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ORIGINAL ARTICLE

Biomineralisation of fipronil and its major metabolite, fipronil sulfone, by *Aspergillus glaucus* strain AJAG1 with enzymes studies and bioformulation

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Abstract Fipronil is a phenylpryazole insecticide which is extensively used for the protection of agricultural yields. However, this insecticide poses various threats to the environment. Therefore it is essential to develop an effective method to degrade or eliminate this pollutant from the environment. In this present study, a fungal strain AJAG1 capable of degrading fipronil and its metabolite, fipronil sulfone, was isolated through enrichment technique. Isolated fungal strain was identified as *Aspergillus glaucus* based upon its morphological, and 18S rRNA sequence analysis. Strain AJAG1 could degrade 900 mg L^{-1} of fipronil efficiently in both aqueous medium and soil. In addition, fipronil degradation was tested with various kinetic models and the results revealed that biodegradation in aqueous medium and soil was ascertained by pseudo-first order and zero order rate kinetics, respectively. The infrared spectrum of fipronil degraded sample confirmed the formation of esters, nitro, and alkanes groups. A tentative degradation pathway of fipronil by strain AJAG1 has been proposed on the basis of gas chromatography–mass spectrometry (GC–MS) analysis. The lignolytic enzymes activities were studied during fipronil degradation by strain AJAG1. Further, scanning electron microscopy (SEM) was used to examine the surface morphology of strain AJAG1 after fipronil degradation. In the present investigation, bioformulation of strain AJAG1 was developed using low cost materials such as groundnut shell powder, molasses, and fly ash to remediate the fipronil from agricultural field. These results highlight *A. glaucus* strain AJAG1 may have potential for use in bioremediation of fipronil-contaminated environment.

Keywords *Aspergillus glaucus* strain AJAG1 · Degradation · Kinetic studies · GC–MS · Low cost powder formulation

Introduction

As per the 2014–2015 National Horticulture Broad database, India is the world's second largest producer of fruit and vegetables, after China, in large input agronomic practices. Pesticides and insecticides are repeatedly used to combat insect and significantly increase the crop yield in different agro-ecosystem. Over 98% of insecticides sprayed reach a destination other than their target pest (Miller 2004). Improper application of insecticides has hazardous effect on human health and environment. Fipronil [5-amino-3-cyano-1-(2,6-dichloro 4 trifluoromethylphenyl)-4-trifluoromethyl sulfinyl pyrazole] is a phenyl pyrazole insecticide having broad spectrum activity against insects such as rice stem borer, leaf folder, cockroaches, mosquitoes, locust, ticks and fleas at both their larval and adult stages (Chanton et al. 2001; Aajoud et al. 2003). It has been highly recommended for insects which are resistant or tolerant to conventional insecticides such as pyrethroid, cyclodiene, organophosphorous, and carbamate (Colliot et al. 1992; Bobe et al. 1997). Moreover, its application rates of 0.6–200 g a.i. ha^{-1} are lower than the most other conventional insecticides (e.g., fipronil at 0.6 g a.i. ha^{-1} is effective against grasshoppers in laboratory conditions). Fipronil elicits its toxicity in the central nervous system (CNS) by blocking the passage of chloride

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ions through the gamma-aminobutyric acid (GABA) regulated chloride channel, resulting in a disruption of neuron signaling and eventually the shutdown of CNS which leads to paralysis and death (Gant et al. 1998). There are four main degradation products of fipronil and some of the metabolites are more toxic than fipronil. Fipronil degrades to its major metabolites by reduction to sulfide, oxidation to sulfone, hydrolysis to amide and photolysis to desulfinyl. Studies on the environmental fate of fipronil indicate that its half-life in soil varies from 3 days to 7 months (Tingle et al. 2003). The persistent nature and large scale use of fipronil has resulted in serious environmental contamination problem, and poses a serious threat to the health of human beings and ecosystems. Therefore, it is necessary to investigate a suitable method for cleanup of fipronil contaminated environment. Biodegradation has been attracting much attention in cleanup of the contaminated environments because implementation of conventional physicochemical methods for disposal of persistent pollutants is low in efficiency, expensive and unfriendly to the ecosystem. Biodegradation is generally considered to be the safest, least disruptive, and cost-effective technique to remediate the environment from contaminants. Microbes have a potential to degrade the pesticides resulting in harmless or less toxic byproducts. It has been widely used in degrading recalcitrant contaminants such as, pesticide, plastics, dyes, petroleum, surface agents, etc. Both fungi and bacteria are used for degrading environmental pollutants, however, fungi are particularly more suitable because some pollutants cannot be degraded by bacteria. When compared with bacteria, fungi have an advantage in metabolic versatility and resilience. They are capable of oxidizing various amounts of chemicals and survive in harsh environmental conditions such as low moisture and high concentrations of pollutants. Filamentous fungi have an added advantage than other microbes, as they are able to translocate essential factors like nutrients and water from the intermediate environment. Any pollutants that are found in the environment are degraded and translocated within the mycelium. They release extracellular enzymes that act on a broad array of organic compounds. There are many studies on degradation of the pollutants by ligninolytic fungus. Only a few genera are capable of degrading complex lignin polymers and they provide the highest degree of mineralization (Chen et al. 2008). Among them, *Aspergillus* sp. is often reported as a tolerant organism to many pollutants and they are indicated as a potential bioremediators of various pesticides in the soil (Pinedo-Rivilla et al. 2009). However, there are no reports about fungal strains that can degrade both fipronil and its metabolite, fipronil sulfone in aqueous medium and soil. So far, bacterial strains like *Paracoccus* sp. (Kumar et al. 2012); *Bacillus thuringiensis* (Mandal et al. 2013); *Bacillus firmus* (Mandal et al. 2014); *Stenotrophomonas acidaminiphila* (Uniyal et al. 2016) have been reported to degrade fipronil. Therefore, the present study was conducted to evaluate the degradation potential of fungus on fipronil and fipronil sulfone.

In the present study, a fungal strain, which has the potential to degrade fipronil and its major metabolite, fipronil, has been isolated from an agricultural field and successful degradation was carried out. The objective of this study was to characterize the degradation potential of fungus to degrade fipronil and its major metabolite, fipronil sulfone both in aqueous medium and in soil. Furthermore, kinetics models and enzymatic activity of the isolated potent strain on fipronil degradation was investigated. The degradation pathway of fipronil by the fungus has been proposed. Degradation by the fungal strain would be incomplete if it is not applicable in a formulation mode; hence a simple and cost effective powder bioformulation of isolated potent strain was developed for field application.

Materials and methods

Chemicals and media

Commercial grade fipronil (5% SC) was used in this study which was obtained from M/s Bayer Crop Science India Ltd., Mumbai, India. The technical grade analytical standards of fipronil (purity 97.5%), fipronil sulfone (purity 99.7%) and all enzyme substrates were obtained from Sigma-Aldrich, USA. HPLC grade solvents like water, acetone, acetonitrile were procured from Merck, Darmstadt, Germany. All other chemicals for fungal medium preparation were purchased from Hi-media India Ltd.

Preparation of standard solutions

Standard stock solutions of parent compound fipronil and its main metabolite, fipronil sulfone (1 mg mL^{-1}) were prepared in acetonitrile and acetone, respectively. The working standard solution was prepared from stock solutions. All stock solutions were sterilized by membrane filtration (0.22 μm) and stored at 4 °C before use. The retention time for each of the compounds was as follows: fipronil 8.7 min and fipronil sulfone 9.2 min.

Soil sample collection

The soil sample used in this present study was obtained from *Abelmoschus esculentus* field (commonly known as lady's finger) located in Vellore, Tamilnadu, India (12.98°N, 79.13°E). The sampling site was under intensive agricultural practices for several decades and was exposed

to fipronil treatment for prolonged period of time. Sampling procedure was followed according to the standard method (Gupta 2004). Soil was collected from the surface layer (0–20 cm) and it was air-dried, ground, and passed through *a* <2-mm sieve to remove debris and large particles.

Enrichment, isolation and screening of fipronil degrading fungal strain

The enrichment culture technique was employed for the isolation of efficient fungal strain capable of degrading fipronil. 20 g of soil sample was inoculated in 100 mL of enrichment medium (g L^{-1}: $NaNO_3$ 2 g; KCl 0.5 g; $MgSO_4 \cdot 7H_2O$ 0.5 g; glucose 10 g; $FeCl_3$ 10 mg; $BaCl_2$ 0.2 g; $CaCl_2$ 0.05 g; distilled water 1000 mL at pH 6.8) spiked with 100 mg L^{-1} of fipronil as a sole carbon and energy source and was incubated at 180 rpm on a rotary shaker at 28 ± 2 °C for 7 days. After the incubation period, 5 mL of culture was transferred to 100 mL of fresh enrichment medium containing 100 mg L^{-1} of fipronil for another 7 days. Afterwards, three successive transfers were carried out in fresh enrichment medium containing fipronil as the only carbon source. Then, 100 µL of enriched sample was taken from the last transfer and it was spread on potato dextrose agar (PDA) plates containing 100 mg L^{-1} of fipronil. After 3 days of incubation at 28 ± 2 °C, fungal colonies were obtained and they were purified by streak-plate method.

Gradient plate method

Gradient plate method was performed on Czapek Dox agar to screen the isolated fungal strains for fipronil tolerance as described by Silambarasan and Abraham (2013a, b). Czapek Dox medium contained yeast extract 3 g L^{-1}; peptone 10 g L^{-1}; dextrose 2 g L^{-1}; agar 18 g L^{-1}; and distilled water 1000 mL (pH 6.8). Briefly, the fipronil gradient plate was prepared by adding a base layer of 20 mL Czapek Dox agar to a Petri plate tilted at an angle of 30° and it was allowed to solidify. Onto the solidified base, 20 mL of agar containing fipronil (1000 mg L^{-1}) was poured to give a fipronil gradient across the plate. Fungal isolates were streaked along the fipronil gradient using sterile cotton swab. Then, the inoculated plates were incubated at 28 ± 2 °C for 8 days. After 8 days, the length of fungal growth along the gradient was recorded. Strain AJAG1 showed the highest length of growth on gradient and was selected for further studies.

Minimum inhibitory concentration

Fungal isolates showing the greatest length along the gradient was selected. In order to check the minimum inhibitory concentration of fipronil, a series of 250 mL Erlenmeyer flasks containing 100 mL of M1 medium (composition per liter: $NaNO_3$ 2 g; KCl 0.5 g; $MgSO_4 \cdot 7H_2O$ 0.5 g; glucose 10 g; $FeCl_3$ 10 mg; $BaCl_2$ 0.2 g; $CaCl_2$ 0.05 g; distilled water 1000 mL at pH 6.8) amended with gradually increasing concentration of fipronil from 100 to 1000 mg L^{-1} and was inoculated with 1 mL of strain AJAG1 (10^8 spores mL^{-1}) and these flasks were incubated at 28 ± 2 °C for 8 days at 120 rpm. After 8 days of incubation the flasks were examined for mycelial growth. The flask which showed complete inhibition of fungal mycelial growth was considered as minimum inhibitory concentration of fipronil.

Identification and characterization of fipronil and its metabolite, fipronil sulfone degrading strain AJAG1

Strain AJAG1 was characterized and identified by cultural, morphological and 18S rRNA gene analysis. For cultural and morphological studies, strain AJAG1 was grown on PDA medium. Cultural characters such as color and nature of the growth of the colony were determined by visual observation. Morphological characteristics of the strain AJAG1 like mycelia, conidiophores and conidia were investigated with a light microscope (Olympus, Japan) and scanning electron microscope (Zeiss EVO 18, Germany). Analysis of 18S rRNA was performed for the taxonomic characterization of the strain AJAG1. Fungal genomic DNA was extracted by standard method described by Lee (1988) and Wu et al. (2001). The 18S rRNA gene was amplified using the highly conserved fungal rRNA gene primers ITS1: 5′-TCCGTAGGTGAACCTGCGG-3′ and ITS4: 5′-TCCTCCGCTTATTGATATGC-3′ previously described (White et al. 1990; Gardes and Bruns 1993). The conditions for thermal cycling were as follows: denaturation of the target DNA at 94 °C for 4 min and followed by 30 cycles at 94 °C for 1 min, primer annealing at 52 °C for 1 min and primer extension at 72 °C for 1 min. At the end of the cycling, the reaction mixture was held at 72 °C for 10 min and cooled at 4 °C. The purified PCR products obtained were sequenced by Sanger's dideoxynucleotide chain termination method and carried out at Xcelris Labs Ltd., Ahmedabad, India. The sequence obtained was initially run in BLAST of GenBank to find their closest homologues. The sequences with the highest 18S rRNA partial sequence similarity were selected and compared by CLUSTAL W. The method of Jukes and Cantor (1969) was used to calculate evolutionary distances; phylogenetic dendrograms were constructed by neighbor joining method, and the tree topologies were evaluated by bootstrap analysis of 1000 datasets using MEGA version 5.0 software with Kimura 2-parameter model.

Growth kinetics

Growth kinetics of the strain AJAG1 was performed to check the utilization of fipronil as sole carbon and energy source in 100 mL of modified Czapek's Dox broth. 1 mL of spore suspension (10^8 spores mL^{-1}) of the strain AJAG1 was inoculated into a series of flasks containing modified Czapek's Dox broth which was amended with 900 mg L^{-1} of fipronil. Another series of flasks was inoculated with 1 mL of spore suspension into modified Czapek's Dox broth without fipronil. All the flasks were incubated at 28 ± 2 °C on a rotary shaker at 120 rpm for 5 days. One flask from each series was removed at 24-h intervals. The mycelia mass from each flask was removed from the media by filtration using Whatman filter paper no. 1 and dry weight of fungal biomass was determined by drying for constant weight at 80 °C.

Degradation of fipronil and its metabolite, fipronil sulfone in aqueous medium by strain AJAG1

To study the degradation ability of fipronil and fipronil sulfone by strain AJAG1 in aqueous medium, 100 mL of M1 medium was placed in a 250 mL Erlenmeyer flask. M1 medium was supplemented with 900 mg L^{-1} of fipronil as sole carbon and energy source. 1 mL of strain AJAG1 (10^8 spores mL^{-1}) was inoculated in M1 medium and incubated on a rotary shaker at 120 rpm. A non-inoculated flask was maintained as control. The degradation studies were conducted in triplicate. The study period for the biodegradation of fipronil in M1 medium was 15 days. The samples were aseptically removed at intervals of 24 h for residual analyses to determine insecticide concentration by high performance liquid chromatography (HPLC) (Young Lin, Acme 9000 South Korea).

Degradation of fipronil and its metabolite, fipronil sulfone in soil by strain AJAG1

Degradation of fipronil and its metabolite, fipronil sulfone in soil by strain AJAG1 was performed according to the method described by Pino and Penuela (2011). Briefly, soil sample from which the fungus was isolated was used in this study. Before using the soil for degradation studies, it was sterilized threefold by autoclaving for 30 min at 121 °C. The following experiments were carried out to understand the effect of strain AJAG1 on fipronil and fipronil sulfone degradation. Two experiments were carried out as follows: (1) Addition of fipronil, strain AJAG1, nutrients (C: N: P, in the ratio of 100: 10: 1). The sources of C: N: P were glucose, $(NH_4)_2SO_4$ and K_2HPO_4, respectively. (2) Addition of fipronil, strain AJAG1 devoid of nutrients, which served as a control. 100 g of sterilized soil was placed in a 250 mL Erlenmeyer flask containing nutrients and then it was treated with fipronil (900 mg kg^{-1} soil). After mixing it properly, 30 mL of spore suspension (10^8 spores mL^{-1}) of strain AJAG1 was inoculated. As a soil test control, samples sterilized in an autoclave were used in order to verify that degradation which was mainly due to the action of microbes rather than abiotic factors. All the flasks were kept at room temperature and the soil samples were periodically collected for the determination of fipronil and its metabolite, fipronil sulfone residues using HPLC.

Extraction of fipronil and fipronil sulfone residues

Fipronil and its metabolite, fipronil sulfone residues from the aqueous medium and soil were extracted using the standard extraction method. Periodically recovered aqueous samples from M1 medium were centrifuged at 6000 rpm for 10 min to obtain cell-free supernatants. These cell-free supernatants were used for extraction of fipronil and its metabolite, fipronil sulfone residues. For fipronil residues extraction, 10 mL of cell-free supernatants obtained from aqueous sample were introduced into a 50 mL polypropylene centrifuge tube with 10 mL of dichloromethane (DCM). Fipronil sulfone residues in the aqueous sample were extracted by the addition of equal volume of solvent mixture, DCM and acetone (1:1, v/v) to the cell-free supernatant. Further, the centrifuge tubes were capped and shaken vigorously for 15 min. Then the tubes were set aside to allow clean separation of the phases. Organic layer was extracted from the aqueous layer and was analyzed using HPLC. For the extraction of fipronil and its metabolite, fipronil sulfone residues from soil, 50 mL of acetonitrile, was added to 10 g of soil sample which was kept in rotary shaker for 1 h at 200 rpm and was centrifuged. Finally, 1 mL of organic layer was taken in an Eppendorf and kept in a refrigerator prior to analysis.

HPLC conditions and analysis

Residues of fipronil and fipronil sulfone were analyzed on a HPLC (Young Lin, Acme 9000 South Korea) equipped with 2487 UV detector (Waters 2695, Waters Corp., USA). A reverse-phase column C_{18} (Waters, Reliant 4.6 mm × 250 mm × 5 μm) was used for chromatographic separations and was maintained at 30 °C throughout the analysis. The mobile phase consisted of 60% acetonitrile and 40% water (v/v) with 0.1% phosphoric acid at the flow rate of 1.0 mL min^{-1}. The injection volume was 10 μL and the detector wavelength was fixed at 220 nm. Before injecting the samples in HPLC, all the samples were filtered through nylon syringe filter (0.45 μm).

Analysis of fipronil degradation metabolites by gas chromatography mass spectrometry (GC–MS)

The intermediated produced during degradation of fipronil and fipronil sulfone was analyzed by GC–MS. The potential fipronil degradation pathway was also identified. A 1 µL aliquot of extracted sample was injected and analyzed on an Elite-5MS capillary GC column (length 30.0 m; ID 0.25 mm; film thickness 250 µm; Perkin). The GC condition were: split injection (injector temperature 280 °C, split 1/8 for samples and 1/20 for standard samples); oven temperature programmed from 60 °C (held for 2 min) to 300 °C (15 min) at 10 °C min^{-1}. The carrier gas used was helium at a flow rate of 1 mL min^{-1}. The ion trap was operated at 70 eV with a scan range of *m/z* from 50 to 600. MS was performed in electron ionization mode, with electron energy 70 eV, ion source temperature 230 °C. Data were acquired in full-scan (*m/z* 50–600) and scanning acquisition modes with solvent delay 4 min and analyzed based on the National Institute of Standards and Technology (NIST) library database.

FTIR analysis

FTIR spectra of analytical grade standards of fipronil and its degraded products were analyzed to determine the vibrational frequency changes in their functional groups. The spectra were recorded on FT-IR spectrometer (Avatar 300 model; Thermo Nicolet Co., USA). Infrared spectra of pure fipronil, fipronil sulfone and degraded products were determined in the range of 4000–400 cm^{-1} at a spectral resolution of 4 cm^{-1} by KBr pellet method.

Enzyme assays

The activities of fipronil degrading enzymes were checked in the strain AJAG1 at different time intervals. Strain AJAG1 was grown in the M1 medium spiked with 900 mg L^{-1} concentration of fipronil as sole source of carbon and energy. For cell-free extract, spore of strain AJAG1 was harvested and washed in 100 mM Tris–HCl (pH 7.4) and was re-suspended in the same buffer. Finally, the cells were incubated in ice, followed by sonication in three pulses of each 10 s. This cell-free extract was used for enzyme studies. The enzyme activity in culture supernatant was also estimated simultaneously. The crude extracts from fungal spores grown in M1 without fipronil was used as controls. The enzyme activities were expressed as units per milliliter, where 1 U was defined as 1 mmol of substrate oxidized per min. All enzymes assays were conducted in triplicates and were recorded on a double beam UV–VIS spectrophotometer (AU-2701, Systronics).

Laccase (Lac) activity was determined by the oxidation of 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfoniuc acid) diammonium salt (ABTS) method as described by More et al. (2011). Reaction mixture contained 0.5 mM ABTS, 2.8 mL of 0.1 M sodium acetate (pH 4.5), and 100 µL of culture supernatant and was incubated for 5 min. Absorbance was noted at 420 nm in a spectrophotometer against a suitable blank.

Manganese peroxidase (MnP) activity was measured by the oxidation of $MnSO_4$. The reaction mixture was conducted in 3 mL cuvette containing 2.5 mL of 20 mM sodium tartrate buffer (pH 4.5), 1 mL of 1.0 mM $MnSO_4$, 1 mL of enzyme extract and 0.5 mL of 2.0 mM H_2O_2. Absorbance was measured at 238 nm (Husaini et al. 2011).

Lignin peroxidase (LiP) activity was assayed using veratryl alcohol as a substrate. Reactions were carried out in 3 mL cuvette containing 1.25 mL of 50 mM sodium tartrate buffer (pH 2.5). 0.5 mL of enzyme extract, 0.25 mL of 2.0 mM veratryl alcohol and 0.5 mL of 500 µM H_2O_2. The reaction was monitored at 310 nm (Husaini et al. 2011).

Scanning electron microscopic (SEM) analysis

The fungal strain AJAG1 was subjected to SEM (Zeiss EVO 18, Germany) examination to study the surface morphology of fungal spores before and after degradation of fipronil. Strain AJAG1 treated with 900 mg L^{-1} of fipronil and, control devoid of fipronil were observed. Care was taken that the fungal spores were completely dried on the stubs prior to coating with thin film of gold in vacuum evaporator inorder to avoid charging.

Kinetics studies on fipronil and fipronil sulfone degradation

Degradation of fipronil and its metabolite, fipronil sulfone in aqueous medium and soil has been applied to various kinetics models like zero order, first order, second order and pseudo-first order, respectively, to determine the rate constant (k). The time in which the insecticide concentration in M1 medium or soil was reduced by 50% (DT_{50} values) was calculated from the linear equation obtained from the regression between $C_t - C_0$ (zero order model), $\ln (C_t/C_0)$ (first order model), $\ln C_t$ (pseudo-first-order model) $1/C$ (second order model), t/Ct (pseudo-second-order model) of the chemical data and time. Kinetic model equations were described by:

$$C_t - C_0 = kt \,(\text{zero order model}) \quad (1)$$

$$C_t/C_0 = e^{-kt} (\text{first order model}) \quad (2)$$

$$\ln C_t = -kt + \ln C_0 (\text{pseudo first order model}) \quad (3)$$

$$1/C = kt + 1/C_0 (\text{second order model}) \quad (4)$$

$$t/Ct = t/Ce + 1/kCe^2 (\text{pseudo} - \text{second order model}) \quad (5)$$

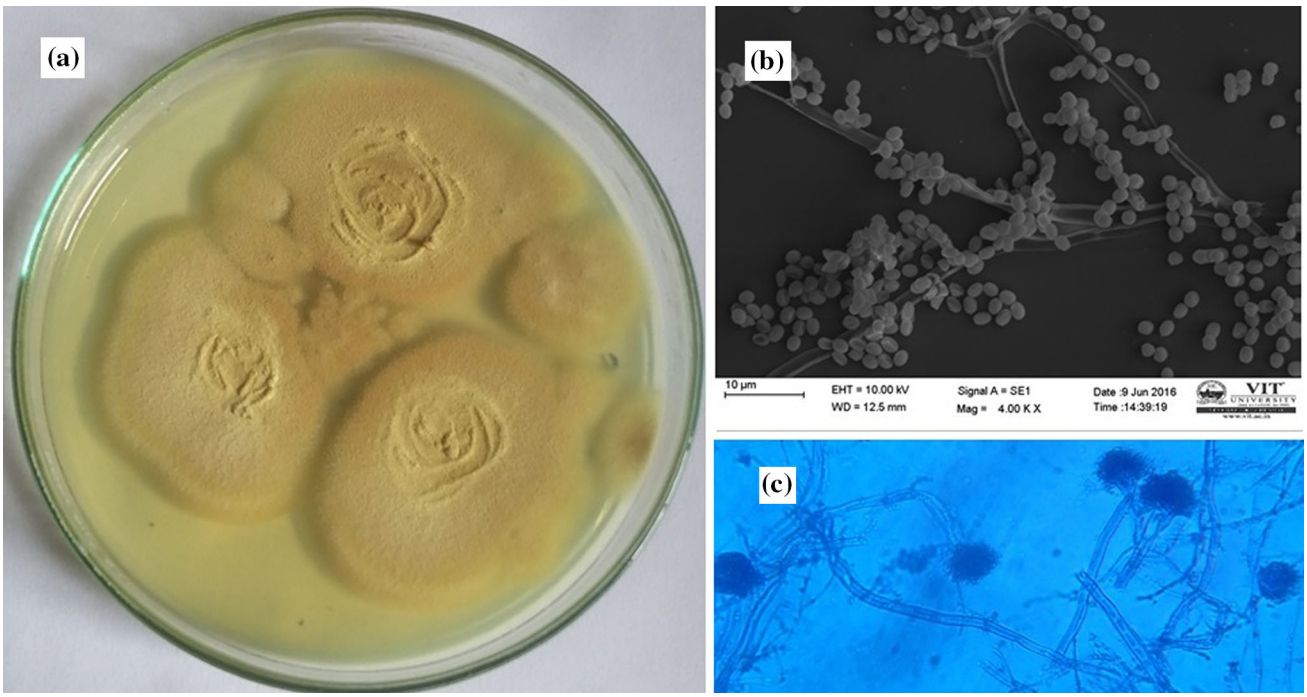

Fig. 1 Morphological characteristics of *Aspergillus glaucus* strain AJAG1 (**a**) on PDA media after 48 h, (**b**) under scanning electron microscope (4000 X) (**c**) under bright field microscope (X 100)

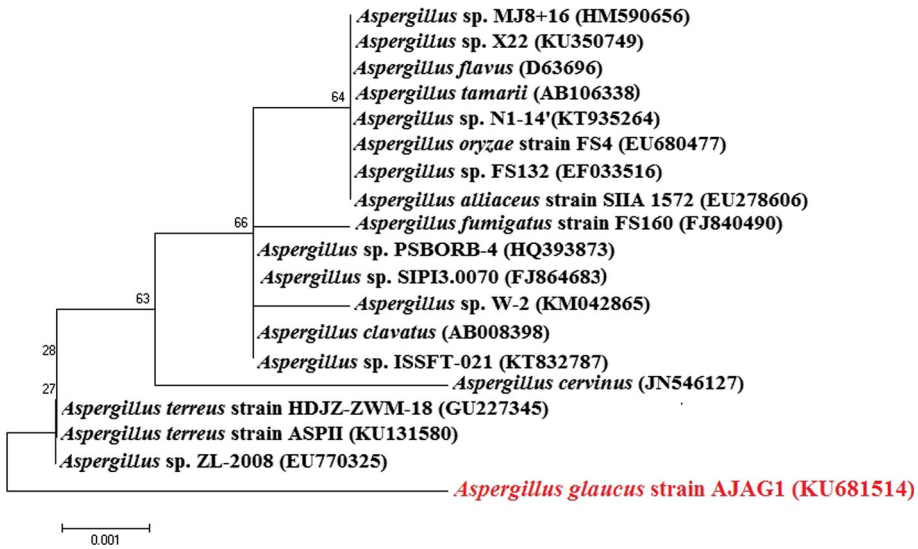

Fig. 2 Phylogenetic tree based on the 18S r RNA sequences of *Aspergillus glaucus* strain AJAG1 using MEGA version 5

whereas C_0, *Ce* are the amount of the insecticide in M1 or soil at time zero time and C_t, *C*, *Ct* are the amount of insecticide in M1 or soil at the time '*t*'. *k* and *t* are the rate constant (d^{-1}) and degradation time in days, respectively.

Preparation of low cost powder bioformulation

Low cost powder bioformulation of strain AJAG1 was prepared according to the method described by Silambarasan and Abraham (2014). In this method, fly ash was used as carrier material for immobilization of strain AJAG1. The carrier material was improved by incorporation of groundnut shell powder and then the final mixture consisted of groundnut shell powder-soil-5% molasses in a ratio of 15:5:1 whereas in the second set a mixture of groundnut shell powder-soil-nutrients (nutrients: C/N/P) in a ratio of 15:5:1. In order to avoid the bacterial contamination, 75 mg nalidixic acid kg^{-1} material was added to the formulation bag. The mixture was filled in a clear, transparent sterile autoclave bags and autoclaved at 121 °C for 20 min and further one part of stock culture was added followed by shaking for uniform distribution. To test the shelf life of the formulation with regard to CFU load and contamination by other microbes, the packets were sealed and stored at room

temperature for 13 weeks. After incubation period, CFU load was determined using dilution plate method.

Results and discussion

Isolation and screening of fipronil degrading fungal strain

The enrichment technique resulted in four morphologically different strains of fungus and it was designated as FA1, FA2, FA3 and AJAG1. Their degradation potential upon fipronil was confirmed by using streak plate method and pure culture was obtained by repeated streak plate technique. Gradient plate technique was performed to screen the potential strain for degradation among FA1, FA2, FA3 and AJAG1. The results showed that strain AJAG1 possessed the good degradation capacity. In addition, strain AJAG1 was also metabolize fipronil and use it as carbon and energy source when grown in M1 medium. Therefore, further studies were carried out with strain AJAG1.

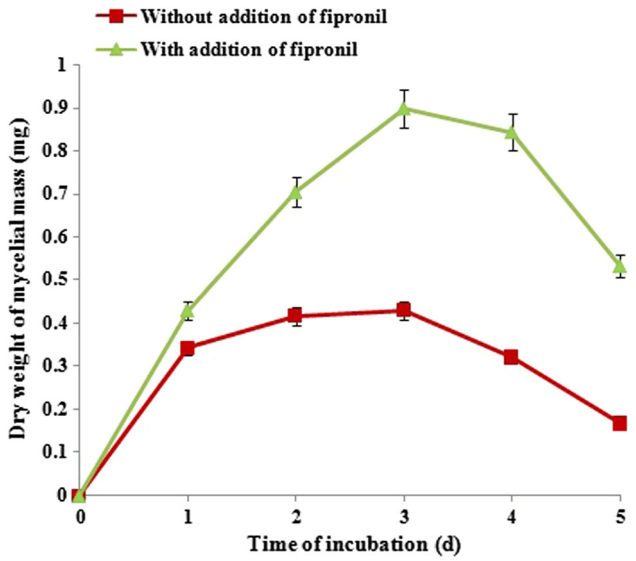

Fig. 3 Growth pattern of *Aspergillus glaucus* strain AJAG1 in the presence and absence of fipronil (900 mg L^{-1}). Each value is the mean of three replicates, with error bars representing the standard deviation of the mean

Identification and characterization of strain AJAG1

Strain AJAG1 was identified based on its morphological, cultural characteristics and 18S rRNA analysis. Fungal strain AJAG1 colony characteristics were observed on PDA medium. Slow growth of broad and flat colonies was noticed and the color of the colonies was turquoise to deep green with yellow central portions, while reverse plate showed yellow in color (Fig. 1). Most of the morphological characters of the strain AJAG1 coincided with the known features of *Aspergillus glaucus*. Molecular identification of strain AJAG1 was done by 18S rRNA analysis. 18S rRNA gene sequence of fipronil degrading strain AJAG1 obtained in this study was submitted to GenBank database (http://www.ncbi.nlm.nih.gov) under the accession number KU681514. The blast analysis result closely exhibited with *A. glaucus*. Therefore, the strain AJAG1 was identified as *A. glaucus* and it was designated as *A. glaucus* strain AJAG1. In the phylogenetic tree based on the neighbour joining method, the strain AJAG1 fell within the cluster comprising *A. glaucus* (Fig. 2).

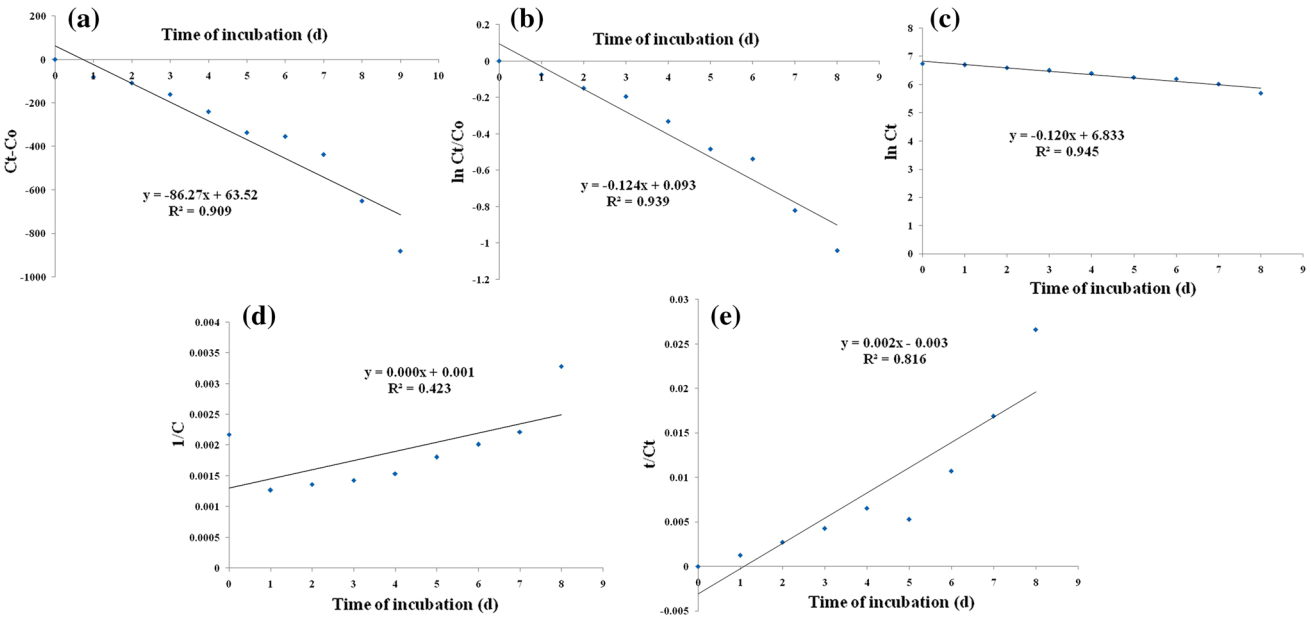

Fig. 4 Degradation kinetics of (**a**) zero order (**b**) first order (**c**) pseudo-first (**d**) second order and (**e**) pseudo-second order for fipronil by strain AJAG1 in M1 medium

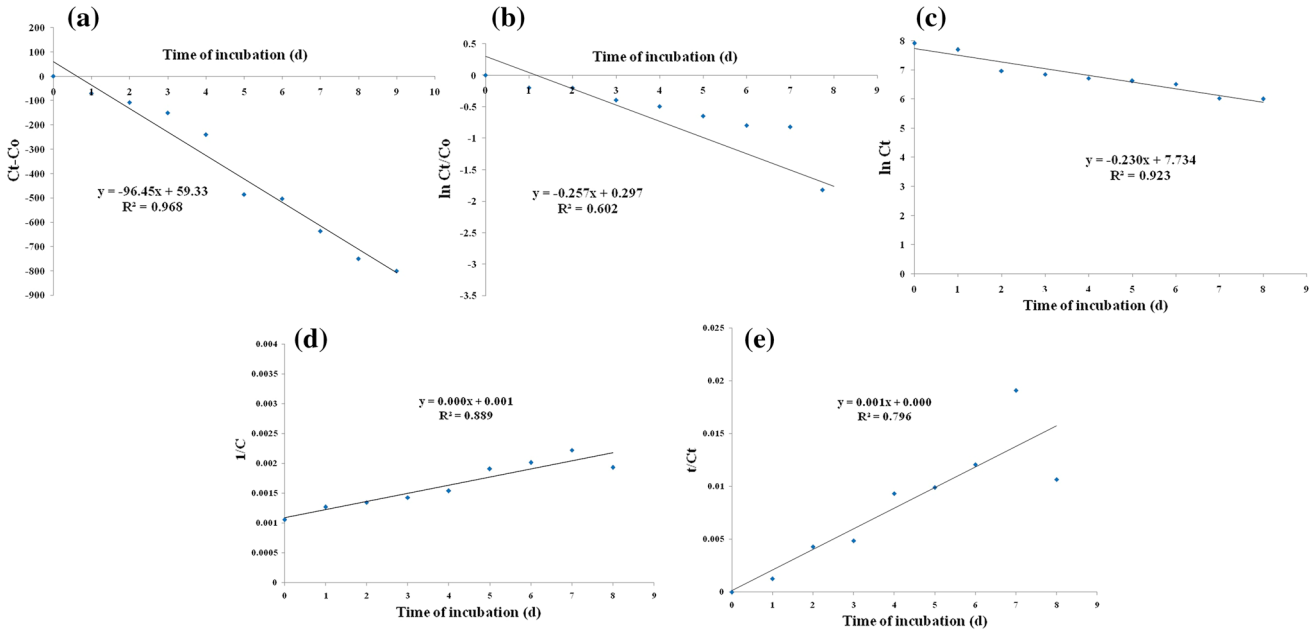

Fig. 5 Degradation kinetics of (**a**) zero order (**b**) first order (**c**) pseudo-first (**d**) second order and (**e**) pseudo-second order for fipronil by strain AJAG1 in soil

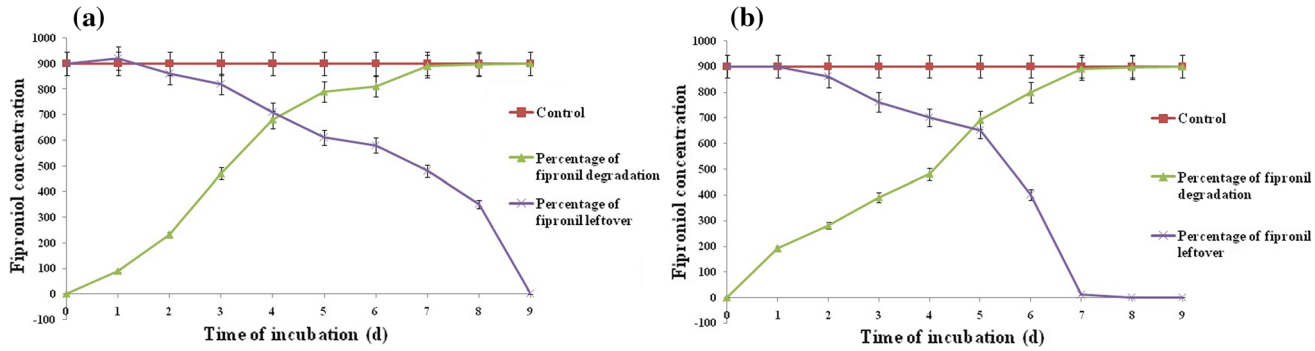

Fig. 6 Percentage calculation of fipronil by strain AJAG1 (**a**) M1 medium (**b**) soil

Minimum inhibitory concentration and growth of strain AJAG1

The Minimum inhibitory concentration was noted as the concentration of fipronil resulting in complete inhibition of microbial growth. Minimum inhibitory concentration results revealed that the strain AJAG1 could tolerate up to 1100 mg L^{-1} and showed efficient growth rate at 900 mg L^{-1} of fipronil in M1 medium. The strain AJAG1 could tolerate 1100 mg L^{-1} of fipronil. Therefore strain AJAG1 was further processed for the degradation of fipronil at 900 mg L^{-1}. Figure 3 depicts the growth of strain AJAG1 in the presence and absence of fipronil. The metabolism of fipronil by strain AJAG1 was indicated by a visible increase in mycelia mass with time. The growth of strain AJAG1 was effective in Czapek Dox broth supplemented with fipronil. Initially, the growth was found to be suppressed in the presence of fipronil, but after acclimatization to fipronil the culture could grow rapidly and exhibited high growth rate. In later stages, the amount of biomass produced in the medium containing fipronil was much higher when compared to the growth in absence of fipronil. The results clearly confirmed that the strain AJAG1 acclimatized in the medium and utilized fipronil as sole source of carbon and energy. Similar results were observed by earlier researchers (Jadhav and David 2016; Gajendiran and Abraham 2015; Siripattanakul-Ratpukdi et al. 2015).

Biodegradation of fipronil and fipronil sulfone

Very few bacterial strains which degrade fipronil have been isolated and characterized (Kumar et al. 2012; Mandal et al. 2013, 2014; Uniyal et al. 2013, 2016). In the present

investigation, degradation of fipronil and its metabolite, fipronil sulfone by *A. glaucus* strain AJAG1 was achieved both in aqueous medium and in soil. Due to the growth of strain AJAG1 and the degradation of fipronil, a slight decrease of pH in the M1 medium was observed. For detailed study on fipronil degradation, the experimental data were compared to reveal the impact of strain AJAG1 on fipronil degradation in aqueous medium and soil. For modeling the degradation kinetics of fipronil and to estimate the degradation rate constant, well-known kinetics models, viz., zero order, first order, pseudo-first order, second order and pseudo-second order have been used. The degradation dynamics of fipronil from M1 medium exhibited the best fit with the pseudo-first-order model which has shown the highest regression coefficient (R^2) (Fig. 4). The degradation process of fipronil in M1 medium was characterized by a rate constant of 0.120 d^{-1} and DT_{50} was 5.8 days. Fipronil degradation by strain AJAG1 in soil with nutrients showed a very good compliance with degradation kinetics of the zero order model (Fig. 5). This could be due to the R^2 values which were higher than the other kinetic models. The degradation process of fipronil by strain AJAG1 in soil with nutrients was characterized by a rate constant of 96.45 d^{-1} and $T_{1/2}$ was 4.15 days. The percentage rate of fipronil residue depleted by strain AJAG1 over incubation period and the rate of percentage of fipronil residue leftover were calculated (Fig. 6). This observation is extremely important from the perspective of environmental pollution control. The degradation rate constant (k) and half-lives ($T_{1/2}$), and kinetics equations for degradation of pesticides in aqueous medium and soil with addition of nutrients, respectively, are described in Table 1. In the present study, strain AJAG1 was found to degrade not only fipronil but also its main metabolite, fipronil sulfone as well. Uniyal et al. (2016) studied the kinetics of fipronil degradation in soil by *Acinetobacter* sp. and

Table 1 Kinetic parameters for the degradation of fipronil by *Aspergillus glaucus* strain AJAG1

Kinetic model	Parameters	Treatments	
		M1 + fipronil + strain AJAG1	Soil + N + fipronil + strain AJAG1
Zero order	k (d^{-1})	86.27	96.45
	$T_{1/2}$	5.25	4.15
	R^2	0.909	0.968
First order	k (d^{-1})	0.124	0.257
	DT_{50}	5.82	3.828
	R^2	0.939	0.602
Pseudo-first order	k (d^{-1})	0.120	0.230
	DT_{50}	5.82	3.70
	R^2	0.945	0.923
Second order	k (d^{-1})	0.000	0.000
	DT_{50}	0.000	0.000
	R^2	0.423	0.889
Pseudo-second order	k (d^{-1})	0.002	0.001
	DT_{50}	231.1	346.5
	R^2	0.816	0.796

N nutrients such as glucose, $(NH4)_2SO_4$ and K_2HPO_4

Table 2 Enzyme activity (U/mL) in the culture supernatant and cell free extracts of *Aspergillus glaucus* strain AJAG1

Enzyme activity	Day 2		Day 4		Day 6		Day 8		Day 10	
	A	B	A	B	A	B	A	B	A	B
Laccase	–	–	–	–	–	–	–	–	–	–
Lignin peroxidase	2.42	1.059	3.45	1.95	0.84	0.24	0.58	0.04	0.06	0.01
Manganese peroxidase	3.76	1.25	2.93	1.07	1.50	0.79	0.74	0.30	0.04	0.007

A enzyme activity in the culture supernatant, *B* enzyme activity in cell lysate

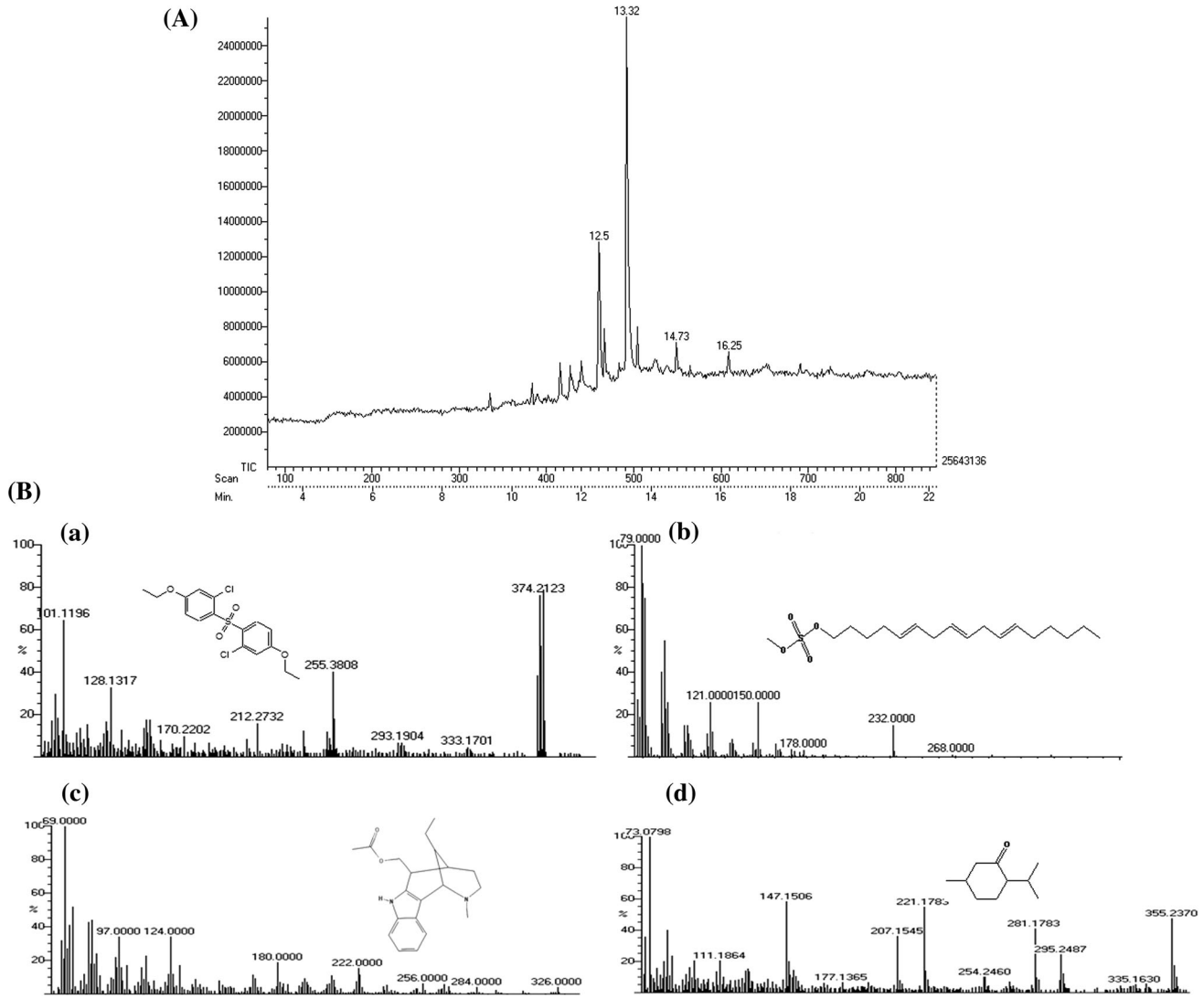

Fig. 7 (**A**) GC-MS chromatogram of the metabolites produced during fipronil degradation by strain AJAG1 (**B**) GC-MS identification of the four main metabolites produced during fipronil degradation by strain AJAG: (**a**) Bis[2-chloro-4-ethoxyphenyl]sulfone; (**b**) sulfuric acid, 5,8,11-heptadecatrienyl methyl ester; (**c**) D asycarpidan-1-methanol, acetate (ester); and (**d**) Isomenthone

reported that degradation of fipronil followed pseudo-first-order kinetics with the rate constant value between 0.041 and 0.051 d $^{-1}$. Similarly, the residues of fipronil were found to persist only up to 10 days in soils fortified with fipronil at the rate of 20 µg kg^{-1} and amended with *Paracoccus* sp., while in the soils fortified at the rate of 80 µg kg^{-1} fipronil, residues persisted up to 20, 30 and 30 days in loamy sand, sandy loam, and clay loam, respectively (Kumar et al. 2012). Masutti and Mermut (2007) suggested that biodegradation seems to be dependent on the bioavailability of the fipronil. Uniyal et al. (2016) studied the characterization of *Stenotrophomonas acidaminiphila* in fipronil degradation. The bacterial strain was able to metabolize 25 mg L^{-1} of fipronil in Dorn's media under optimum condition. Tingle et al. (2003) reported that fipronil degrades slowly in soil and water, with a half-life ranging between 36 h and 7.3 months depending on substrate and conditions. Result of the present study revealed that the half-life of fipronil degraded by strain AJAG1 was rapid both in M1 medium and in soil when compared to the earlier findings. It was further observed that no residues of fipronil or its metabolite, fipronil sulfone were detected in the samples collected after 9 days from both in M1 medium as well in soil. Different species of *Aspergillus* have been reported to degrade many pollutants (Silambarasan and Abraham 2013a, b; Gajendiran and Abraham 2015; Gajendiran et al. 2016). As we clearly discussed above, *A. glaucus* strain AJAG1 has many advantages on fipronil degradation but it also has some disadvantage such as environmental conditions.

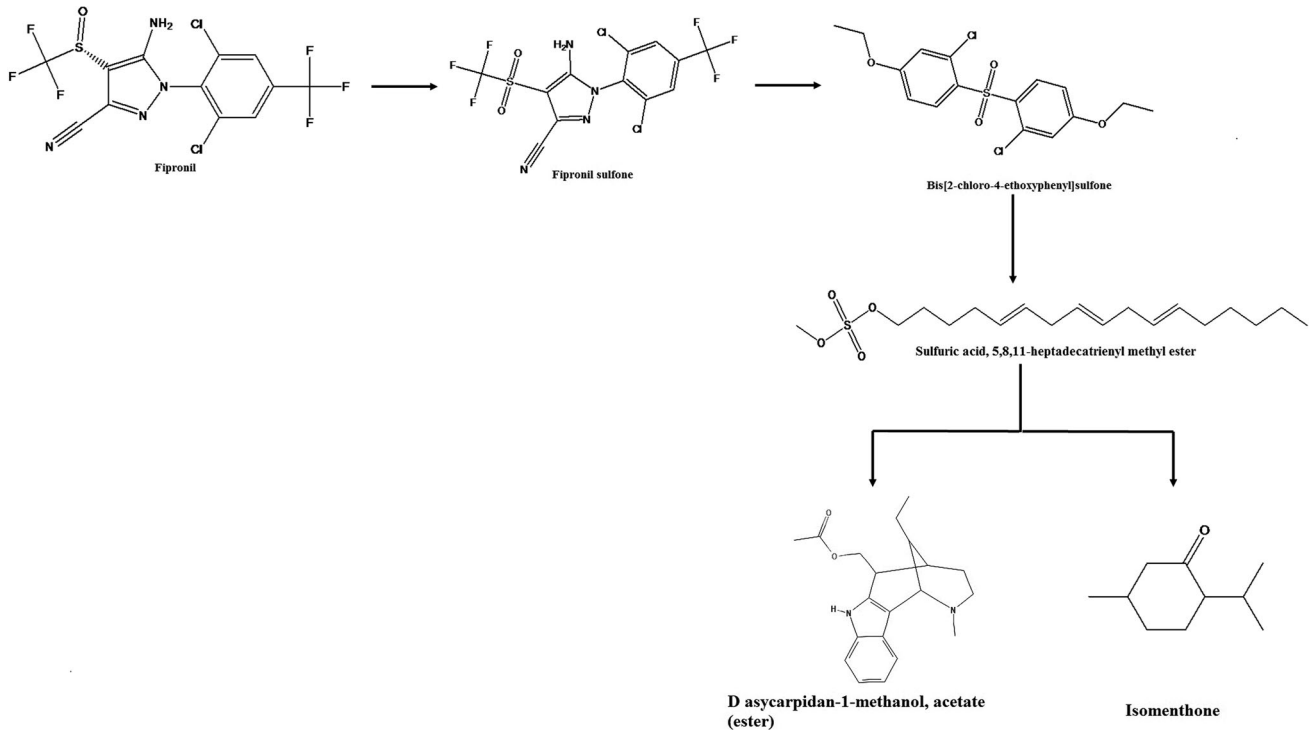

Fig. 8 The proposed pathway for fipronil degradation by *Aspergillus glaucus* strain AJAG1

Though, strain AJAG1 has extracellular enzymes activities on fipronil degradation. Environmental factors like pH, temperature, soil salinity, soil organic matters and, nutrients play a vital role in biodegradation process. For future study, the influence of environmental conditions on fipronil degradation by strain AJAG1 should be performed.

Enzymatic activity

The extracellular multienzyme complexes of fungi are involved in the breakdown of pollutants by their hyphal system. Furthermore, hyphal system is able to colonize and penetrate the substrates rapidly and transport the nutrients within their mycelium (Matavulji and Molitoris 2009). Ligninolytic enzymes (Lac, MnP, and LiP) are successful at breaking down the complex structures of many pesticides and has been reported and used in bioremediation (Castillo et al. 2008, Pizzul et al. 2009; Pant and Adholeya 2009). Gondim-Tomaz et al. (2005) investigated the degradation rate of herbicides, diuron and py rithiobacsodium by 30 fungal strains. Their results revealed that the highest degradation rate was by ligninolytic enzymes. Similarly, Jauregui et al. (2003) reported that more than 15 fungal strains were capable of degrading different organophosphate pesticides up to 96% by enzyme catalyzed pathways. Therefore in this present study, Lac, MnP, and LiP activities were studied to understand the role of strain AJAG1 in fipronil degradation. This is the first report on the involvement of these three enzymes during fipronil degradation in fungal species. In this study, fipronil biodegradation by strain AJAG1 did not show any laccase activity. However, strain AJAG1 showed activities on both MnP, and LiP. Table 2 represents the enzyme activities in the culture supernatant and cell lysate during fipronil degradation by strain AJAG1. On the initial phase of fipronil degradation, MnP, and LiP enzymes activities was at maximum in culture supernatant as seen in Table 2 which gradually decreased with increasing incubation periods. The higher MnP enzymes activity was detected when compared to LiP enzyme activity suggesting that MnP might be predominating enzyme during fipronil degradation by strain AJAG1. The enzymatic activity was more in culture supernatant than in the cell lysate which implicates the extra cellular nature of these enzymes. The low levels of LiP enzyme activity detected compared to MnP activity showed that LiP might be less significance in fipronil biodegradation by *A. glaucus* strain AJAG1.

Biodegradation pathway for fipronil by strain AJAG1

GC–MS analysis of the metabolites produced during fipronil degradation by strain AJAG1 revealed four peaks with retention time of 12.5, 13.32, 14.73, 16.25 min (Fig. 7a). The metabolites were identified as Bis[2-Chloro-4-ethoxyphenyl]sulfone, sulfuric acid-5,8,11-heptadecatrienyl

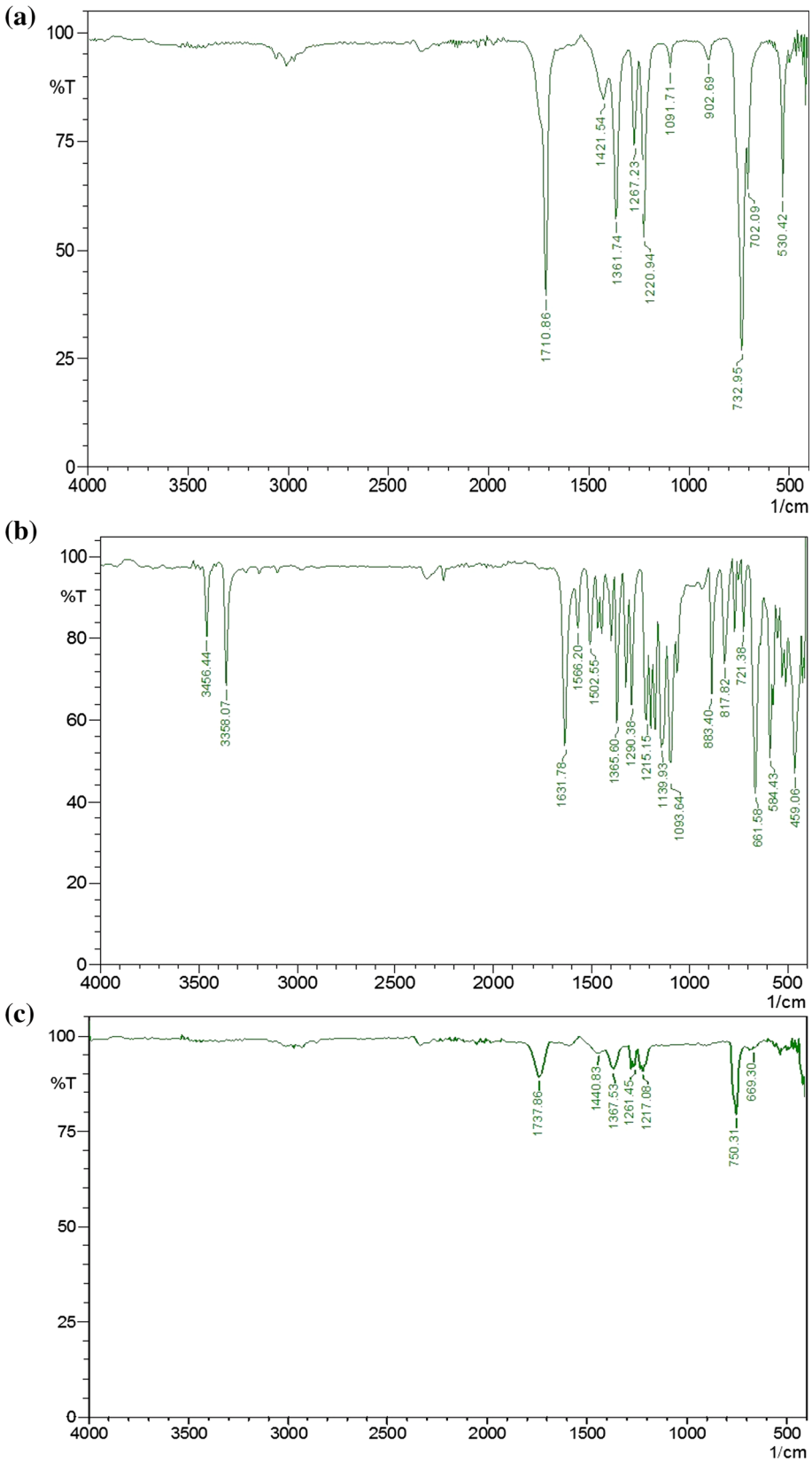

Fig. 9 FTIR spectrum (**a**) standard condition of fipronil (**b**) standard condition of fipronil sulfone (**c**) FTIR spectrum for the degraded sample by strain AJAG1

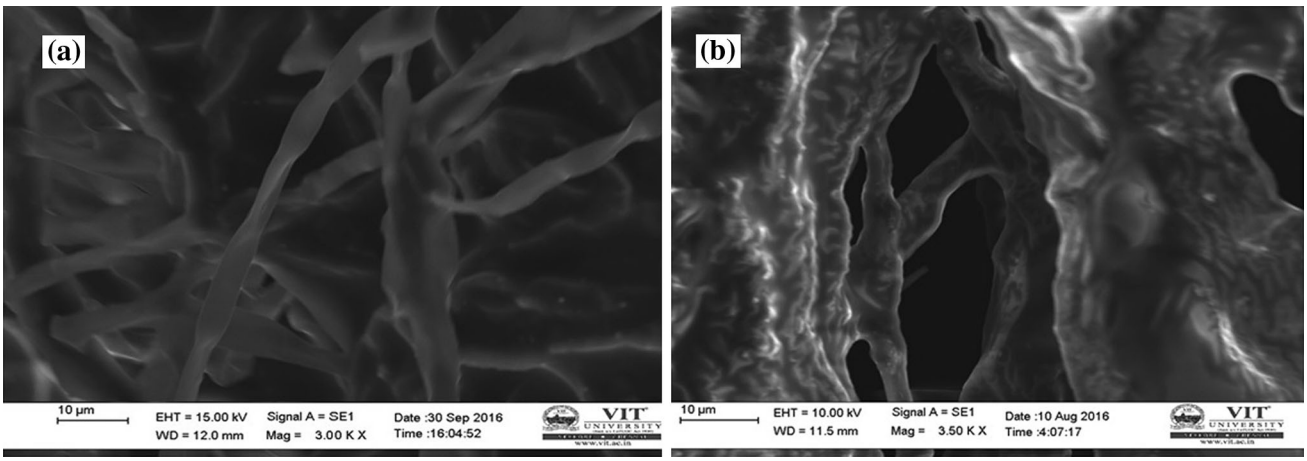

Fig. 10 SEM micrographs of *Aspergillus glaucus* strain AJAG1 (**a**) before degradation (**b**) after degradation of 900 mg L^{-1} fipronil by strain AJAG1

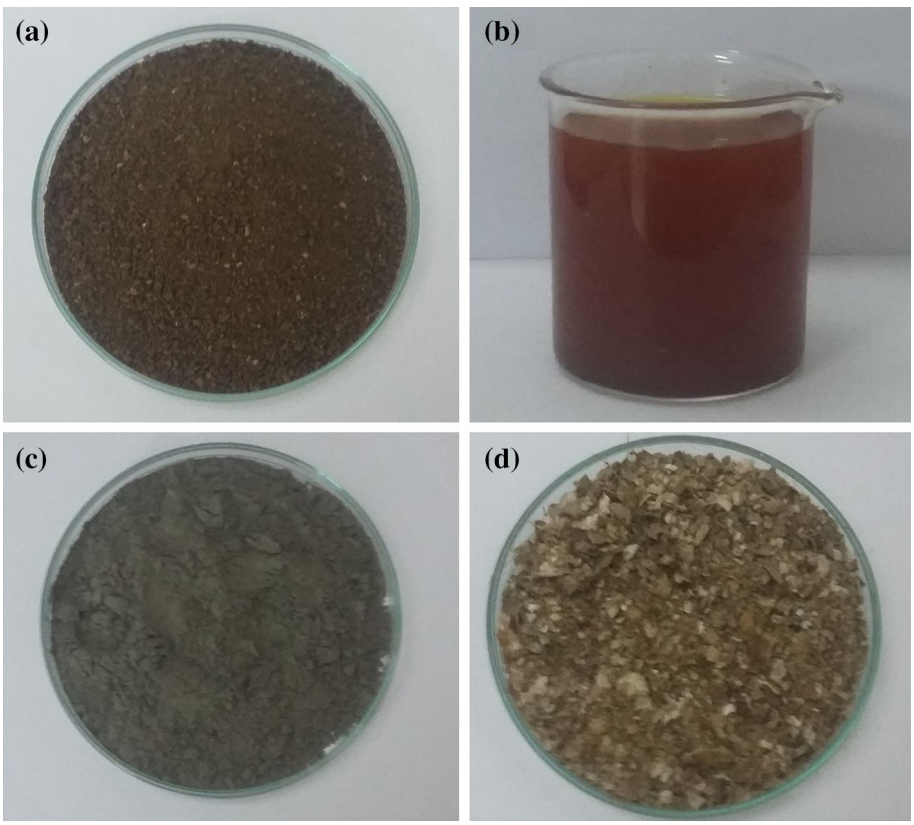

Fig. 11 Components used in formulation of *Aspergillus glaucus* strain AJAG1 (**a**) soil (**b**) molasses (**c**) fly ash (**d**) groundnut shell powder

methyl ester, D-asycarpidan-1-methanol, and isomenthone, respectively, using NIST library identification program (Fig. 7b). The possible fipronil biodegradation pathway adopted by strain AJAG1 was proposed as shown in Fig. 8.

FTIR analysis

FTIR spectra of degraded sample showed the various structural changes when compared to standard spectra of fipronil and its metabolite, fipronil sulfone, (Fig. 9). Spectra of degraded sample showed significant change in positions of peaks when compared to standard fipronil spectrum. The formation of acid group in the final degraded samples confirms the degradation of pesticides. This result was well in accordance with the observation of Guerin (2005). The infrared spectrum of fipronil degraded sample showed a band at 1737.86 cm^{-1} which corresponds to C=O asymmetric stretch represents for esters group. A band presented at 1440.83 cm^{-1} corresponds to H–C–H

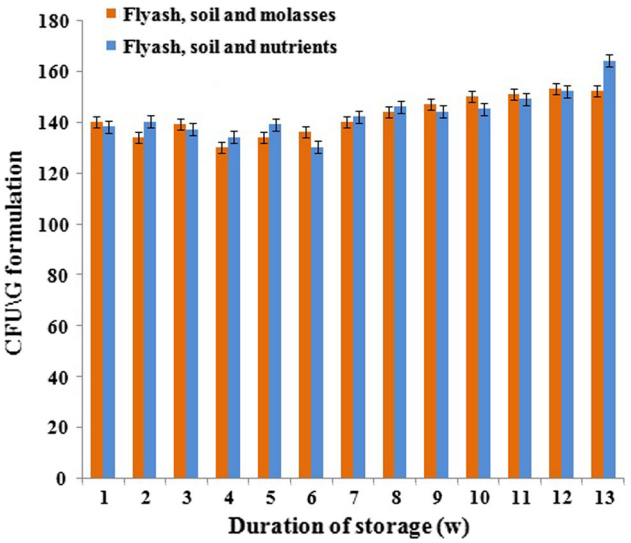

Fig. 12 Shelf life of *Aspergillus glaucus* strain AJAG1 showing CFU load per gram of formulations stored at room temperature. Each value is the mean of three replicates, with error bars representing the standard deviation of the mean

stretch and represents the alkanes group. The peak position at 1367.53 cm^{-1} corresponds to N=O stretch and represents the nitro group. Bands presented at 1261.45 and 1217.08 cm^{-1} correspond to C–O stretch represent the ester group. Comparison of FTIR spectrum of control with extracted metabolites after complete degradation clearly indicated the biodegradation of fipronil and its metabolite.

SEM analysis

Surface morphology of the strain AJAG1 incubated without fipronil was treated as positive control. The morphology is conspicuously different from that of the insecticide exposed fungal strain. The smooth surface of mycelia was found to be rough and irregular after the degradation process could be clearly seen in the Fig. 10

Formulation of *Aspergillus glaucus* strain AJAG1

Figure 11 depicts the low-cost solid materials used in the formulation of *A. glaucus* strain AJAG1. Bioformulation shelf life and the microbial contamination rate were investigated for 13 weeks at room temperature. The results revealed that the strain AJAG1 remained viable during the study period and also the CFU load g^{-1} in formulation was increased (Fig. 12). Contamination rate by other microbes was below the detection limit. Abraham and Silambarasan (2016) developed the powder bioformulation for degrading chlorpyrifos presented in agricultural field. The results revealed that bioformulation of *A. glaucus* strain AJAG1 can be used in the agricultural field and also it is eco-friendly.

Conclusion

To the best of our knowledge, this is the first report on the detailed degradation of fipronil and its metabolite, fipronil sulfone both in aqueous medium and in soil by *A. glaucus* strain AJAG1. Kinetic studies reveal a very good compliance with the pseudo-first-order model and zero order model in M1 medium and soil, respectively. Enzyme studies revealed the involvement of Laccase (Lac), Mn peroxidase and lignin peroxidase activity during fipronil degradation. Moreover, low-cost powder bioformulation was developed which is less expensive as they are developed from agricultural waste. The results of our experiments confirm that strain AJAG1 qualifies as a potential candidate for biodegradation of fipronil, which can be used for environmental cleanup. In addition, further research is needed to investigate the transformation product of fipronil degraded by *A. glaucus* strain AJAG1.

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Compliance with ethical standards

Conflict of interest The authors declare that there is no conflict of interest regarding the publication of this article.

References

Aajoud A, Ravanel P, Tissut M (2003) Fipronil metabolism and dissipation in a simplified aquatic ecosystem. J Agric Food Chem 51(5):1347–1352

Abraham J, Silambarasan S (2016) Biodegradation of chlorpyrifos and its hydrolysis product 3, 5, 6-trichloro-2-pyridinol using a novel bacterium *Ochrobactrum* sp. JAS2: a proposal of its metabolic pathway. Pest Biochem Physiol 126:13–21

Bobe A, Coste CM, Cooper JF (1997) Factors influencing the adsorption of fipronil on soils. J Agric Food Chem 45:4861–4865

Castillo MDP, Torstensson L, Stenström J (2008) Biobeds for environmental protection from pesticide use-a review. J Agric Food Chem 56:6206–6219

Chanton PF, Ravanel P, Tissut M, Meyran JC (2001) Toxicity and bioaccumulation of fipronil in the non target arthropodan fauna associated with subalpine mosquito breeding. Ecotoxicol Environ Saf 52:8–12

Chen F, Xie G, Yu H, Peng D, Yu M, Huang H, Zeng G (2008) Development of lignin-biodegrading inoculant for composting. Shengtai Yu Nongcun Huanjing Xuebao 24(2):84–87

Colliot F, Kukorowski KA, Hawkins DW, Roberts DA (1992) Fipronil: a new soil and foliar broad spectrum insecticide. In: Brighton crop protection conference: pests and diseases, British Crop Protection Council, Bear Farm, UK

Gajendiran A, Abraham J (2015) Degradation of monocrotophos by *Aspergillus oryzae* SJA1 isolated from oryza sativa field soil. J Pure Appl Microbiol 9:2303–2312

Gajendiran A, Krishnamoorthy S, Abraham J (2016) Microbial degradation of low-density polyethylene (LDPE) by *Aspergillus clavatus* strain JASK1 isolated from landfill soil. 3 Biotech 6(1):1–6

Gant DB, Chalmers AE, Wolff MA, Hoffman HB, Bushey DF (1998) Fipronil: action at the GABA receptor. Rev Toxicol 2:147–156

Gardes M, Bruns TD (1993) ITS primers with enhanced specificity for basidiomycetes-application to the identification of mycorrhizae and rusts. Mol Ecol 2(2):113–118

Gondim-Tomaz MA, Franco TT, Durrant LR (2005) Biodegradation of diuron and pyruthiobac-sodium by white-rot and soil fungi. Contam Soil Sediment Water 9:21–32

Guerin TF (2005) Natural attenuation of metabolites of a chlorinated pesticide in soil. Int J Environ Stud 62:235–248

Gupta PK (2004) Methods in environmental analysis-water, soil, and air. Agrobios, Jodhpur, India. ISBN:978-81-7754-308-7

Husaini A, Fisol FA, Yun LC, Hussain MH, Musid S, Roslan HA (2011) Lignocellulolytic enzymes produced by tropical white rot fungi during biopulping of *Acacia mangium* wood chips. J Biochem Tech 3(2):245–250

Jadhav SS, David M (2016) Biodegradation of flubendiamide by a newly isolated *Chryseobacterium* sp. strain SSJ1. 3 Biotech 6(1):1–6

Jauregui J, Valderrama B, Albores A, Vazquez-Duhalt R (2003) Microsomal transformation of organophosphorus pesticides by white rot fungi. Biodegradation 14(6):397–406

Jukes TH, Cantor CR (1969) Evolution of protein molecules. Mamm Protein Metab 3(21):132

Kumar R, Singh B, Gupta VK (2012) Biodegradation of fipronil by *Paracoccus* sp. in different types of soil. Bull Environ Contam Toxicol 88:781–787

Lee SM (1988) A rapid high yield mini-prep method for isolation of total genomic DNA from fungi. Fungal Genet Newsl 35:23–24

Mandal K, Singh B, Jariyal M, Gupta VK (2013) Microbial degradation of fipronil by *Bacillus thuringiensis*. Ecotoxicol Environ Saf 93:87–92

Mandal K, Singh B, Jariyal M, Gupta VK (2014) Bioremediation of fipronil by a *Bacillus firmus* isolate from soil. Chemosphere 101:55–60

Masutti CS, Mermut AR (2007) Degradation of fipronil under laboratory conditions in a tropical soil from Sirinhaém Pernambuco, Brazil. J Environ Sci Health B 42(1):33–43

Matavulj M, Molitoris HP (2009) Marine fungi: degraders of poly-3-hydroxyalkanoate based plastic materials. Zb Mat Srp Prir Nauk 116:253–265

Miller GT (2004) Sustaining the earth: an integrated approach. Thomson/Brooks/Cole, Salt Lake City, pp 211–216

More SS, Renuka PS, Pruthvi K, Swetha M, Malini S, Veena SM (2011) Isolation, purification, and characterization of fungal laccase from *Pleurotus* sp. Enzyme Res. doi:10.4061/2011/248735

Pant D, Adholeya A (2009) Concentration of fungal ligninolytic enzymes produced during solid-state fermentation by ultra filtration. World J Microbiol Biotechnol 25:1793–1800

Pinedo-Rivilla C, Aleu J, Collado IG (2009) Pollutants biodegradation by fungi. Curr Org Chem 12:1194–1214

Pino N, Peñuela G (2011) Simultaneous degradation of the pesticides methyl parathion and chlorpyrifos by an isolated bacterial consortium from a contaminated site. Int Biodeterior Biodegrad 65(6):827–831

Pizzul L, Castillo MDP, Stenström J (2009) Degradation of glyphosate and other pesticides by ligninolytic enzymes. Biodegredation 20:751–759

Silambarasan S, Abraham J (2013a) Ecofriendly method for bioremediation of chlorpyrifos from agricultural soil by novel fungus *Aspergillus terreus* JAS1. Water Air Soil Pollut 224(1):1369

Silambarasan S, Abraham J (2013b) Mycoremediation of endosulfan and its metabolites in aqueous medium and soil by *Botryosphaeria laricina* JAS6 and *Aspergillus tamarii* JAS9. PLoS One 8(10):e77170

Silambarasan S, Abraham J (2014) Halophilic bacterium JAS4 in biomineralisation of endosulfan and its metabolites isolated from *Gossypium herbaceum* rhizosphere soil. J Taiwan Inst Chem Eng 45(4):1748–1756

Siripattanakul-Ratpukdi S, Vangnai AS, Sangthean P, Singkibut S (2015) Profenofos insecticide degradation by novel microbial consortium and isolates enriched from contaminated chili farm soil. Environ Sci Pollut Res 22:320–328

Tingle CC, Rother JA, Dewhust CF, Lauer S, King WJ (2003) Fipronil: environmental fate, ecotoxicology, and human health concerns. Rev Environ Contam Toxicol 176:1–66

Uniyal S, Rawat M, Rai JPN (2013) Cadmium biosorption by *Stenotrophomonas humi* and *Micrococcus luteus*: kinetics, equilibrium and thermodynamic studies. Desalin Water Treat 51:1–13

Uniyal S, Paliwal R, Verma M, Sharma RK, Rai JPN (2016) Isolation and characterization of fipronil degrading *Acinetobacter calcoaceticus* and *Acinetobacter oleivorans* from rhizospheric zone of Zea mays. Bull Environ Contam Toxicol 96(6):833–838

White TJ, Bruns TD, Lee SB, Taylor JW (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. PCR protocols : a guide to methods and applications. Academic Press, San Diego, pp 315–322

Wu ZH, Wang TH, Huang W, Qu YB (2001) A simplified method for chromosome DNA preparation from filamentous fungi. Mycosystema 20:575–577