature, the impact of fipronil and other pesticides has been investigated for theie resource recovery potential. It was concluded that the microbial strains have the potential to use fipronil as a nutritional source and can make natural sites clean (Bhatt et al., 2021). The maximum soil fipronil residue was found in the top layers as compared to the bottom layers (Chatterjee and Gupta, 2010). Fipronil was recovered from contaminated water and soil, in nature habitats (Hayasaka et al., 2012). Different extraction methods can be used for the recovery of fipronil from wastewater and contaminated soils (Kurz et al., 2013). Previously, the QuEchERS

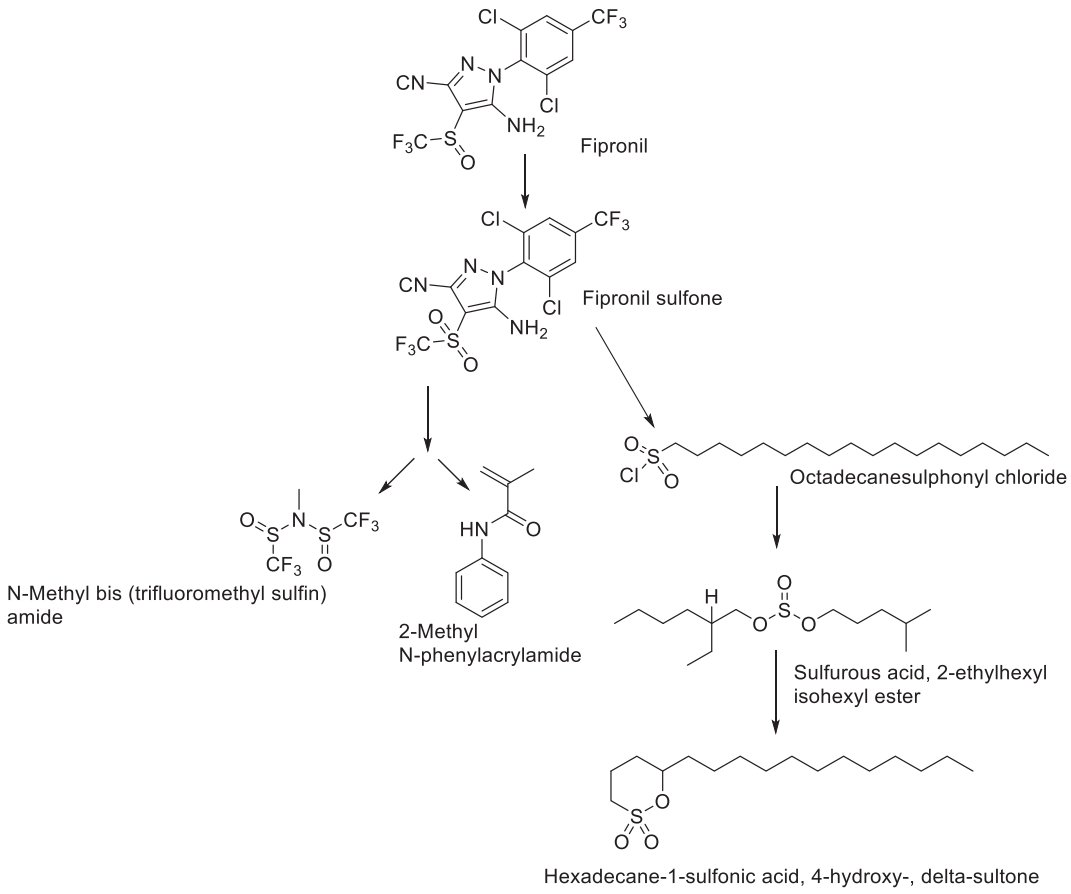


Fig. 7. Proposed metabolic degradation pathway of fipronil in strain FA4.

approach and high-performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) were used for the recovery analysis of fipronil and its metabolites in seeds, plants, and soil (Wu et al., 2017). Fipronil residue and its metabolites were recovered from cauliflower and identified using GC–MS (Duhan et al., 2015). The results of the present work confirmed the resource recovery potential of the strain FA4 from contaminated environments.

4. Conclusions

The *Bacillus* sp. strain FA4 has been investigated for its fipronil degradation potential. The strain FA4 was able to use fipronil as a carbon and energy source in MSM and soil. A significant reduction in half-life ($t_{1/2}$) of fipronil was obtained with strain FA4. Immobilization of the bacterium with agar discs and sodium alginate beads confirmed the enhancement of degradation. Furthermore, the metabolites of fipronil degradation were identified and a degradation pathway has been proposed. The strain FA4 was also used for the recovery of fipronil from the MSM and soil samples. On the basis of the performance of strain FA4, it could be used for the large-scale remediation of phenylurea herbicides from various contaminated sites.

Declaration of competing interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2021.130156.

Credit author statement

This work was mainly planned by PB, SG, GK, AS and SC and experiments were done in the lab by PB. The experimental data was also analyzed and interpreted by PB, ERR, AJK, AS and SC. PB wrote the manuscript, analyzed the data, draw the figures. ERR, AJK, AS, WZ and SC edited and revised the manuscript.

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