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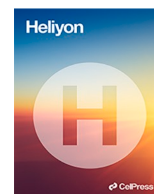

Research article

Biodegradation of monocrotophos, cypermethrin & fipronil by *Proteus myxofaciens* VITVJ1: A plant - microbe based remediation

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A B S T R A C T

Current study was focused on the degradation of pesticides such as Monocrotophos, Cypermethrin & Fipronil (M, C & F) using phyto and rhizoremediation strategies. The isolate *Proteus myxofaciens* (VITVJ1) obtained from agricultural soil was capable of degrading M, C & F. The bacteria exhibited resistance to all the pesticides (M, C & F) up to 1500 ppm and was also capable of forming biofilms. The degraded products identified using Gas Chromatography-Mass Spectroscopy (GC-MS) and FTIR was further used for deriving the degradation pathway. The end product of M, C & F was acetic acid and 3-phenoxy benzoic acid which was confirmed by the presence of functional groups such as C=O and OH. Seed germination assay revealed the non-toxic nature of the degraded products with increased germination index in the treatments augmented with degraded products. The candidate genes such as *opdA* gene, *Est* gene and *MnP1*gene was amplified with the amplicon size of 700bp, 1200bp and 500bp respectively. *P. myxofaciens* not only degraded M, C & F, but was also found to be a plant growth promoting rhizobacteria. Since, it was capable of producing Indole Acetic acid (IAA), siderophore and was able to solubilize insoluble phosphate. Therefore, VITVJ1 upon augmentation to the rhizoremediation setup aided the degradation of pesticides with increase in plant growth as compared to that of the phytoremediation setup. To our knowledge this is the first study where *P. myxofaciens* has been effectively used for the degradation of three different classes of pesticides, which could also enhance the growth of plants and simultaneously degrade M, C & F.

1. Introduction

Pesticides are widely used in agriculture to control pests, weeds and insects. In the contemporary world, pesticides have become an essential part of agriculture [1]. Globally, the use of pesticides has increased up to approximately 3 billion kilograms every year and the widespread use of pesticides has enhanced crop yield [2]. However, extensive usage has led to a major environmental threat that can cause adverse effects on the environment as well as human health which is mentioned in Table 1. The requirement for agricultural food crops is increasing with the increase in the human population and anthropologism [3]. The increase in population of India, has also led to increase in the requirement for food [4].

Organophosphorus pesticides are widely used in agriculture to control pests, these pesticides are effective but non-selective, which can harm both target and non-target pest insects. OPs act by inhibiting acetylcholinesterase (AChE) which leads to the accumulation of acetylcholine (ACh) at nerve synapses resulting in cholinergic neurons, sympathetic neuron and parasympathetic nervous system

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which results in bronchospasm, respiratory muscle paralysis and ultimately respiratory failure [5]. Monocrotophos, an organophosphorus pesticide (OPs) is also commonly known as azodrin is extensively used for agricultural purposes worldwide due to its effectiveness and broad-spectrum properties towards various pests [6]. Monocrotophos accounts for more than 34 % of total insecticide merchandise across the globe [7]. Uncontrolled application of pesticides lead to pollution of saturated and unsaturated zones leading to deleterious effect on the environment and health by causing ill effects such as neurotoxicity, diarrhea, genotoxicity and respiratory failure which ultimately causes death [8]. The lethal dosage when applied in the field is 0.25–1.5 kg ha^{-1} and it has an LD_{50} of 18–20 mg/kg for mammals with a half-life of 17–96 days [6]. Organophosphorus pesticides were 1st introduced in Germany during the 2nd world war and it has aryl or alkyl compounds, these compounds get bonded with phosphorous by forming phosphonates or through sulphur/O_2 atoms. They are commonly employed to protect crops such as cotton, rice, sugarcane, vegetables, tobacco, groundnut and maize from pest infestation [9].

Cypermethrin is a pyrethroid class of insecticide that is extensively used for insect control in vegetable crops, cotton and in the treatment of cattle and livestock [10]. The major role of pyrethroid insecticide is to control the vector borne disease which attacks most crops and their usage has led to pollution of agricultural soil worldwide. Pyrethroid accounts for 23.4 % of the global insecticide market and the expected growth rate in the sales market is 6.6 % until 2030 [11]. The United States National Pesticide Telecommunication Network, 2008 has reported the half-life of cypermethrin in soil as 30 days. Pankaj Bhatt in Ref. [12] reported the ability of indigenous bacterial consortia in the treatment of cypermethrin in *Zea mays*. In a study by Shaohua Chen in Ref. [13] co-culturing of *Streptomyces aureus* and *Bacillus cereus* enhanced the cypermethrin degradation. In another study by Shaohua Chen in Ref. [14] used the same isolates and obtained PhenoxyBenzoic Acid (3PBA) as the degraded product. The half-life of 3PBA is reported to be 120–180 days and when it enters the water bodies, it is absorbed by the sediments thereby leading to the increase in half-life. In a study by Pankaj Bhatt in Ref. [10], *Bacillus thuringiensis* was capable of degrading cypermethrin up to a concentration of 85 %. The hydrophobicity of the active ingredient present in the pesticide and the continuous exposure may lead to the accumulation of these toxic pollutants in the sediments, crops, waterbodies and in humans [15].

The broad spectrum fipronil pesticide which comes under the class phenyl pyrazole is extensively used for agricultural purposes. It causes damage to the epithelial walls of insects which inhibits the gamma aminobutyric acid (GABA) leading to paralysis, convulsions and ultimately death to the insects [16]. Fipronil is also considered a toxic insecticide with lipophilic and most persistent in nature [17]. The fipronil residues are also found in lower organisms and vertebrates [18]. Fipronil with a similar mode of action showed higher toxicity and binding affinity to the GABA receptors of the insects [19]. Zhe Zhou in Ref. [20] described that fipronil degradation is similar in different bacteria as they undergo oxidation, reduction, hydrolysis, photolysis and form fipronil sulfone, fipronil sulfide and fipronil amide respectively. Pankaj Bhatt in Ref. [21] described the effective fipronil degradation by *Bacillus* sp immobilized in sodium alginate beads for accelerated degradation. Pankaj Bhatt in Ref. [22] also used *Bacillus* sp. (FA3) for the degradation of fipronil. Bonmatin in Ref. [23] described the half-life of fipronil to be 3–7.3 months in field which majorly depends on the physio-chemical characteristics of soil.

Plant microbe interactions are mediated naturally in some cases and are practiced using by the application of plant growth promoting rhizobacterial (PGPR) strains which possess traits such as IAA, siderophore, HCN production etc., which enhances the growth of plants thereby enhancing the degradation potential. Phyto-rhizo remediation using plants can remediate the contaminants directly by the uptake in roots and detoxification by phyto-transformation [24]. Depending on the nature of contaminant, several strategies such as phytoextraction, phytodegradation, phytostabilization, phytovolatization, etc., can be employed. Phytoremediation employs biological, natural and biological methods to degrade and detoxify the pesticides [25]. There are several reports on the enhancement of pesticide degradation upon supplementation of microbes to the rhizosphere region which attributes to the rhizoremediation strategy. A study by Dash in Ref. [26] revealed the augmentation of *Kosakonia oryzae* (VITPSCQ3) in degradation of monocrotophos. In a study performed by Murugesan Chandrasekaran in Ref. [27] it was evident that the rhizoremediation could be an effective method for degrading pesticides.

The present study focuses on the biodegradation of monocrotophos, cypermethrin and fipronil by indigenous bacteria obtained from agricultural soil. Though there are several reports involved in the degradation of pesticide. To our knowledge, this is the first study emphasizing the ability of *Proteus myxofaciens* in the degradation of 3 different classes of pesticides such as organophosphorus,

Table 1
Symptoms from exposing pesticides (Roberts and Reigart, 2013; EXTOXNE, 2006).

S. no.	Active Ingredient	Symptoms
1	Monocrotophos (Organophosphate)	Headache, excessive tearing, blurred vision, nausea, convulsions, loss of consciousness
2	Chlorpyrifos (Organophosphate)	Headache, respiratory depression, pinpoint pupils, muscle twitching
3	Endosulfan (Organochlorine)	Burning, Tingling of skin, lack of coordination, tremor, seizures
4	Malathion (Organophosphate)	Headache, excessive salivation, Diarrhea, respiratory depression
5	Cypermethrin (Pyrethroid)	Irritability to sounds, abnormal facial sensation, numbness, fatigue
6	Fipronil (Phenylpyrazoles)	Excessive sweating, nausea, headache, abdominal pain, agitation, weakness

pyrethroid and phenyl pyrazole. This study also will be of importance for the scientific community working on phyto and rhizoremediation of pesticides.

2. Materials and methods

2.1. Pesticides & chemicals

Chemicals used for the study were procured from HiMedia & SRL India. Pesticides such as Monocrotophos, Cypermethrin and Fipronil (M, C & F) were obtained from the local markets in Vellore, Tamil Nadu, India. The stock concentration for M, C & F was 10,000 ppm. The other chemicals used for the preparation of Liquid Glucose Ivo (LGI) media was procured from HiMedia.

2.2. Sample collection

Wetland paddy soil was collected from the agricultural fields located in Thiruvalam, Vellore, India (12.9758°N, 79.26318°E) by random sampling method. A total of 9 soil samples were collected for the isolation of pesticide degrading bacteria.

2.3. Isolation and identification of M, C & F degrading bacteria

Isolation of bacteria was carried by direct plating and enrichment. For the direct plating wetland paddy rhizosphere soil samples were pooled and were serially diluted and plated from 10^{-5}onto modified LGI medium (K_2HPO_4 0.2 g/l, KH_2PO_4 0.6 g/l, $MgSo_4.7H_2O$ 0.2 g/l, $CaCl_2$ $2H_2O$ 0.02 g/l, Na_2MoO_4 0.002 g/l, $FeCl_3.6H_2O$ 0.01 g/l, glucose 10 g/l, Yeast extract 10 g/l, pH 6.8) supplemented with 250 ppm of M, C & F and 2 % NaCl. For enrichment technique soil samples were transferred in LGI broth and the flasks were incubated in a rotary shaker for 7 days at 120 rpm. Further, it was serially diluted and plated on LGI agar [28].

2.4. Determination of maximum tolerance concentration (MTC) for pesticides

MTC was performed using both plate and broth assay. For plate assay LGI medium was supplemented with all the three pesticides M, C & F individually with concentrations ranging from 100 to 500 ppm. The pH was maintained at 6.8. The isolates were patch inoculated and the plates were incubated for 48 h [26]. Visible growth of the isolates indicated the ability of the organism in tolerating M, C & F. For broth assay, 10 ml of LGI broth was augmented with M, C & F with varying concentrations of 500–1500 ppm. Tubes were inoculated and incubated with 1 % of inoculum for 24–48 h. Uninoculated broth served as a control [29].

2.5. Optimization of the effective isolate for pesticide degradation

Optimization of the pesticide degrading parameters includes three different factors such as pH (4.5–8.5), carbon (glucose, sucrose and glycerol) and nitrogen sources (yeast extract, ammonium sulfate & urea) with the concentration of 1 % being constant throughout the experiment. Upon initial optimization carbon, nitrogen and varied pH was studied using Response Surface Methodology (RSM) design expert statistical software. The preliminary experiments were performed to fix the range [30]. RSM was carried out using a quadratic model approach. The data was analyzed by considering one factor as reference and with other two factors varied with three different levels (−1, 0, +1) [31]. The pesticide degradation has been denoted as variable R1. Twenty experimental runs were performed to validate the model. Output was analyzed using Analysis of Variance (ANOVA) and results were analyzed using 3D contour plots [32,33].

2.6. Growth kinetics

Ability of the isolate to grow with the optimized parameters consisting of pH, carbon and nitrogen sources and in the presence of M, C & F supplemented with 250 ppm concentration of each of the three pesticides. From the seed culture, 2 % inoculum (0.3OD) was added and the flasks were incubated in dynamic condition at 120 rpm at 28 ± 2 °C. The difference in the changes in various growth phases with and without treatment was studied by taking absorbance at 600 nm for every 12 h interval till the cells reached the decline phase [26].

2.7. Molecular characterization of effective isolate

The purified effective isolate was molecularly characterized by 16S rRNA gene sequencing and primers 27F (5′ GT TAC CTT GTT ACG ACT/T3′) and 1492R (5′-AGA GTT TGA TCC TGG CTC AG-3′) was used for amplification [34]. Further the sequences were compared with NCBI sequence database using BLAST [35]. Phylogenetic tree was constructed using neighbor joining method and was bootstrapped using MEGA4 software [36].

2.8. Biodegradation of M, C & F

For the biodegradation of all the three pesticides, a modified LGI medium with optimized parameters was used in three different

250 ml Erlenmeyer flasks. Seed culture (2 % 0.3OD) was inoculated in all three flasks and uninoculated broth served as control. Flasks were incubated at 120 rpm and 28 ± 2 °C for 5 days. Upon incubation, the samples were centrifuged at 10,000 rpm for 8 min, cell free extracts were used to test the concentration of pesticides using high performance liquid chromatography (HPLC), C18 reverse phase column as mentioned in Hina Jabeen in Ref. [37] to monitor the biodegradation of M, C & F. The flow rate was maintained at 1 ml/min, Cell free extract was also used to detect the percentage degradation and was calculated using the following formula

$$MCF\ \%\ degradation = \frac{Initial\ absorbance - Final\ absorbance}{Initial\ absorbance}$$

2.9. *Identification of metabolites and prediction of pathways*

The degraded metabolites were extracted at 24 h intervals for five days. For extraction, ethyl acetate was mixed in the ratio of 1:1 (liquid: liquid) and solvent phase was separated for analysis using gas chromatography mass spectroscopy (GCMS) (Clarus 680, PerkinElmer). The degraded products obtained were extracted using ethyl acetate and the samples were injected in GCMS. The temperature used for melting was 260 °C (Clarus 680, PerkinElmer). The carrier gas used was helium and the flow rate used was 1 ml/min and the degraded metabolites were identified using the RTE integrator library search. The presence of functional group of the predicted compound was analyzed using Fourier transform infra-red spectroscopy (FTIR). The frequency of vibration provided the presence of various functional groups. Based on the compounds predicted a pathway for the degradation of pesticide was developed using PATHPRED software [38].

2.10. *Enzymatic analysis*

2.10.1. *Estimation of phosphatase*

Phosphatase assay was performed by inoculating VITVJ1 into LGI media supplemented with 250 ppm of M, C & F and was incubated at 120 rpm for 24 h. The broth was centrifuged at 10,000 rpm for 10 min and the cell free supernatant was used for the detection of phosphatase enzyme using p-nitrophenyl phosphate (p-NPP) as a substrate and the amount of p-nitrophenol released in unit time was measured spectrophotometrically at 400 nm [26,39].

2.10.2. *Estimation of esterase*

The esterase enzyme assay was analyzed by the addition of 20 μl of cell free extract to 0.1M sodium acetate buffer (pH 6.5) and 1 mM p nitrophenyl acetate and was incubated for 15 min at 37°c. The esterase activity was determined spectrophotometrically at 405 nm. The assay was estimated by the amount of p-nitrophenol released in unit time [40].

2.11. *Amplification of the M, C & F functional genes*

Bacterial DNA was harvested from overnight bacterial culture using phenol-chloroform method. Target genes for the degradation of monocrotophos (*opd*A gene), Cypermethrin (*Est* gene) and Fipronil (MnP1) were amplified using gene specific primers and the amplicons were run in 1.8 % agarose gel [41]. The list of primers has been mentioned in Table 4.

2.12. *Toxicity assessment*

Upon the degradation studies of M, C & F degraded broth with and without bacteria was subjected to toxicity assessment.

2.12.1. *Seed germination assay*

The toxicity of the degraded metabolites was tested against *glycine max, Vigna radiata* and *Vigna mungo*. The seeds were surface sterilized with sodium hypochlorite and was placed on a petriplate layered by filter paper soaked with sterile distilled water, untreated pesticides and M, C & F degraded broth respectively [42]. The germination rate was observed for 7–14 days. The toxicity assessment was measured based on the length of plumule and radical [43]. The germination index was calculated by the relative seed germination and relative root germination.

$$\mathrm{RSG\%} = \frac{\text{Number of seeds germinated in treatment}}{\text{Number of seeds germinated in control}} \times 100$$

$$\mathrm{RRG\%} = \frac{\text{Mean root length in treatment}}{\text{Mean root length in control}} \times 100$$

$$\mathrm{GI\%} = \frac{\mathrm{RSG} \times \mathrm{RRG}}{100}$$

2.13. *Characterization of PGPR*

The efficient M, C & F degrading bacteria VITVJ1 was assessed for its PGPR traits such as IAA, siderophore, ammonia production

and phosphate solubilization both qualitatively and quantitatively. LGI broth was supplemented with 5 mM of tryptophan and was inoculated and incubated for 48 h. The supernatant was recovered upon centrifugation at 10,000 rpm and to the supernatant, Salkowski reagent was added and was further incubated for 30min to observe pink coloration which indicated the production of IAA and it was also quantified spectrophotometrically at 530 nm [44].

Siderophore production was carried out by inoculating VITVJ1 in Fiss minimal media with ion limiting conditions. Upon 48 h of incubation the broth was centrifuged at 10,000 rpm for 10min and the supernatant was added to CAS agar wells. Formation of halo zone indicated the production of siderophore [45]. Ammonia production was performed by inoculating VITVJ1 in peptone water and incubated for 48 h. The amount of ammonia produced was detected by adding 500 μl of Nessler's reagent. Brown coloration indicated the production of ammonia. Phosphate solubilization was determined by solubilizing insoluble tricalcium phosphate in Pikovskya agar by the formation of halozone [46]. Solubilization index was calculated using the following formula

$$SE\% = \frac{\text{Diameter of solublization zone} - \text{Diameter of the colony}}{\text{Diameter of the colony}} \times 100$$

2.14. Pot culture studies

Pot culture study was performed to observe the efficiency of the effective isolate in enhancing as well as uptake M, C & F. Based on the fibrous root system and growth at extreme conditions, the fresh plantlets of *Chrysopogon zizanioides* were selected and was collected from the VIT nursery. The plants were washed thoroughly and were pruned to 5 cm of root and shoot length prior to planting. 2 kg garden soil was air dried and sieved with 2 mm pore size mesh to obtain even sized particles. Further, the soil was packed into the LDPE bags. The treatments were differentiated as phytoremediation i.e., without the augmentation of effective bacteria and rhizoremediation i.e., with the augmentation of effective bacteria. The soil was artificially amended with 50, 100 & 150 ppm of M, C & F. The rhizoremediation pots were bioaugmented with 5 ml of the effective isolate at an equal interval of 15 days. Pot culture study was carried out for 45 days in VIT green house. The average moisture content was maintained by watering the plants every two days [47].

2.14.1. Analysis of various parameters

2.14.1.1. Assessment of physiological plant growth. The plant samples were uprooted at every 15 days and was measured for its shoot height and root length.

2.14.1.2. Chlorophyll content. Fresh leaves of 1 g was homogenized with 80 % acetone and was centrifuged. The supernatant was measured at 645 nm and 663 nm. The chlorophyll *a* & b was determined by Arnow's equation [48].

2.14.2. Analysis of M, C & F biodegradation in soil

20 g of soil was air-dried and was extracted using ethyl acetate which was filtered using 0.2 μm syringe and was analyzed using UV–Vis spectrophotometer at 214, 220 & 280 nm [49,50]. Residual M, C & F and its compounds was identified by GC-MS analysis [51].

2.15. Statistical analysis

All the statistical analysis was performed using Origin software V 8.0. The data was assessed for optimization using Design expert software V 12.0. RSM was carried out using a quadratic model approach, where the data was analyzed by considering one factor as reference and with other two factors being varied with three different levels (−1, 0, +1) and output was analyzed using Analysis of Variance (ANOVA) and results were interpreted using 3D contour plots.

3. Results and discussion

3.1. Isolation of bacteria

A total of 8 morphologically distinct isolates were obtained from paddy rhizosphere soil using LGI agar medium supplemented with M, C & F (250 ppm each). The isolates obtained were named as VITVJ1 to VITVJ8. A similar study carried out by Dash in Ref. [26] also revealed that bacteria obtained from wet land paddy rhizosphere soil was also capable of degrading pesticide. It is understandable that sampling location plays a major role in obtaining the indigenous M, C & F degrading bacteria [52]. Eleni Chanika in Ref. [53] revealed the indigenous bacteria have the ability to tolerate and degrade pesticides.

3.2. Maximum tolerance concentration (MTC) for the effective strain

MTC assay showed that the isolates VITVJ1, VITVJ3 and VITVJ7 were capable of growing in concentrations above 1200 ppm of M, C & F. Whereas, isolate VITVJ1 was capable of surviving at 1500 ppm concentration of M, C & F (Fig. 1). A similar study by Shaohua Chen in Ref. [54] reported that *B. cereus* DG-02 was capable of growing in the presence of fenpropathrin a pyrethroid class of pesticide up to the concentration of 1200 ppm. Another study by Yu-Hong Huang in Ref. [55] reported the potential of *B. subtitlis* in tolerating dibutyl phthalate (DBP), a plasticizer up to a concentration of 200 ppm. Jie Liu in Ref.[56] revealed that *Bacillus pumilus* (HZ-2) was

also capable of metabolizing 200 ppm of mesotrione with leaving 2.8 ppm of residue. In our study, VITVJ1 was capable of resisting cypermethrin up to a concentration of 1500 ppm which was similar to the study carried out by Ying Xiao in Ref. [57] where *B. subtilis* was capable of degrading β-cypermethrin (BSF01) up to50 mg/l within 7 days. In another study *Bacillus cereus* AKD1 is capable of tolerating cypermethrin up to 500 ppm; however, there was a decrease in biodegradation efficacy [58]. Shaohua Chen in Ref. [59] used *B. thuringiensis* ZS-19 for the degradation of cyhalothrin up to 100 μg/ml within 72 h and Xiaozhen Wu in Ref. [34] reported *B. thuringiensis* to secrete proteins that could possess larvicidal activity against lepidopteran and coleopteran insects. Isolates VITVJ1 was capable of tolerating monocrotophos up to a concentration of 1500 ppm. A study by Dash in Ref. [26] reported the ability of VITPSCQ3 to tolerate and degrade monocrotophos. Bhadbhade in Ref. [39] reported that *Arthrobacteria* sp. and *Bacillus* sp. have the ability to tolerate monocrotophos up to 1000 ppm. This could be due to the synthesis of enzymes upon exposure to high concentration of pesticides. Pankaj Bhatt in Ref. [60] revealed fipronil degradation was maximum at 50 ppm in 12 days. However, in our study VITVJ1 was able to resist up to 1500 ppm and was capable of degrading fipronil 250 ppm. Therefore, VITVJ1 was considered as effective strain.

3.3. Optimization of the growth parameters of the effective isolate VITVJ1

RSM with Box Behnken design (BBD) was used to optimize the experimental conditions for pesticide degradation. RSM is a powerful statistical tool used in the experimental designs for optimization [61]. BBD is performed when the number of factors is more than 2 and less than 5 (Fig. 2). Carbon and nitrogen sources along with the pH are the major factors influencing the pesticide degradation. Study by Shimei Pang in Ref. [62] described the optimal conditions for methomyl degradation depended on pH, temperature and inoculum concentration to enhance the degradation. By varying these factors at different levels and to observe the response (pesticide degradation percentage) with the help of this model, maximum pesticide degradation can be achieved upon optimization. A contour plots and the surface diagrams aids to visualize the relationship between the factor and responses. RSM with BBD helps in optimization with lesser number of trials as compared to the traditional method. A study by Shao-Fang Chen in Ref. [5] described the increased biodegradation efficiency of chloroacetamide herbicides is by optimizing the degradation conditions with the help of RSM an BBD model for fixing the factors in lesser number of trials.

The Box-Behnken design (BBD) model facilitated the construction of contour plots and 3D surface diagrams (Fig. 2a–f). the validation of actual and predicted response values was conducted through ANOVA quadratic model (Table 2). The analysis revealed significant F values of 44.18, 1.04 and 0.0607 for the three responses supported by *P* values (<0.0001, 0.0323, 0.8104) indicating the significance of two response with *P* values less than 0.05. adequate precision indicating signal to noise ratio above 4 was measured at 76.0966, affirming the reliability of the model. Similar study by Ziqiu Lin in Ref. [63] revealed the *P* value to be less than 0.05 (0.0003) which indicated that the model is significant and the adequate precision of greater than 4 (18.327) indicated adequate signal. The 3D diagrams (Fig. 2a–c) and contour plots (Fig. 2d–f) illustrates the optimal values of input variables showing their substantial influence on pesticide degradation through various combinations. The RSM design encompassed 20 experimental runs (Table 3) with factors A (pH), B (carbon source), C (nitrogen source), AB, AC, BC, A2, B2 and C2 identified as significant. Utilizing coded factors, the derived BBD equation for pesticide degradation% $= 86.23 – 1.19A + 0.1817B – 0.0439C – 0.1250AB – 0.2500AC – 0.2250BC – 9.39 A^2 – 2.88 B^2 + 2.42C^2$. This equation enables the prediction of responses and assessment of factors. Consistency between obtained responses and statistical values which are validated using RSM models is effective in maximizing the pesticide degradation by VITVJ1. Previous studies [32,33,64] have similarly demonstrated the efficacy of RSM optimization in bioremoval studies.

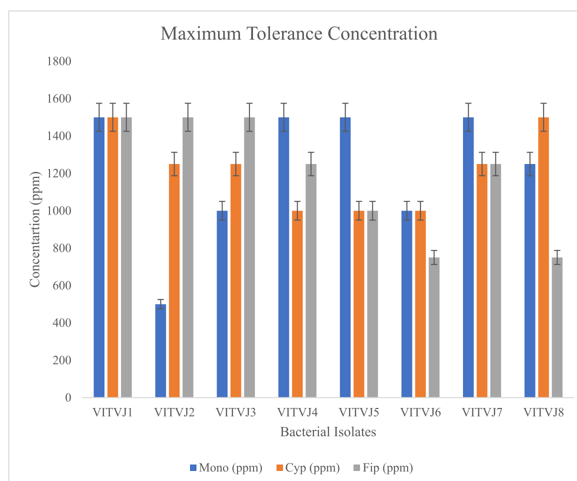

Fig. 1. Graph indicating MTC of the isolates.

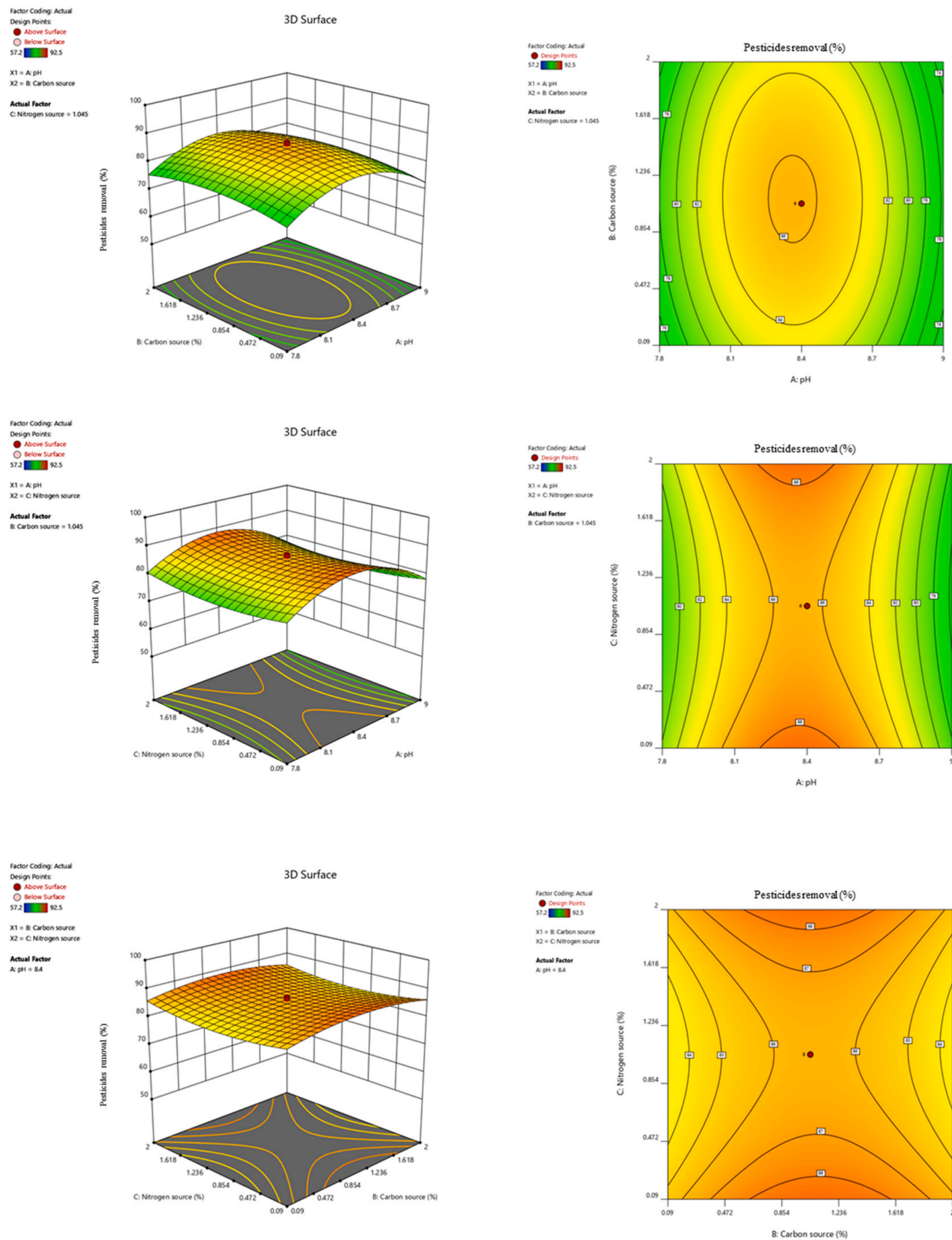

Fig. 2. 3D surface diagram and contour plots showing the interaction between different optimized parameters with respect to M,C, F degradation by *Proteus myxofaciens* (VITVJ1).

3.4. Molecular characterization of the effective strain by 16S rRNA gene sequencing

Based on 16S rRNA gene sequencing of the effective strain, VITVJ1 was characterized and identified to be the closest neighbor of *Proteus myxofaciens* and it also exhibited 99.86 % homology (Supplementary (SM) Fig. 1). The obtained nucleotide sequence was submitted to Gen Bank with the accession number OM250031. To our knowledge, there has been no report about pesticide degradation by *P. myxofaciens* till date.

Table 2
ANOVA for RSM - quadratic model – heavy metal removal.

Source	Sum of Squares	*df*	Mean Square	*F*-value	*p*-value	
Model	1528.65	9	169.85	390.93	<0.0001	Significant
A-pH	19.19	1	19.19	44.18	<0.0001	
B-Carbon source	0.4510	1	0.4510	1.04	0.3323	
C-Nitrogen source	0.0264	1	0.0264	0.0607	0.8104	
AB	0.1250	1	0.1250	0.2877	0.6034	
AC	0.5000	1	0.5000	1.15	0.3086	
BC	0.4050	1	0.4050	0.9322	0.3571	
A^2	1269.78	1	1269.78	2922.55	<0.0001	
B^2	119.64	1	119.64	275.37	<0.0001	
C^2	84.54	1	84.54	194.57	<0.0001	
Residual	4.34	10	0.4345			
Lack of Fit	3.59	5	0.7183	4.77	0.0558	Insignificant
Pure Error	0.7533	5	0.1507			
Cor Total	1533.00	19				
Std. Dev.						0.6591
Mean						79.51
C.V.						0.8290
R^2						0.9972
Adjusted R^2						0.9946
Predicted R^2						0.9816
Adeq Precision						76.0966

Table 3
Run table.

Run Order	Actual Value	Predicted Value	Residual	Leverage	Internally Studentized Residuals	Externally Studentized Residuals	Cook's Distance	Influence on Fitted Value DFFITS	Standard Order
1	77.40	76.84	0.5650	0.670	1.492	1.605	0.451	2.285(1)	1
2	78.30	77.90	0.4015	0.670	1.060	1.067	0.228	1.520	3
3	87.00	86.23	0.7667	0.166	1.274	1.320	0.032	0.590	15
4	78.20	77.70	0.5029	0.670	1.328	1.388	0.357	1.976	5
5	86.40	86.23	0.1667	0.166	0.277	0.264	0.002	0.118	16
6	76.10	75.78	0.3226	0.670	0.852	0.839	0.147	1.195	4
7	86.10	86.23	−0.1333	0.166	−0.221	−0.211	0.001	−0.094	17
8	86.00	86.23	−0.2333	0.166	−0.388	−0.371	0.003	−0.166	18
9	75.70	75.21	0.4861	0.670	1.283	1.332	0.334	1.897	2
10	92.50	93.16	−0.6576	0.607	−1.592	−1.748	0.392	−2.174(1)	13
11	75.00	74.74	0.2605	0.670	0.688	0.668	0.096	0.952	8
12	86.10	86.23	−0.1333	0.166	−0.221	−0.211	0.001	−0.094	20
13	78.00	78.39	−0.3893	0.607	−0.943	−0.937	0.137	−1.165	12
14	78.20	77.86	0.3394	0.670	0.896	0.886	0.163	1.262	7
15	92.50	93.01	−0.5098	0.607	−1.234	−1.272	0.236	−1.582	14
16	77.00	77.78	−0.7781	0.607	−1.884	−2.225	0.549	−2.767(1)	11
17	57.20	57.69	−0.4899	0.607	−1.186	−1.214	0.218	−1.509	10
18	86.00	86.23	−0.2333	0.166	−0.388	−0.371	0.003	−0.166	19
19	75.50	75.08	0.4239	0.670	1.119	1.135	0.254	1.617	6
20	61.00	61.68	−0.6775	0.607	−1.640	−1.820	0.416	−2.263(1)	9

3.5. Growth kinetics

Growth kinetics of the effective isolate VITVJ1 in the presence of M, C & F revealed extended lag phase which could be due to the acclimatization of the effective strain to selective pressure induced due to M, C & F. A similar study by Dash in Ref. [26] revealed the increase in lag phase when supplemented with monocrotophos. Fo-Ting Shen in Ref. [65] also reported that the cells of *B. aryabhattai* reached decline phase at 35th h. The various concentrations of chlorpyriphos stimulated the growth of *P. stuartii* (MS09) and remained in a prolonged log phase [66]. Study by Shaohua Chen in Ref. [59] revealed the growth of the strain ZS-19 *Bacillus thuringiensis* with cyhalothrin as the sole carbon source where in initial 24 h there was rapid increase in the cell numbers with 61.8 % degradation.

3.6. Biodegradation of M, C & F by VITVJ1

Degradation of pesticide was carried out individually for M, C & F (250 ppm). There was a drastic reduction in the area of the peaks in HPLC with the retention time of 2.5, 3.3 & 9.0 min in HPLC (Fig. 3). The degradation of monocrotophos was observed through a gradual reduction in peak height (Fig. 3b) as compared to Fig. 3a, and for cypermethrin (Fig. 3c & d) and fipronil (Fig. 3e & f) there was

a complete disappearance of peak indicating the degradation C & F. Similarly, a study by Liu et al., 2012 reported the degradation of monocrotophos and cypermethrin at RT of 2.5min and 3.3min showed a complete degradation. HPLC analysis revealed the fipronil degradation and its metabolite fipronil sulfone which is more toxic than fipronil [67]. The microbial degradation of M, C & F is a complex study as it contains pesticides, solvents, bacterial cells and its metabolites.

GC-MS analysis led to the identification of intermediate products from biodegradation of M, C & F. The confirmation of M, C & F degradation relied on their respective *m/z* value of the degraded products which was 223, 416 and 437 (Fig. 5a–c & e). The formation of intermediate product for monocrotophos is monomethyl phosphate (*m/z* 113) which was previously reported by Dash in Ref. [26]. For cypermethrin, 3-phenoxybenzaldehyde (*m/z* 198) was obtained upon extraction using ethyl acetate which was similar to the work carried out by Kaidi Hu in Ref. [68]. β-Cypermethrin degradation using *B. licheniformis* indicates that 3- phenoxy benzoic acid (PBA) at the RT of 6.5 and β-cypermethrin at the RT of ∼ 15.0. The products identified were acetonitrile, 3-phenoxy benzaldehyde, 3-phenoxy benzoic acid and cypermethrin isomers [69]. Fipronil degradation is reported to obtain fipronil sulfide (*m/z* 421) as the intermediate product which is mentioned in Fig. 5b–d & f. For instance, *Proteus* sp. acquires numerous metabolic abilities to adapt in different toxic environments [70]. The ability of the bacteria such as *Pseudomonas* sp., *Klebsella* sp., *Proteus* sp. and *Acinetobacter* sp. to tolerate and utilize the toxic compound has been previously reported by Nancy pino in Ref. [71].

FTIR spectrum with and without bacteria (control) as mentioned in Fig. 4a & b exhibited peaks at 3381, 3271.27, 3068.75 cm^{-1} indicating the presence of O-H stretching. Nitro groups, chlorine groups, sulfonyl chloride, and phosphate groups showed peaks at 1514.12 (N-O), 769.60 (C-Cl), 1402.25 (S=O), 990 (P=O) which were absent in the test treated with VITVJ1. The absence of characteristic peaks from the parent compound provided evidence of degradation. FTIR results also revealed that peak at 2973 cm^{-1} which corresponded to vinyl bonds which was completely disappeared in the test and the shift in peak was observed at 2928 cm^{-1} inorganic phosphate which indicated degradation of monocrotophos [29,72]. Cypermethrin degradation showed major peak shift in the range 100–1650 and 2259-3431 cm^{-1} [73]. Fipronil biodegradation reveals that peak 1735 cm^{-1} corresponds to the ester group which confirming the degradation of fipronil as the product was fipronil sulfide.

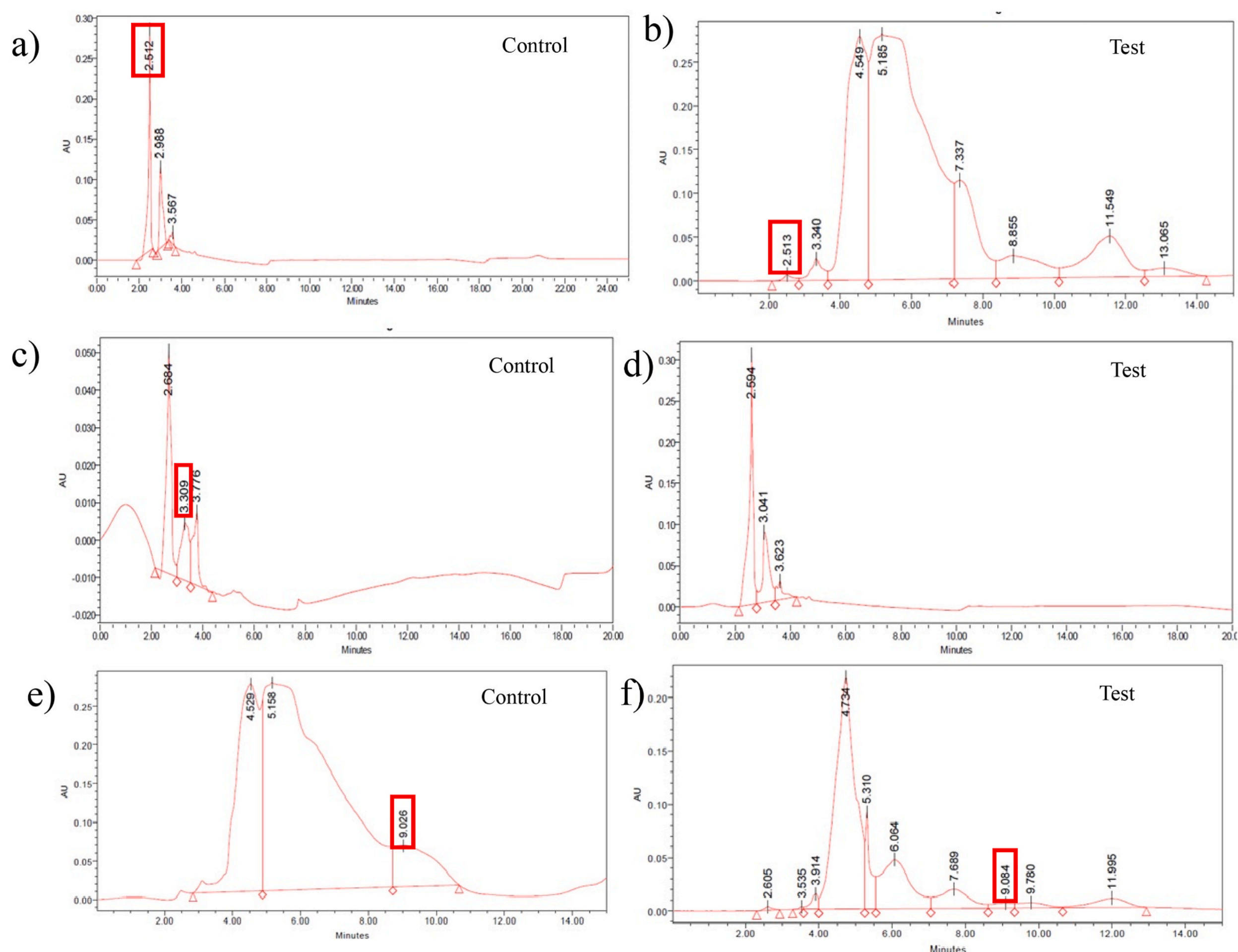

Fig. 3. Analysis of M, C & F biodegradation by HPLC.

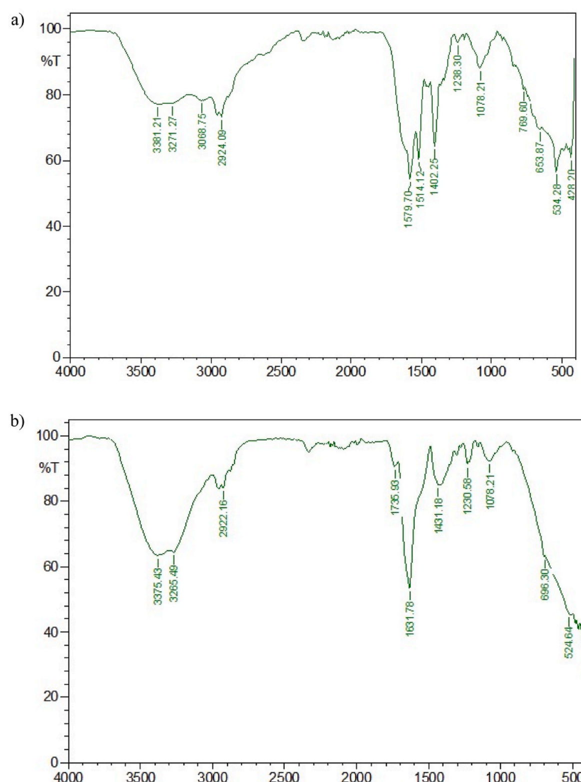

Fig. 4. FTIR spectrum of M, C & F degradation by *Proteus myxofaciens* (VITVJ1).

3.7. Pathway prediction

Bacterial degradation of xenobiotic compounds are recognized by their ability to breakdown or bioconvert a diverse array of end products [74]. The presence of these products upon breakdown of M, C & F by VITVJ1 was confirmed by GC-MS analysis. The major biodegradable product was acetic acid for M & F. For fipronil, the degraded product was phenoxybenzoic acid (Fig. 6). The bioconversion of M, C & F underwent numerous degradation pathways such as styrene degradation, glycoxylate metabolism and further gets utilized by TCA cycle. The pathway suggested the possible biodegradation of M, C & F. Dimethylcyclopropane carboxylate is an important component of monocrotophos formulation which was detected in degradation (Fig. 6a). Further, biodegradation of Cypermethrin was confirmed by the degradation and disappearance of cyclopropane carboxylate and formation of 2-hydroxy-2, (3-phenoxy phenyl) acetonitrile, a nitrile compound which was formed by the dehydration of amide group (Fig. 6b). Sungmin Han in Ref. [75] revealed that fipronil was converted to 2-(2,5, dichlorophenoxy) acetic acid by the oxidation of acetaldehyde (Fig. 6c). However in our study fipronil was broken down into benzenesulfonic acid. Ying Xiao in Ref. [57] described the biodegradation pathway of the β-cypermethrin by the *B. subtilis* (BSF01), which was metabolized by the hydrolysis of ester linkage to yield cyclopropane carboxylate and phenoxy benzyl acetonitrile and further by oxidation it gets converted into phenoxy benzaldehyde. Subsequent oxidation results in 3 phenoxy benzoic acid, leading to the detoxification of β-cypermethrin.

3.8. Enzymatic analysis

The activity of phosphatase was accelerated with the increase in monocrotophos concentration of 150 ppm (SM Fig. 3). Whereas, activity of esterase was found to be increased in cypermethrin and fipronil concentration. The phosphatase activity was determined by measuring the inorganic phosphate from monocrotophos [26]. The phosphatase exhibited its highest activity at 47.84 U ml^{-1} with monocrotophos as a substrate confirmed that VITVJ1 had the ability to degrade monocrotophos and also to produce phosphatase [39]. Whereas, cypermethrin and fipronil degradation was mediated by esterase enzyme. Active site of the enzyme contained certain amino acids which play a vital role in the catalysis of pyrethroid and phenyl pyrazole.

3.9. Molecular amplification of candidate gene

The amplicons upon PCR showed products with varying size i.e.., 700bp, 1200bp and 500bp indicating the presence of *OPDA* (SM Fig. 4a), Est (SM Fig. 4b) and MnP1 (SM Fig. 4c) candidate genes. The opdA gene is the most widely sequenced marker gene responsible

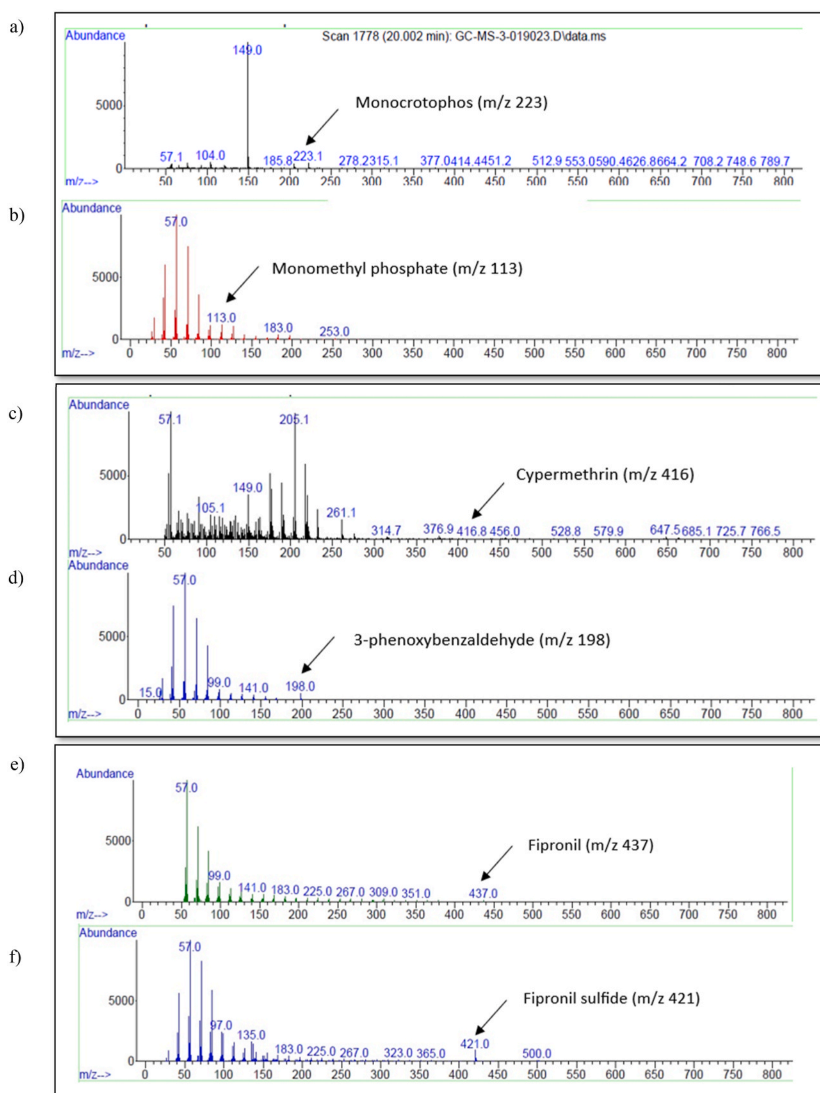

Fig. 5. GCMS analysis of MCF degradation by *Proteus myxofaciens* (VITVJ1).

for encoding for the protein responsible for the degradation monocrotophos [26]. Pankaj Bhatt in Ref. [76] amplified Est gene responsible for the degradation of cypermethrin and MnP1 gene encoded for the gene responsible for fipronil degradation. The presence of these genes in the effective strain VITVJ1 confirmed their role in the degradation of M, C & F.

3.10. Toxicity assessment

3.10.1. Seed germination assay

The phytotoxicity assessment by the seed germination assay using the seeds of *Vigna radiata, Vigna mungo* and *Gycine max* revealed the non-toxic nature of the degraded metabolites. The seeds which were soaked in untreated M, C & F solution did not show significant difference whereas, there was a reduction in the germination index with only 6.3 % with the seeds treated with M, C & F. However, when VITVJ1 was treated with M, C & F there was a significant difference in the germination index of 52.72 and 100 % germination was observed in seeds treated with distilled water (Fig. 7) (Table 5).

The phytotoxicity assessment indicated that the pesticides treated with VITVJ1 showed less toxicity levels as compared to the untreated pesticides [42]. Hence, the degradation of pesticides by VITVJ1 was found to be effective and non-toxic to the environment. Allan Pretti Ogura in Ref. [77] described the herbicides used for sugarcane cultivation can inhibit the seed germination and the result indicated higher phytotoxicity as it inhibited root and shoot growth.

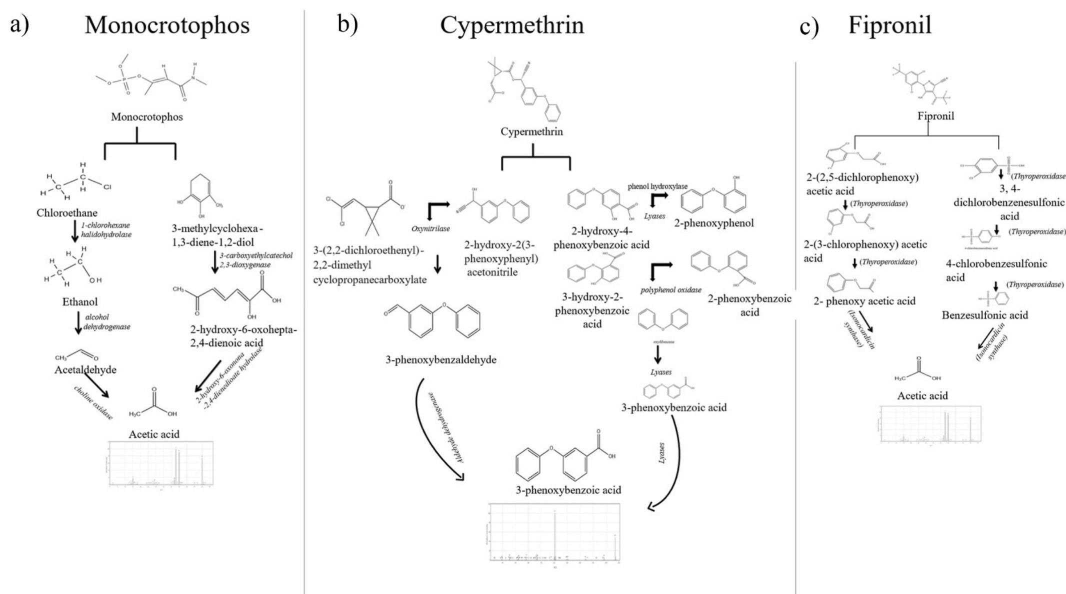

Fig. 6. Degradation pathway in artificially contamination broth with a) Monocrotophos, b) Cypermethrin c) Fipronil.

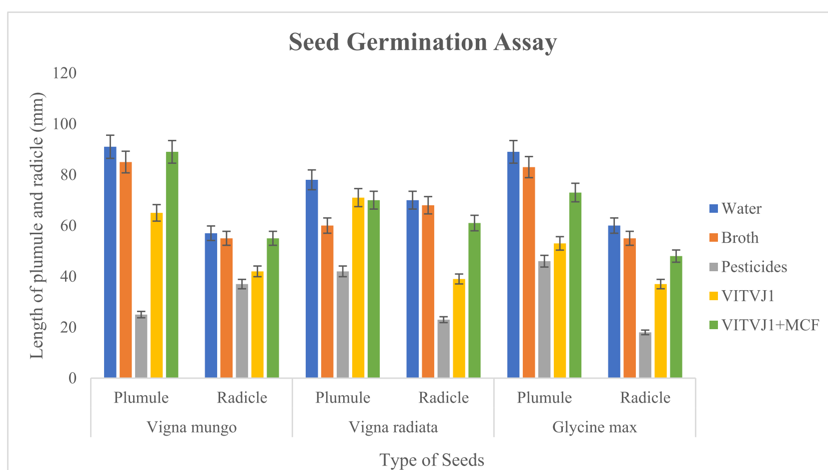

Fig. 7. Seed germination assay.

Table 4
List of primers used for the detection of MCF degradation genes by molecular traits.

Target	Primer name	Primer sequence 5′–3′
Monocrotophos	OpdA	5′-TTCATCCTGAAAGACACTGCCGGA-3′ 5′-ACGCGCTTCCAGACCAACCTATTT-3′
Cypermethrin	Est	5′-TGAATTGCTCGGCTGACACCTC-3′ 5′-ATCTCCTTCGACCGGCATTATTTC-3′
Fipronil	MnP 1	5′-GGATCCCTGTCTGGTCTTCTACAC-3′ 5′-CGCGTATGATGGTCGCGTGGTGGTC-3′

3.11. Screening of biofilm and analysis of PGPR traits

Qualitatively biofilm formation was confirmed by the visible lining on the walls of the test tubes. In quantitative analysis by microtiter plate assay there was a positive correlation to the qualitative assay. VITVJ1 showed 33 % of enhanced biofilm with 250 ppm of M, C & F (Table 6). Therefore, biofilm formation in the current study could aid in the development of film by VITVJ1 to the

Table 5
Seed germination.

Isolates	RSG%	RRG%			GI
		Vigna mungo	*Vigna radiata*	*Glycine max*	
Water	100	100	100	100	100
VITVJ1	80	73.68421	55.71429	61.66667	57.31955
VITVJ1+MCF	60	96.49123	87.14286	80	52.72682
MCF	15	64.91228	32.85714	30	6.388471

rhizoplane of *C. zizanioides.* Therefore, it could enhance the plant growth and simultaneously degrade the pesticides supplemented to the soil [76].

VITVJ1 was capable of producing siderophore and IAA, solubilize insoluble phosphate which resulted in the enhancement of plant growth (Table 6). Quantitative analysis of IAA by VITVJ1 showed the ability to produce tryptophanase which was used to convert tryptophan into IAA revealed production of 63 μg/ml when 5 mg/ml of tryptophan was supplemented. A similar study by Joseph Williamson in Ref. [78] showed the ability of bacteria in the production of phytohormones. The presence of halo zone around the colonies in NBRIP medium confirmed the conversion of insoluble phosphate into soluble form with 72.3 % efficiency. The solubilization of insoluble phosphate had a direct impact on plant growth and a study by Pratibha Rawat in Ref. [79] enhanced grain yield when phosphate solubilizing bacteria (PSB) was augmented. Siderophore production was confirmed by halo zone formation of 1.238 cm. It is observed that siderophores secreted by bacteria can bind to ion specific ligands and help in the sequestration and mobilization of ion for bacteria [80]. Ammonia production was found to be negative as there was negative in the addition of Nessler's reagent. Since, VITVJ1 was found to be positive for the tested traits and it was considered as PGPR which was further used for the augmentation in rhizoremediation setup during pot culture.

3.12. Assessment for plant growth parameters

In both phyto and rhizo remediation setups, there was noticeable increase in root and shoot length. Vetiver was capable of surviving at 250 ppm of M, C & F without the augmentation of VITVJ1. However, the growth rate was notably higher in rhizoremediation, attributed to the enhancement provided by PGPR strain VITVJ1 (Fig. 8). It is already been reported that plant bacterial association could enhance the pesticide degradation upon treatment with rhizobacteria which assists their host plants to survive in the contaminated environment [27,81]. The reduction in pesticide concentration can be correlated with plant growth and the production of non-toxic end products such as acetic acid, ethanol, 3-phenoxy benzoic acid and phenoxy acetic acid (Table 7). It also reveals the production of chlorophyll content to be 62 μgml^{-1} in rhizo and 20.1 μgml^{-1} in phytoremediation setup (Fig. 9) which reveals that VITVJ1 aided the growth and metabolism of pesticides in higher concentration. The synergistic effect of phyto-rhizoremediation while coupling the PGPR bacteria with the *C. zizanioides* enhanced the uptake of pesticides and promoted the plant growth.

It also increases the soil fertility by releasing the organic compounds in the environment. Pesticides mostly exists in soil in insoluble form[82]. PGPR bacteria increases their bioavailability by changing the pH of rhizosphere and increases the bioavailability [83]. The pesticides are sorbed into the root surface and moves into the root cells. The uptake of pesticides into roots by apoplastic and symplastic pathway indicates the passive and active transport mechanism in plants [84]. Upon entering into the roots, the pesticides form complex with ion chelators. The complex are sulfate, phosphate and carbonate precipitate, these complexes are further immobilized in intra and extracellular spaces (vacuoles). The complex further moves into the xylem are moved into shoots [85]. *C. zizanioides* commonly known as vetiver, has been reported for its phytoremediation potential [47]. Therefore, augmentation of *P. myxofaciens* to the rhizospheric region enhanced the degradation of M, C & F along with the enhancement of plant growth. There was a considerable increase in the root length and shoot height of rhizoremediation setup as compared to the phytoremediation. A similar study by Ankita Itusha in Ref. [86] also reported the increase in the root and shoot height when PGPR bacteria was augmented in soil treated with pesticides.

3.13. Biodegradation of M, C & F in soil

Phyto-rhizoremediation studies revealed the degradation potential of VITVJ1 in the soil revealed that the rate of M, C & F degradation was rapid at 15 days as compared to other intervals which is due to the growth and survivability of the effective strain in the rhizosphere region. A study by Singh in Ref. [87] described the different types of soil such as clayey, red, sandy and composted soil was used for the endosulfan biodegradation by mixed bacterial culture, the degradation efficiency of 95.48 % in sandy soil was observed. However, in the present study of rhizoremediation treatment of monocrotophos, the degradation rate was up to 93 > 98 > 96 % in 150, 100 and 50 ppm respectively. A study by Paramjeet K. Sidhu in Ref. [88] described monocrotophos degradation when persisted for 17d in the presence of *P. synxanthla* in soil. Whereas, the degradation rate was up to 89 > 92 > 99 % in 150, 100 and 50 in rhizoremediation treatment of cypermethrin. Similarly, Haoyu Zhao in Ref. [89], revealed the biodegradation of cypermethrin in soil by *Catellibacterium* sp. and the degradation efficiency was 97 % in 100 mg/l cypermethrin supplemented soil. In the rhizoremediation treatment of fipronil, degradation rate was up to 92 > 93 > 96 % in 150, 100 and 50 ppm. Rajinder Kumar in Ref. [90] described the fipronil degradation in soil by *Paracoccus* sp. and gamma proteobacteria. *Paracoccus* sp. was best in degradation of soil when compared to gamma proteobacteria. The fipronil residues persist only for 10 days with the supplementation of 20 μg/kg.

Table 6
Quantification data of PGPR traits by VITVJ1.

Assay	VITVJ1
Biofim (tube assay)	+++
Biofim (plate assay)	33 %
Phosphate solubilization (SE)	33.3 %
IAA (μg ml^{-1})	61 μg/ml
Siderophore	1.728 cm
Ammonia production	–

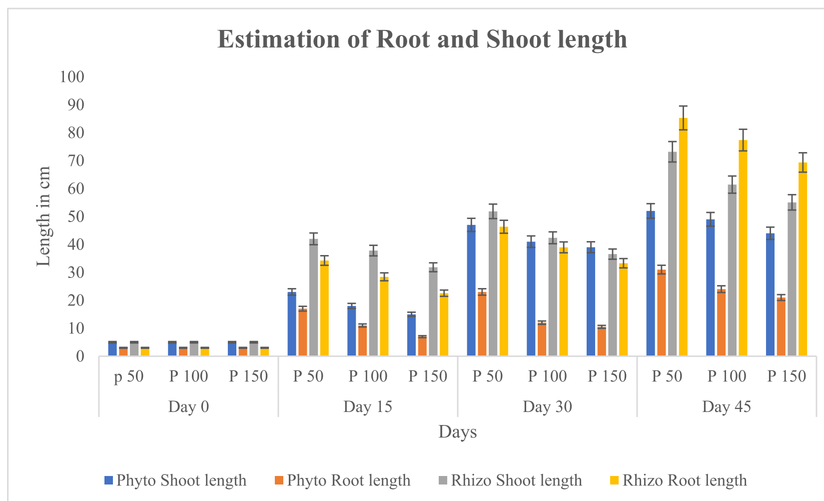

Fig. 8. Estimation of root and shoot length.

Table 7
Metabolites of M, C & F biodegradation identified by GC-MS.

RT	CHE formula	Che name PPB	M/Z
7.327	$C_4H_{10}OS$	1-propanol, 3-(methylthio)-	61
8.543	C_8H_8O	Benzeneacetaldehyde	92
9.083	C_5H_9NO	2-pyrrolidinone, 1-methyl-	98
9.508	C_8H_8O	Phenylethyl Alcohol	92
10.984	$C_6H_{12}O_2S$	Propanoic acid, 2-mercapto-, 1-methylethyl ester	148
13.291	$C_{10}H_{10}O$	1-(2-Vinylphenyl)ethanone	103
13.954	$C_{15}H_{16}O$	Benzeneethanol, .alpha.-(phenylmethyl)-	91
14.205	$C_{17}H_{30}OSi$	Phenol, 2,4-bis(1,1-dimethylethyl)-	57
16.319	$C_{12}H_{14}O4$	Diethyl Phthalate	177
18.764	CH_3COOH	Acetic acid	79
19.205	$C_{10}H_{11}NO$	Tryptophol	161
19.49	$C_{17}H_{24}O_3$	7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-d	71
20.01	$C_{32}H_{54}O_4$	Phthalic acid, nonyl pentadecyl ester	57.1
20.438	$C_{12}H_{30}NO_2$	1H-Indole-3-ethanol, acetate (ester)	130
20.874	$C_{16}H_{14}N_2$	6-Methyl-4-phenyl-quinazoline	57.1

The process of phytoremediation was not found to be effective as compared to rhizoremediation when M, C & F was supplemented to respective treatments. Whereas, rhizoremediation process was a symbiotic relationship between VITVJ1 and *C. zizanioides* which led to cometabolism, thereby reducing the concentration of M, C & F and above 90 % of degradation of all the pesticides (Fig. 10a & b). The mechanism of the pesticide degradation is described in Fig. 11. Ishwar Chandra Yadav in Ref. [91] mentioned that 90 % of the pesticides do not reach the target, instead it gets distributed in the soil contaminating soil, crops, air and water. Therefore, supplementation of VITVJ1 along with vetiver could possibly enhance in the degradation of pesticides present in non-targeted site of the soil [92,93].

4. Conclusion

From the present study it can be concluded that the isolate VITVJ1 (*Proteus myxofaciens*) obtained from polluted agricultural soil

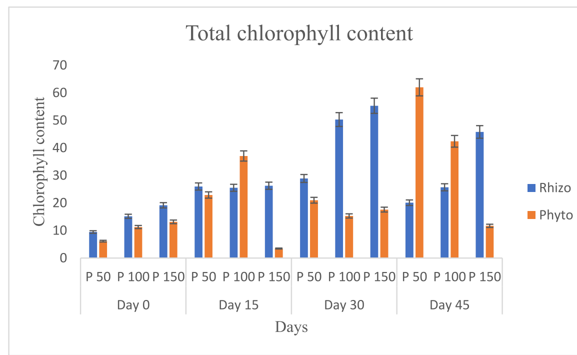

Fig. 9. Total chlorophyll content.

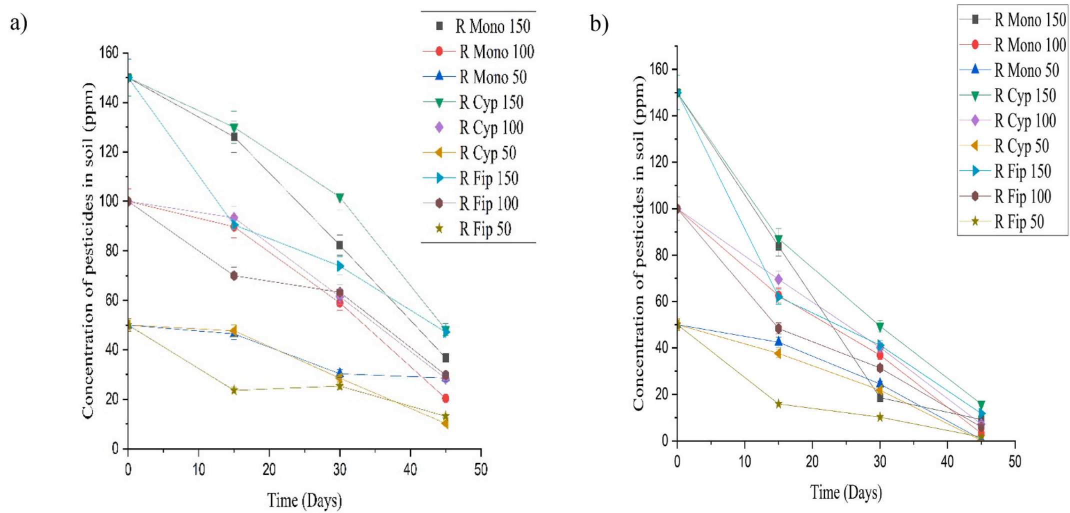

Fig. 10. Analysis of biodegradation of M, C & F in soil a) phytoremediation b) rhizoremediation.

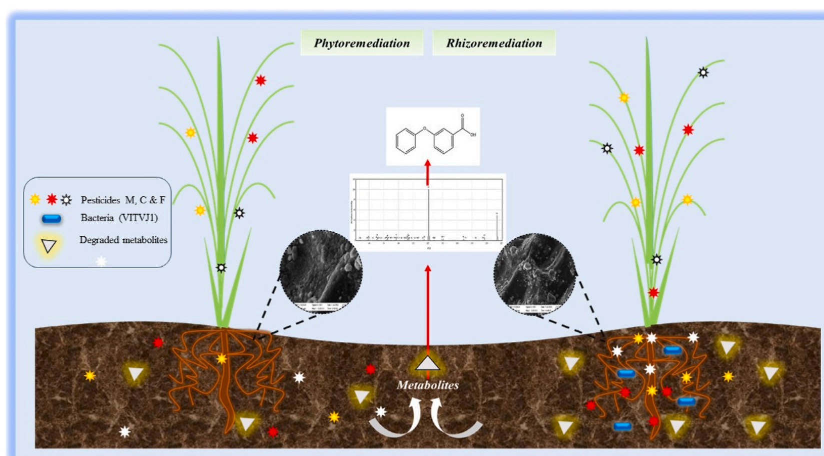

Fig. 11. Mechanism of pesticide degradation.

was capable of resisting and degrading M, C & F at a concentration of 250 ppm. The isolate was also capable of developing biofilm and showed plant growth promoting traits such as indole acetic acid, phosphate and siderophore production. The molecular characterization by 16S rRNA gene sequencing revealed the isolate to be the closest neighbor of VITVJ1. Further, augmentation of effective

strain in pot culture study showed significant potential in degrading M, C & F, especially when VITVJ1 was supplemented to the rhizosphere region of *C. zizanioides*. Therefore, the current strategy i.e. application of plant along with effective strain VITVJ1 can be used for the large-scale degradation of pesticides upon development of formulation using effective strain.

Ethical approval

Not applicable.

Consent to participate

Not applicable.

Consent to publish

Not applicable.

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Data availability statement

Question: Sharing research data helps other researchers evaluate your findings, build on your work and to increase trust in your article. We encourage all our authors to make as much of their data publicly available as reasonably possible. Please note that your response to the following questions regarding the public data availability and the reasons for potentially not making data available will be available alongside your article upon publication.

Has data associated with your study been deposited into a publicly available repository?

Response: No.

Question: Please select why. Please note that this statement will be available alongside your article upon publication. as follow-up to "Data Availability Sharing research data helps other researchers evaluate your findings, build on your work and to increase trust in your article. We encourage all our authors to make as much of their data publicly available as reasonably possible. Please note that your response to the following questions regarding the public data availability and the reasons for potentially not making data available will be available alongside your article upon publication.

Has data associated with your study been deposited into a publicly available repository?

Response: Data included in article/supp. material/referenced in article.

CRediT authorship contribution statement

Jeevanandam Vaishnavi: Writing – review & editing, Writing – original draft, Validation, Methodology, Formal analysis, Data curation, Conceptualization. **Jabez William Osborne:** Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e37384.

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