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Biodegradation of the pyrethroid pesticide cyfluthrin by the halophilic bacterium *Photobacterium ganghwense* isolated from coral reef ecosystem

V. S. JAYASREE*, K. S. SOBHANA, PRIYANKA POULOSE*, KEERTHI R. BABU*, S. JASMINE, L. RANJITH, R. SARAVANAN, H. JOSE KINGSLY, K. R. SREENATH, K. K. JOSHI, RANI MARY GEORGE AND A. GOPALAKRISHNAN

ICAR-Central Marine Fisheries Research Institute, P. B. No. 1603, Ernakulam North P. O., Kochi - 682 018, Kerala, India
**Cochin University of Science and Technology, Cochin University P. O., Kochi - 682 022, Kerala, India*
e-mail: sobhanapradeep11@gmail.com

ABSTRACT

A halophilic bacterial strain T14 isolated from the mucus of coral *Acropora formosa* was found to be highly effective in degrading the pyrethroid pesticide, cyfluthrin. T14 was identified as *Photobacterium ganghwense* (GenBank Accession No. MT360254) based on phenotypic and biochemical characteristics as well as by 16S rRNA gene sequence analysis. The pyrethroid degrading efficiency of *P. ganghwense* T14 strain was examined under different culture conditions. It was observed that *P. ganghwense* T14 was able to utilise cyfluthrin as a sole carbon source and was found to grow on mineral medium with pesticide concentrations ranging from 10 to 100 mg l^{-1}. Optimal temperature and pH conditions for efficient cyfluthrin degradation by *P. ganghwense* T14 were determined as 30°C and 8 respectively. Degradation of cyfluthrin by *P. ganghwense* T14 was quantitated by gas chromatography-tandem mass spectrometry (GC-MS/MS). Mineral medium supplemented with 100 mg l^{-1} cyfluthrin and inoculated with *P. ganghwense* T14 (10^6 cells ml^{-1}) recorded 92.13% pesticide decomposition within 120 h. Cytotoxicity assay on a fish cell line EM4SpEx derived from the grouper *Epinephelus malabaricus,* revealed a drastic reduction in cyfluthrin toxicity as evidenced by reduction in the intensity of cell destruction as well as morphological changes when exposed to *P. ganghwense* T14 treated filtrate, in comparison with that of parent cyfluthrin filtrate. Results of the study clearly indicated potential bioremediative use of *P. ganghwense* T14 in cyfluthrin contaminated sites.

Keywords: Biodegradation, Cyfluthrin, Cytotoxicity, Halophilic bacterium, Pesticide degradation, *Photobacterium ganghwense,* Pyrethroid

Introduction

Tropical marine ecosystems including coral reefs, seagrass meadows and mangrove forests are of major concern when considering the threatening impact of chemical contaminants (Ferguson and Johannes, 1975). Growing urban affluence caused human populations to expand adjacent to these ecosystems causing profound environmental hazards including worsening of water quality and problems of waste disposal (Connell and Hawker, 1991). Both tropical and temperate marine ecosystems share common sources of chemical pollutants primarily the terrestrial runoff from rivers, agricultural, industrial and domestic activities (Esther *et al*., 1997). Near shore coastal areas are severely susceptible to these runoffs, especially during the monsoon season (Lewis *et al*., 2009). Considering the inevitable role of chemical pesticides in agricultural fields and to minimise the hazardous after-effects, use of organochloride pesticides were controlled in the beginning of 2000 leading to a significant increase in the use of pyrethroid pesticides. Owing to the comparatively faster degradation of organic insecticide pyrethrins, their chemical analogs called pyrethroids were synthesised. Nowadays, pyrethroids contribute more than 25% of the world's total pesticide market (Chen *et al*., 2011). Thus the run offs started showing more of the presence of pyrethroids than organochlorides.

Pesticide pollution in aquatic ecosystems attract increasing attention owing to bioaccumulation of pesticides in aquatic organisms, which would lead to food safety and human health issues. Further, the impact of chemical stressors on mangrove forests, seagrass meadows and coral reefs are being studied and discussed seriously in the context of ecosystem management and ecological risk assessment. Among the variety of pollutants assessed in tropical marine ecosystems; heavy metals, petroleum and pesticides received significant attention. Pesticides from agricultural and land runoff can affect coral reproduction, growth and other physiological processes including recruitment of a variety of organisms (Esther *et al*., 1997).

Herbicides, in particular, can destructively interfere with the symbiotic algae resulting in coral bleaching. Until late 1960's, islands and coastal waters of the tropics and subtropics were considered as highly productive, unique and speciose ecosystems. But in early 1970's, as the exploitation of natural resources started extending more towards the tropic regions from the temperates, these economically and culturally valuable pristine ecosystems started declining especially the shallow water tropical marine regions (Johannes and Betzer, 1975). Once pesticides or other chemical pollutants reach the coral vicinity, the response towards the pollutant varies with the coral morphology. For example, pesticide susceptibility of branching species of corals would be more than the massive corals (Stebbing and Brown, 1984). Similarly, large polyped species are more resistant to heavy metal exposure than small polyped species (Scott, 1990). Difference in the orientation, growth form and mucus production of the coral species also have influence on their ability to withstand sediment particles falling on their surfaces (Wijsman, 1974). The lipid layer that covers the coral skeleton can facilitate the direct uptake of lipophilic chemicals like pesticides thereby making the coral colonies more susceptible to contaminants dissolved in seawater or adsorbed on particles.

Published literature suggests occasional presence of pesticides in coral reefs, sometimes in high concentrations. For instance, in a baseline study conducted on the presence of organochloride pesticides in the Great Barrier Reef, an alarming presence of lindane was consistently detected with concentrations of 0.05 to 0.39 ng g^{-1} wet weight in coral reef organisms (Olfson, 1978). Shockingly, coral tissues collected off Florida had the presence of DDT (Dichloro-diphenyl-trichloro-ethane) at 3 to 12 ng g^{-1} wet weight and dieldrin at 0.260 to 0.320 ng g^{-1} wet weight (McCloskey and Chesher, 1971). A frightening observation was that, 100% of the gorgonian corals and 96.6% of the scleractinian corals sampled off the northern Florida Keys showed presence of pesticides at concentrations upto 7.6 mg g^{-1} wet weight (Glynn *et al*., 1989) with chlordane being the most frequently encountered pesticide. Glynn (1995) conducted a study on sediment and biota samples from Pennekamp Coral Reef State Park and Key Largo National Marine Sanctuary, Florida, USA and their results revealed presence of organochloride pesticides in sediments and tissues of sponges, corals, crustaceans and fishes.

Coral reefs harbour a wide array of microorganisms and persistent influx of chemical stressors would cause ecological disturbance on these microbial communities leading to development of resistant strains. Pesticides reaching the coral reefs can have either beneficial or detrimental effect on the associated microflora. Beneficial communities would utilise pesticides as a source of nutrients facilitating their growth and survival whereas sensitive ones might get decimated (DeLorenzo *et al*., 2001). During the course of our study on marine bacterial diversity associated with coral reefs, we isolated two strains (T14 and T25) of the halophilic bacterium, *Photobacterium ganghwense* from mucus of the scleractinian coral *Acropora* spp. Wang *et al*. (2019) reported pyrethroid degradation potential of *P. ganghwense* strain 6046 (PGS6046) isolated from an offshore seawater environment of Ningbo, China and therefore we attempted to probe in to the pyrethroid degradation potential of the two bacterial strains T14 and T25 using cyfluthrin.

Cyfluthrin ($C_{22}H_{18}Cl_2FNO_3$) is a broad spectrum synthetic ester pyrethroid commonly recommended against *Lepidopteran* pests affecting solanaceous crops. Light sensitivity of first generation pyrethroids necessitated a second generation which overcame most of its shortcomings and became a suitable candidate for agricultural, veterinary, greenhouse and other public facilities (Laskowski, 2002). Pyrethroids are the most common active ingredient in commercially available insect sprays and are the domain pesticide for malaria control. Widespread and long-term use of cyfluthrin has led to substantial pesticide residue levels in aquatic ecosystems as well as aquatic products affecting both human and aquatic health (Almakkawy and Madbouly, 1999). Based on their toxicological and physical properties, pyrethroids are categorised into two separate classes *viz*., Type I and Type II. Type I pyrethroids do not have a cyano group and Type II pyrethroids have a cyano group in their structure (Laskowski, 2002). Difficulty in degradation along with its critical environmental impacts has heightened the necessity of an efficient bioremediative strategy for cyfluthrin contaminated sites. The present study investigated the ability of *Photobacterium ganghwense* isolated from coral reef ecosystem to biodegrade cyfluthrin pesticide.

Materials and methods

Chemicals and microbiological media

All chemicals used in the study were of analytical grade and procured form Hi Media India Pvt. Ltd., Mumbai, India. The microbiological media used were procured from BD-Difco, USA. Difco Nutrient agar and Nurient broth were supplemented with 2.2% NaCl to get a final concentration of 3%. Mineral Medium (MM) containing $(NH_4)_2SO_4$ - 2g l^{-1}; $MgSO_4$ - 0.2 g l^{-1}; $CaCl_2$ - 0.01 g l^{-1}; $FeSO_4$ - 0.005 g l^{-1}; $MnCl_2$ - 0.002 g l^{-1}; K_2HPO_4 - 10.5 g l^{-1} and KH_2PO_4 - 4.5 g l^{-1} was prepared in seawater. The pyrethroid pesticide cyfluthrin (Solfac WP 10) having 10% active ingredient was procured from Bayer (India) Ltd., Hyderabad, India.

Sample collection and isolation of bacterial strains

Two bacterial strains designated as T14 and T25 were isolated from coral mucus of *Acropora formosa* and *Acropora hyacinthus* respectively from the coral reef ecosystem in Thoothukudi (8° 59.279 'N and 078° 15.008 'E). Predominant colonies observed growing on Difco Nutrient Agar (NA) medium, having light cream, opaque, round and bulged appearance were subjected to further investigations. Well isolated single colonies were re-streaked several times on NA plates so as to obtain pure colonies. The isolated strains were routinely cultivated aerobically at 28±2°C in Difco Nutrient Agar medium and were preserved in double strength Nutrient Broth with 50% glycerol at -80°C and also in semisolid agar at room temperature using mineral oil overlay method (Hartsell, 1953).

Phenotypic and biochemical characterisation

The isolated bacterial colonies were identified on the basis of their morphological, physiological and biochemical characteristics following Baumann and Baumann (1984). Unless otherwise stated, all of the biochemical and physiological tests used Difco Nutrient Agar as the basal medium and for all the tests performed, incubation was carried out at 28±2°C for 48 h.

Molecular and phylogenetic analysis

DNA extraction, PCR 16S rDNA amplification and sequencing

Bacterial genomic DNA was extracted using standard phenol chloroform extraction method (Sambrook and Russell, 2006). 16S rRNA gene was amplified with the universal primer pair 27F (5' AGAGTTTGATCCTGG CTCAG 3') and 1492R (5' TACGGCTACCTTGTTACGACT T 3'). The reaction mixture had 10 pmol of each primer, 50-100 ng of DNA template and 12.5 µl of 2xDream Taq Green PCR Master Mix (Thermo Fischer). DNA was amplified by PCR cycling profile comprising an initial denaturation at 94°C for 5 min followed by 35 cycles of denaturation at 94°C for 60 s, annealing at 58°C for 60 s, elongation at 72°C for 75 s and a final extension at 72°C for 10 min (Karlsen *et al*., 2014) in a Thermal cycler (Applied Biosystems, USA). The PCR products were subjected to agarose gel electrophoresis, stained with ethidium bromide (EtBr) and visualised in a gel documentation system (Azure Biosystems, USA). The amplified products were sequenced in both forward and reverse directions with the same set of primers in Applied Biosystems AB 3730 capillary sequencer at the sequencing facility. The raw DNA sequences were edited using BioEdit sequence alignment editor version 7.0.5.2 (Hall, 1999). Sequence analysis for homology comparison used the European Nucleotide Archive, ENA (https://www.ebi.ac.uk/ena); Ribosomal Database Project, RDP (https://rdp.cme.msu.edu); EzBioCloud16S database (www.ezbiocloud.net) and National Centre for Biotechnology Information GenBank Database (using the Basic Local Alignment Search Tool, BLAST). For species level identification, only similarity score ≥99% with the reference sequence of a classified species was considered. A phylogenetic tree was constructed by neighbour-joining method using MEGA X software (Tamura, 2011). Distance matrices were calculated using Kimura's 2-parameter correction followed by performing bootstrap analysis (1000 replicates) to check the robustness of groupings.

Optimisation of cyfluthrin degradation test

In order to assess the cyfluthrin degrading potential of the bacterial strains T14 and T25, both strains were allowed to grow in a medium containing cyfluthrin. Cyfluthrin stock (100 mg l^{-1}) was prepared by emulsifying in sterile distilled water at 20°C for maximum solubility (Tomlin, 1997) using a sterile disposable syringe holding a 21 guage needle. Bacterial cells at 10^6 cells ml^{-1} were inoculated in 50 ml of mineral medium (MM) (pH - 7.0) supplemented with cyfluthrin at concentrations of 0, 10, 20, 50 and 100 mg l^{-1} (each dose in triplicates) which were incubated at 28°C (pH 7) on a rotary shaker at 180 rpm for 120 h (5 days) and the bacterial cell density was monitored by measuring OD at 600 nm (OD_{600}) at every 24 h interval (Grant *et al*., 2002) in a spectrophotometer (Nanodrop 1C; Thermo Scientific, USA).

The strain T14 showing highest biomass increase in the presence of cyfluthrin was selected and growth conditions (temperature and pH) were optimised for this strain to facilitate further analyses. For optimisation of temperature, cells of the selected strain T14 (10^6 cells m l^{-1}) was inoculated in MM (pH 7) supplemented with 100 mg l^{-1} cyfluthrin (which showed highest growth of T14 strain among the 4 different concentrations tested) and incubated at different temperatures (25, 30, 37 and 45°C) at pH 7 and the cell density was monitored by measuring OD_{600} at 24 h interval up to 120 h. Subsequently, optimal pH for growth was assessed by monitoring bacterial cell density in MM at varying pH levels (6, 7, 8 and 9), supplemented with 100 mg l^{-1} cyfluthrin and incubated at 30°C (which recorded the highest growth of T14 strain, among the 4 different temperatures tested). Growth was monitored by measuring OD_{600} at 24 h interval up to 120 h.

Evaluation and quantification of in vitro cyfluthrin degradation by P. ganghwense T14

Efficacy of cyfluthrin degradation by T14 strain was evaluated by inoculating 10^6 cells ml^{-1} in 50 ml MM at optimised pH (8), supplemented with 100 mg l^{-1} cyfluthrin and incubated for 120 h at the optimised temperature of 30°C (Test T). Degradation test was performed in amber bottles and incubated in dark condition in order to extend the half life of cyfluthrin. MM inoculated with bacterial strain T14 (10^6 cells ml^{-1}) without cyfluthrin (Control C1) and uninoculated MM supplemented with 100 mg l^{-1} cyfluthrin (Control C2) were maintained as controls and incubated under similar conditions. At every 24 h of incubation, cell density was monitored by measuring OD_{600}. At the end of 120 h of incubation period, the samples were centrifuged at 2500 *g* for 10 min and the supernatants were collected and filtered sequentially in 0.45 µm and 0.22 µm filters using sterile membrane filtration units (Millipore, USA), so as to ensure complete removal of bacterial cells. The filtrates were stored in amber bottles under refrigerated conditions until further analyses. The treated filtrate from test T along with the control filtrate C2 were analysed by gas chromatography-tandem mass spectrometry (GC-MS/MS) to quantify cyfluthrin content post-treatment, in order to ascertain cyfluthrin degradation efficacy of *P.ganghwense* T14. The filtrates from T, C1 and C2 were also tested on a fish cell line to evaluate comparative cytotoxicity, in order to test if there is any reduction in cyfluthrin cytotoxicity owing to T14 treatment.

GC-MS/MS analysis

Analysis was performed as per EN 15662:2009 QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) method (Anastassiades *et al*., 2003; AOAC 2007; AFNOR, 2009) which is based on liquid-liquid partitioning with acetonitrile followed by a cleanup step with dispersive-SPE (Solid Phase Extraction). Ten microlitre of acetonitrile cooled at -18°C was added to 10 ml of sample (filtrate), vortexed for 1 min and then 4 g of $MgSO_4$, 1 g of NaCl, 1 g of $C_6H_3Na_3O_7 \cdot 2H_2O$ and 1 g of $C_6H_6Na_2O_7 0.1 \cdot 5H_2O$ were added followed by manual shake for 1 min and centrifugation at 3500 rpm for 8 min. After this, a LTP step was applied to precipitate interferences where 3 ml of the supernatant was transferred to a 15 ml tube and placed in freezer (-18°C) for 3 h. For d-SPE clean-up step, 2 ml of the resultant supernatant was transferred to another 15 ml tube containing 300 mg of $MgSO_4$, 100 mg of primary secondary amine (PSA), 100 mg of graphitised carbon black (GCB) and 100 mg of C18. The tube was vortexed (1 min) and centrifuged at 3500 rpm for 8 min. Gas chromatography analysis coupled to tandem mass spectrometry (GC-MS/MS) was used for determination in an Agilent 7000 S GCMS TRIPLE QUAD GC SYSTEM 7890. Internal standard (Heptachlor epoxide B) was added at a concentration of 1 µg ml^{-1} in both extracts, prior to injection. Detector used was Q Trap MS/MS system. The chromatograms were recorded and analysed. Cyfluthrin degradation rate was calculated using the formula:

$$\text{Degradation rate (\%)} = [(C_{C2} - C_T)/C_{C2}] \times 100$$

where, C_{C2} and C_T represent cyfluthrin concentration in C2 and T filtrates, respectively.

Comparative cytotoxicity evaluation of T, C1 and C2 filtrates using fish cell line EM4SpEx

EM4SpEx cell line derived from *Epinephelus malabaricus* developed at ICAR-Central Marine Fisheries Research Institute (ICAR-CMFRI), Kochi was used for cytotoxicity evaluation of the filtrates. Initially, to check cyfluthrin induced cytotoxicity on EM4SpEx cell line, cell monolayers (at 90% confluency) were treated with different concentrations of the pesticide cyfluthrin (0.25, 0.5 and 1µg m l^{-1} of the active ingredient) prepared in Leibovitz L-15 [containing 2% foetal bovine serum (FBS) and 20 mm HEPES buffer] in 6-well tissue culture plates (in triplicates) along with control cells without exposure to pesticide treatment and incubated at 28°C in dark for 72 h (3 days) in a refrigerated incubator (Barnstead-Labline, USA). Cells were monitored under phase contrast objective of an inverted microscope (Nikon TS100). Evidence of cytotoxicity in the form of cell destruction was observed in all the treatments in a dose-dependent manner to cyfluthrin exposure and complete cell destruction was observed in 72 h. Control cells remained intact without any cell damage. Subsequently, filtrates from T, C1 and C2 were used for evaluating comparative cytotoxicity on EM4SpEx cell line. EM4SpEx cell monolayers (at 90% confluency) were treated with 500 µl of filtrate from T, C1 (no cyfluthrin) and C2 in L-15 medium (supplemented with 2% FBS and 20 mm HEPES) along with control cells (C0) without addition of filtrate (inoculated with 500 µl of sterile MM alone) and incubated at 28°C in dark for 72 h in a refrigerated incubator. All the treatments were done in triplicate wells in 6 well plates. Changes in morphology of the cells were monitored under inverted microscope under phase contrast objective. Photomicrographs were taken after 72 h of incubation and cell morphologies were compared.

Statistical analysis

Statistical analysis of the data was performed using one-way analysis of variance employing SPSS software ver. 20.0 (SPSS Inc., Chicago, Illinois, USA) and $p<0.05$ was considered statistically significant.

Results and discussion

Isolation and identification of bacterial strains

Phenotypic and biochemical characteristics

Both the bacterial strains isolated *viz.,* T14 and T25 showed growth in nutrient agar and formed slightly cream coloured circular (3 mm dia) smooth convex shiny and opaque colonies of entire margin, when incubated for 48 h at 30°C (Fig. 1a, b). Both strains are motile, aerobic Gram negative rods which grows only at NaCl concentration ranging from 1 to 7% with optimum being 6%. Based on phenotypic and biochemical characteristics, both T14 and T25 strains were identified as *Photobacterium ganghwense* (Table 1). Characteristics of the type strain *P. ganghwense* FR1311^T were used for taxonomic comparison.

The type strain *P. ganghwense* sp. nov. FR1311^T reported by Park *et al*. (2006), had optimum temperature and pH as 35^{0}C and 8 - 9 respectively. Also it did not grow below 10 °C and above 45°C and had a pH range between 5-11. But for T14 and T25 strains, optimum growth was observed at a comparatively lower temperature of 30°C; however, pH tolerence was similar to the type strain *i.e.,* between 5-11 with optimum at 8, above pH 11 no growth was observed. Both strains T14 and T25 exhibited significant morphological, physiological and biochemical similarities among themselves and with the type strain. Difference in the temperature optimum can be attributed to the variability of sources. Agler *et al*. (2016) reported on the genetical and physiological acclimatisation of microbiota in response to various biotic and abiotic factors.

Molecular characterisation and phylogenetic analysis

Based on 16S rRNA gene sequence analysis and homology search using BLAST in the NCBI GenBank database as well as with the EZ Biocloud, ENA and RDP nucleotide sequence databases, the strains T14 and T25 were identified as *P. ganghwense.* The genus *Photobacterium* was first described by Beijerinck in 1889. Based on phylogenetic analysis, *Photobacterium* genus showed close relation to the genus *Vibrio* (Anzai *et al*., 2000). *P. ganghwense* sp. nov. (designated as strain FR1311^T) was first described by Park *et al.* (2006) as a halophilic bacterium isolated from seawater samples from Ganghwa Island, South Korea. Our strains T14 and T25 have similarity scores of 99.79 and 99.58% respectively, with the type strain *P. ganghwense* FR1311^T. Sequences of both the strains T14 and T25 were deposited in NCBI GenBank with Accession Nos. MT360254 and MT360255 respectively. Fig. 2 depicts the phylogenetic tree constructed by neighbourjoining method using MEGA X software.

Optimisation of conditions for cyfluthrin degradation

Of the two strains of *P. ganghwense* tested, T14 was found to have better efficiency in cyfluthrin utilisation for growth which was noticeable from a dose dependent increase in cell density of T14 at all the four concentrations

(a) (b)

Fig. 1. Colony morphology of the bacterial strains (a) T14 and (b) T25 on Nutrient Agar plates

Table 1. Phenotypic and biochemical characteristics of *P. ganghwense* strains T14 and T25 in comparison with Type strain *P. ganghwense* FR1311^T

Phenotypic and biochemical tests	*P. ganghwense* T14 (GenBank Acc. No. MT360254)	*P. ganghwense* T25 (GenBank Acc. No. MT360255)	Type strain (*P. ganghwense* FR1311^T)
Gram reaction	-ve	-ve	-ve
Cell morphology	Rod	Rod	Rod
Swarming on solid media	-ve	-ve	-ve
Growth on TCBS agar	+ve (Green colony)	+ve (Green colony)	+ve (Green colony)
Motility	+ve	+ve	+ve
VP*	-ve	-ve	-ve
MR**	+ve	+ve	+ve
Indole	+ve	+ve	+ve
ONPG	+ve	+ve	+ve
Esculin hydrolysis	-ve	Slight +ve	-ve
Sodium gluconate	-ve	-ve	-ve
Glycerol	-ve	-ve	-ve
Citrate utilisation	+ve	+ve	+ve
Nitrate reduction	+ve	+ve	+ve
Growth in % NaCl			
1	+ve	+ve	+ve
3	+ve	+ve	+ve
5	+ve	+ve	+ve
7	+ve	+ve	+ve
8	-ve	-ve	-ve
Growth at (°C)			
4	-ve	-ve	-ve
10	+ve	+ve	+ve
30	+ve	+ve	+ve
35	+ve	+ve	+ve
45	+ve	+ve	+ve
50	-ve	-ve	-ve
Growth at pH			
4	-ve	-ve	-ve
5	+ve	+ve	+ve
7	+ve	+ve	+ve
11	+ ve	+ ve	+ ve
12	- ve	- ve	- ve
Amino acid utilisation			
Arginine	+ve	+ve	+ve
Ornithine	-ve	-ve	-ve
Lysine	-ve	-ve	-ve
Phenylalanine deaminase	-ve	-ve	-ve
Tryptophan deaminase	-ve	-ve	-ve
Acid from sugar			
Adonitol	-ve	-ve	-ve
Arabinose	-ve	-ve	-ve
Arabitol	Ve	- ve	-ve
Cellobiose	-ve	-ve	-ve
D-Arabinose	-ve	-ve	-ve
Dextrose	+ve	+ve	+ve
Dulcitol	-ve	-ve	-ve
Erythritol	-ve	-ve	-ve
Fructose	+ve	+ve	+ve
Galactose	+ve	+ve	+ve
Inositol	+ve	+ve	+ve
Inulin	-ve	-ve	-ve

*Voges-Proskauer; **Methyl Red

Contd.....

Phenotypic and biochemical tests	*P. ganghwense* T14 (GenBank Acc. No. MT360254)	*P. ganghwense* T25 (GenBank Acc. No. MT360255)	Type strain (*P. ganghwense* FR1311^T)
L-Arabinose	-ve	-ve	-ve
Lactose	-ve	-ve	-ve
Malonate	-ve	-ve	-ve
Maltose	+ve	+ve	+ve
Mannitol	+ve	+ve	+ve
Mannose	weak +ve	+ve	weak +ve
Melibiose	-ve	-ve	-ve
Melzitose	-ve	-ve	-ve
Raffinose	-ve	-ve	-ve
Rhamnose	-ve	-ve	-ve
Salicin	-ve	-ve	-ve
Sorbitol	-ve	-ve	-ve
Sorbose	-ve	-ve	-ve
Sucrose	-ve	-ve	-ve
Trehalose	+ve	+ve	+ve
Xylitol	-ve	-ve	-ve
Xylose	-ve	-ve	-ve
α-methyl-D-Glucoside	-ve	-ve	-ve
α- methyl-D-Mannoside	-ve	-ve	-ve
Enzyme production			
Urease	-ve	-ve	-ve
α-Glucoronidase	-ve	-ve	-ve
α-Galactosidase	-ve	-ve	-ve
β-Xylosidase	-ve	-ve	-ve
Gelatinase	+ve	+ve	+ve
Lipase	-ve	-ve	-ve

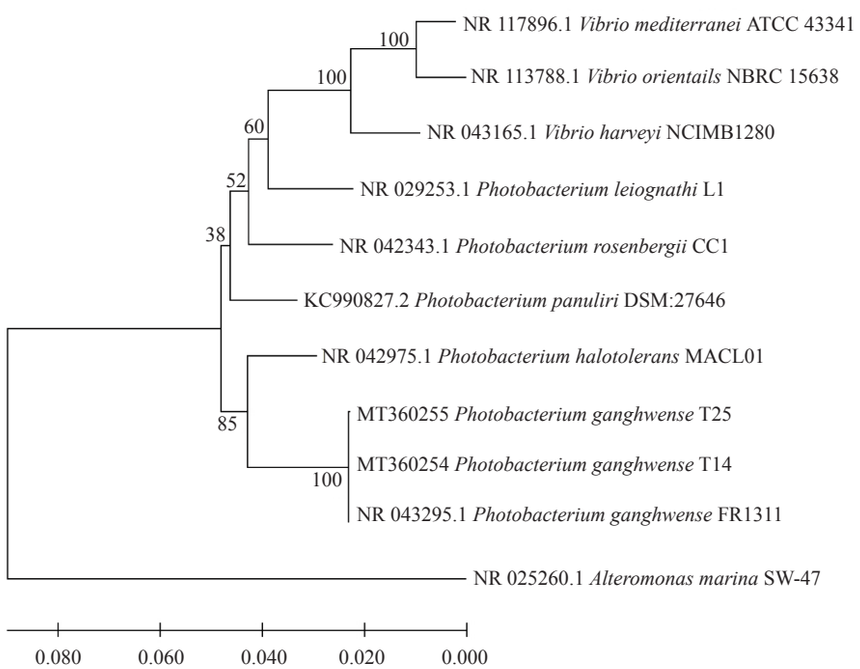

Fig. 2. Neighbor-joining phylogenetic tree based on 16S rRNA gene sequences of *P. ganghwense* T14 and T25 strains. Evolutionary distances were computed using Kimura's 2-parameter method with 1000 bootstrap replicates in MEGA X. Scale bar indicates mean number of substitutions per base. Bootstrap values are displayed at branch points

(10, 20, 50 and 100 mg l^{-1}) of cyfluthrin tested. The cell biomass in all the treatments peaked at 48 h after which a reduction in growth was observed. The highest biomass for both the bacterial strains was recorded at 100 mg l^{-1} cyfluthrin concentration and the lowest for control samples without cyfluthrin (0 mg l^{-1}) (Fig. 3). For the strain T25, increase in cell density was comparatively less as compared to T14 as evident from Fig. 3. In the control samples, which were grown in MM without cyfluthrin (0 mg l^{-1}), both T14 and T25 showed only limited growth (Fig. 3). T14 and T25 exhibited higher growth at all the tested concentrations of pesticide *viz.*, 10, 20, 50, 100 mg l^{-1} with T14 exhibiting comparatively higher growth than T25 at all tested concentrations. Though growth of T14 and T25 were observed in MM even in the absence of cyfluthrin, growth increased by 46 and 33% respectively when cyfluthrin was added. Also the difference in growth of both T14 and T25 in the presence and absence of cyfluthrin was found to be statistically significant ($p<0.05$). This implies the significant role that cyfluthrin plays as the sole carbon source in an otherwise carbon deficit medium (Chen *et al*., 2013). Therefore, for further quantification of cyfluthrin degradation, tests were performed using T14 strain with 100 mg l^{-1} initial cyfluthrin concentration.

After deciding on the strain to be tested (T14) and the concentration to be selected (100 mg l^{-1}), temperature and pH conditions for cyfluthrin degradation were optimised. Park *et al*. (2006) reported the optimum pH of *P. ganghwense* strain FR1311^T as 35°C and pH 8-9. Growth of *P. ganghwense* T14 varied at different temperatures with highest ($p<0.05$) growth recorded in cyfluthrin medium when incubated at 30°C (Fig. 4) which was found to be same as the optimum temperature for the strain in normal growth medium in the absence of cyfluthrin. Temperature plays a crucial role in controlling pyrethroid degradation. Studies by Lin *et al*. (2011) proved that in the biodegradation process, the pesticide degradation rate is consistent with the growth of the strain (Zhang *et al*., 2016). For the degradation to be maximum, growth should be at the highest levels, for which the growth conditions need to be optimum. *Streptomyces* sp. strain HU-S-01 exhibited a degradation rate of about 90% when grown at its optimum temperature range of 26-28°C, whereas above 34°C, the degradation showed a drastic dip to 10% (Lin *et al*., 2011). Similar was the case of *Catellibacterium* sp. CC-5 (Zhao *et al*., 2013), with highest degradation rate observed at the greatest growth of the strain. Temperature dependent degradation studies conducted by Zhang *et al*. (2016) showed that both high and low temperatures are detrimental to pyrethroid degradation process.

pH is another primary factor determining the rate of pyrethroid biodegradation (Chen *et al*., 2012). On optimising the pH conditions of T14, pH of 8 was found to be most favourable for bacterial growth in cyfluthrin medium (Fig. 5). The highest degradation rate at pH 8 could be attributed to the optimum growth of T14 at this pH, as the growth and degradation rate are found to be concomitant factors (Zhao *et al*., 2013). However, growth was observed at all pH levels tested from 6 to 9, with pH 7 and 8 appearing slightly closer in cell densities with highest ($p<0.05$) being at pH 8. The type strain of *P. ganghwense* FR1311^T grows well at pH range of 5-11 with optimum at pH 8-9. T14 strain also showed pH tolerance almost in the same range. Outside this pH tolerance range, the strain would become inactive leading to poor degradation (Zhao *et al*., 2013). Biodegradation studies in liquid media usually favour neutral conditions (Chen *et al*., 2012). However, adaptive pH of the test strain is a critical factor in this regard. For *eg. Streptomyces* HU-S-01 has an adaptive pH value between 6 and 9 with highest

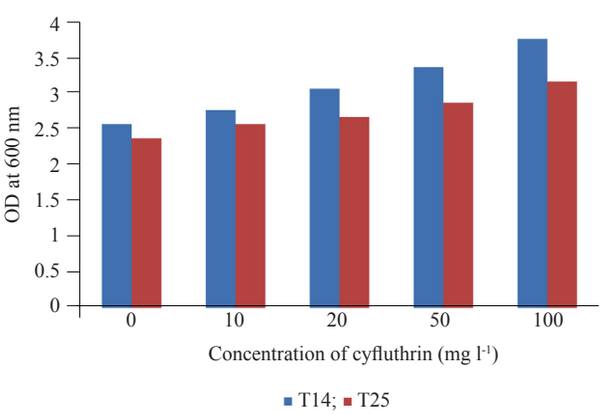

Fig. 3. Growth of *P. ganghwense* T14 and T25 strains in terms of OD_{600} measured after 48 h of incubation, at varying cyfluthrin concentrations (mg l^{-1})

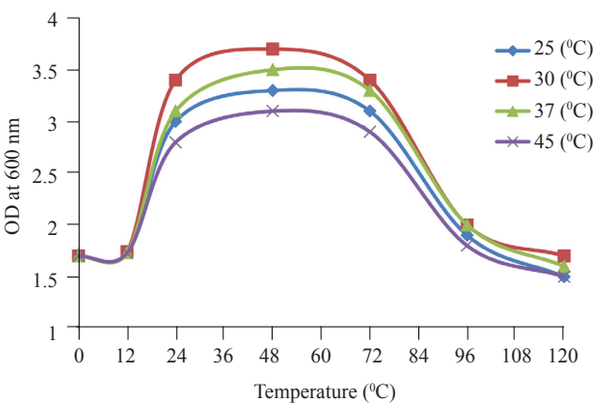

Fig. 4. Growth (in terms of OD_{600} measured after 48 h of incubation), of *P. ganghwense* T14 in mineral medium (pH 7) in presence of 100 mg l^{-1} cyfluthrin at varying temperature levels

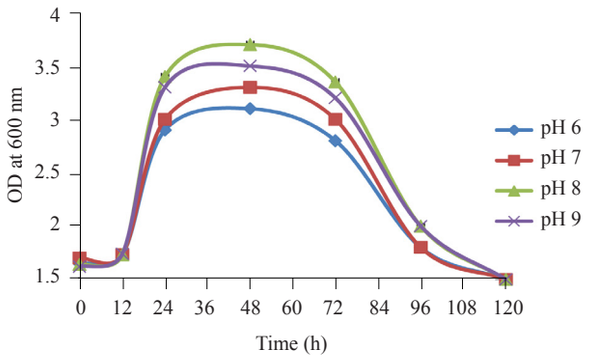

Fig. 5. Growth (in terms of OD_{600} measured after 48 h of incubation at 30^{0}C), of *P. ganghwense* T14 in mineral medium in presence of 100 mg l^{-1} cyfluthrin at varying pH levels

degradation level shown between pH 7-8, with optimum being at pH 7.5 (Chen *et al*., 2012). At pH 5.5 and 10, degradation rates were insignificant. Cypermethrin removal by *Catellibacterium* strain CC-5 was highest at pH 7 followed by 8 and then 6. Below pH 6 and above pH 8, degradation rate was not appreciable (Zhao *et al*., 2013). Certain strains like *Ochrobactrum* DG-S-01 (Chen *et al*., 2011b) and *Stenotrophomonas* strain ZS-S-01 (Chen *et al*., 2011c) showed pyrethroid degradation in a wide pH range from 5-9, with highest degradation rate showed at the highest pH (Chen *et al*., 2011b). Studies have proved that rate of pyrethroid degradation hastens at neutral and alkaline conditions whereas at acidic pH, the process of degradation either stops or delays (Zhang *et al*., 2016). The low degradation rate is attributed to the increase in stability of pyrethroids at acidic pH making them resistant to microbial degradation (Chen *et al*., 2015). Furthermore, it should be noticed that at all the temperature and pH levels tested, growth rate was higher in cyfluthrin supplemented medium than in the control, which further substantiates the significance of cyfluthrin in bacterial growth.

In addition to pH and temperature, several other factors come to role when discussing the factors involved in pyrethroid biodegradation in liquid media. One important factor among these is the addition of carbon source which was reported to have both accelerating (Chen *et al*., 2012) as well as decelerating effects (Tiwary and Dubey, 2016) on degradation rates. For *e.g.* presence of glucose, beef extract and yeast extract enhanced the degradation rate of β-cypermethrin by *Ochrobactrum lupini* DGS-01 by approximately 8% (Chen *et al*., 2011b). Also pyrethroid degrading *Cladosporium* sp. HU (Chen *et al*., 2011a), *S. aureus* HP-S-01 (Chen *et al*., 2011c) and *Bacillus amyloliquefaciens* AP01 (Lee *et al*., 2016) have shown positive response to the addition of carbon source. Here, the growth rate of the pyrethroid degrading bacteria was found to be increased by the co-metabolism with another carbon source leading to enhanced degradation rate. In contrast, biotransformation of cypermethrin by *Bacillus* sp. strain AKD1 and the half-life of cypermethrin, was found to be suppressed by the additional carbon source indicating the inhibitory effect of added compound on pesticide degradation (Tiwary and Dubey, 2016). This inhibition could be related to the decrease in pH caused by the added carbon source (Cycon *et al.,* 2016). Pyrethroid degrading microorganisms were usually unable to initiate utilisation of pesticides as the sole carbon and energy source and therefore additional carbon sources (*e.g.* sucrose, glucose) were needed to start degradation (Lin *et al*., 2011). The ability of the strain T14 to utilise cyfluthrin as a carbon source, along with its potential to initiate proliferation without any other added carbon source indicates its excellent environmental adaptation and suitability as a bioremediant. This is indeed an important feature, of a pesticide degrading microorganism required to be employed for bioremediation in variable contaminated environments.

Fig. 6 depicts the growth curve of T14 (T) in MM supplemented with 100 mg l^{-1} cyfluthrin in comparison with control medium (C1) without supplementation of cyfluthrin at the optimised temperature and pH conditions of 30°C and 8 respectively. Statistically significant differences were observed between the growth of T14 and control ($p<0.05$). It was observed that, in test samples supplemented with cyfluthrin, after a lag phase of about 12 h, the isolate grew rapidly for 36 h and reached its maximum cell density in 48 h and later on declined. Similarly, in the control samples also, bacterial cell density reached maximum in 48 h but with lower biomass as compared to test samples. As the inoculum used in the control was already in the log phase, in the control samples bacteria could easily adapt to the new environment with shorter lag phase. However for test group, a 12 h long lag phase was observed which could be attributed to the acclimatisation time for the bacteria to the high cyfluthrin concentration in the medium. There are reports on the use of pesticides as nutrients by microorganisms and their enzymatic conversion into non-toxic derivatives (Tang, 2018). *O. lupini* was reported to degrade 80% β-cypermethrin when grown in liquid media without any carbon source (Chen *et al*., 2011).

Size of the inoculum is another important concern in the application level. For all the experiments discussed above, the bacterial cells were inoculated at one ml of 10^8 cells ml^{-1} in 50 ml of mineral medium (MM). Studies have proved that microorganisms that are capable of degrading pyrethroids, when inoculated at levels of 10^6-10^{10} cfu ml^{-1}, exhibit an elevated degradation rate and reduced pesticide

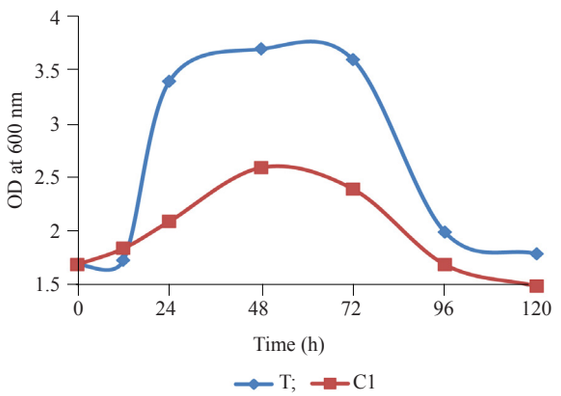

Fig. 6. Growth curve of *P. ganghwense* T14 in mineral medium supplemented with 0 mg l^{-1} (C1) and 100 mg l^{-1} (T) cyfluthrin at pH 8 and temperature 30°C

half-life when compared to the controls (Zhang *et al*., 2016). At lower inoculum densities, the survival rate of the inoculum was found to be lower (Karpouzas and Walker, 2000). This initial decline in inoculum level can be overcome by a higher initial dose so that the survivors can proliferate and degrade pesticide (Karpouzas and Walker, 2000). Cycon *et al*. (2014) have reported that adaptation of the bacterial strain to the high pyrethroid concentration, soil conditions and use of pyrethroid as the carbon source by indigenous bacteria would lead to an initial lag phase even under high inoculum level. Also a high pyrethroid concentration was found to be an important factor influencing microbial growth (Karpouzas and Walker, 2000; Cycon *et al*., 2014).

Apart from the above discussed factors, another most important element for critical consideration that limits the degradation rate is the metabolic byproducts that arise from the parental compound. 3-phenoxybenzaldehyde (PBA) was found to be a common toxic byproduct in almost all Type II pyrethroid degradation process (Chen *et al*., 2011a; Bhatt *et al*., 2016). The antimicrobial property exhibited by this compound would prevent the bacterial proliferation leading to incomplete degradation (Cycon *et al*., 2014). However, this is found to be absent in Type I pyrethroids. In almost all Type II pyrethroids, intermediate PBA is further degraded with diaryl cleavage to form lesser toxic 1, 2-benzenedicarboxylic butyl dacyl ester (Jingjing Yang, 2018).

Chen *et al*. (2013) observed an elevated growth rate of *Brevibacterium aureum* when grown in nutrient rich cyfluthrin medium than in seawater and they opined that the medium of bacterial growth plays a key role in the pesticide degradation process. The authors attributed the exalted growth to the presence of other nutrients and thus cyfluthrin was found to have little effect on bacterial growth when grown in nutrient rich media. Nevertheless, the bacterial growth was higher in seawater with cyfluthrin than in seawater alone (Wang *et al*., 2019). This implies the utilisation of cyfluthrin as a growth nutrient when grown in carbon deficit medium. Therefore, in our study, the bacteria was intentionally put in a nutrient deficit medium *i.e.,* seawater based mineral medium where they had to depend solely on cyfluthrin for the provision of carbon. When the medium is nutrient rich, cyfluthrin would have little effect on bacterial metabolism (Wang *et al.*, 2019). Therefore in bioremediation processes, use of nutrient rich medium would prolong the remediation time as the bacteria would start utilising cyfluthrin only when the other carbon sources become scarce.

Evaluation and quantification of cyfluthrin degradation by GC-MS/MS analysis

Post-120 h incubation, the treated filtrates of T along with the control filtrate C2 were analysed by GC-MS/MS technique to determine the cyfluthrin content. The concentrations of cyfluthrin in C2 filtrate was estimated as 98.2 mg l^{-1} by GC-MS/MS analysis showing 1.8% reduction as compared to the initial level of 100 mg l^{-1}, which could be attributed to auto degradation of cyfluthrin. In filtrate T, cyfluthrin concentration estimated was only 7.72 mg l^{-1}. Fig. 7 shows the GC-MS/MS chromatogram of T filtrate post-120 h treatment of cyfluthrin media with T14 strain. A degradation rate of 92.13% was observed within 120 h (5 days) treatment using 100 mg l^{-1} cyfluthrin concentration calculated using the formula mentioned earlier with C_{C2} as 98.2 mg l^{-1} and C_T as 7.72 mg l^{-1}, which offers an attractive potential of the strain as an effective bioremediative agent in cyfluthrin removal. The half-life of cyfluthrin was reported to be varying with environmental conditions. It has a half-life of about 193 days in water in the absence of sunlight and 12 days under exposure to sunlight. Whereas in soil without oxygen, it takes 34 days and 56 days with oxygen (Casjens, 2002). In the present study, the test was performed in liquid sample under dark condition for 5 days and the loss of cyfluthrin *via* auto-degradation recorded was only 1.8% which can be considered as meagre. It has been reported that the compounds released during the stationary phase also could increase the solubility of the pesticide thereby enhancing the degradation rate (Vyas and Dave, 2011). On measuring the OD_{600}, T and C1 displayed a growth pattern similar to Fig. 6. Obviously, no variation in optical density was observed in uninoculated sample C2.

Cyfluthrin belongs to Type II pyrethroids which have cyano group in their structure (Laskowski, 2002). Metabolomic assessment studies of cyfluthrin degradation by *P. ganghwense* strain PGS6046 elucidated the absence of cyano group in its degradation products which clearly

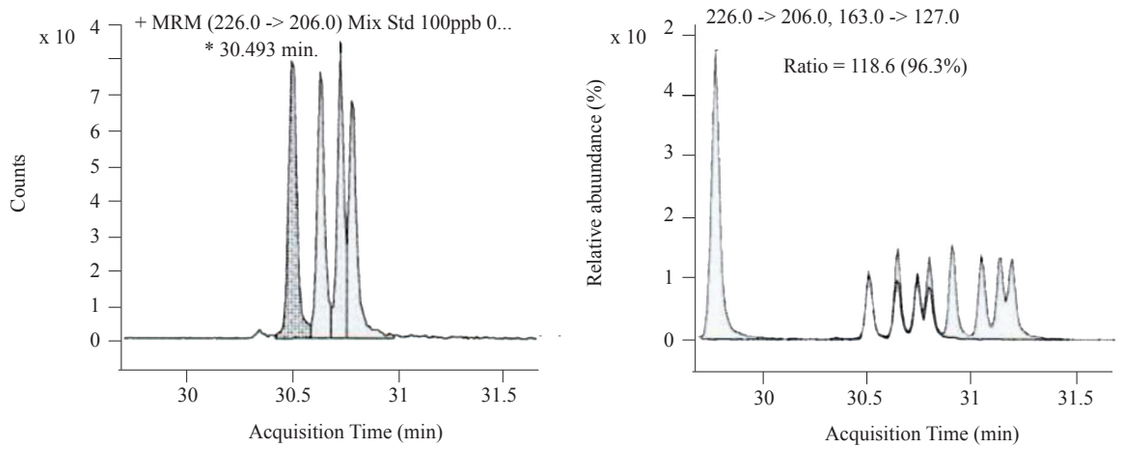

Fig. 7. Extracted ion chromatogram of T filtrate post-120 h treatment of cyfluthrin media with *P. ganghwense* T14 strain from GC-MS/MS analysis. (a) with qualifier and (b) with qualifier and quantifier

indicates the use of cyano group in cyfluthrin as the carbon source leading to its degradation (Wang *et al*., 2019). Thus cyfluthrin was reported to act as a carbon source to *P. ganghwense* when grown in a carbon deficit medium (Wang *et al*., 2019). It was also reported that the cyano group in cyfluthrin is microbiologically metabolised to form cyanoalanine (Yoshikawa *et al*., 2000). Therefore, the reduction in cyfluthrin content when treated with T14 could be possibly due to the utilisation of cyano group in the pesticide. The nature of the byproduct formed by this degradation and its future ecological impacts needs to be studied further. Wang *et al*. (2019), investigated on the metabolic profile of *P. ganghwense* strain 6046 (PGS6046) during cyfluthrin degradation and identified 156 metabolites, none of which were found to be toxic. The type of medium, concentration of cyfluthrin and time of incubation were found to have significant influence on the metabolic profile (Wang *et al*., 2019). Chen *et al.* (2013) reported *B. aureum* DG-12 having 87.4% cyfluthrin degradation (50 mg l^{-1}) potential within a period of 5 days. Chen *et al.* (2015) demonstrated that the degradation rate is higher when the initial concentration of cyfluthrin is lower. As per the reports, degradation rate would further rise if a lower initial concentration of cyfluthrin is used (Chen *et al.,* 2015).

Cytotoxicity evaluation of T, C1 and C2 filtrates using fish cell line EM4SpEx

Fish cell lines act as a valid and reliable means to assess pesticide cytotoxicity (Castano *et al*., 2003). In the initial cytotoxicity assay conducted to assess the toxicity of cyfluthrin to EM4SpEx cell line, the cells were found to respond in a dose-dependent manner to cyfluthrin exposure with maximum cell destruction observed in cell monolayers treated with cyfluthrin at 1µg ml^{-1} of the active ingredient. In the subsequent assay conducted to compare cytotoxicity of filtrates from T, C1 and C2 to EM4SpEx cell line, difference in morphological changes were clearly evident between control cells without exposure to any of the filtrates (C0) and those exposed to T, C1 and C2 filtrates (Fig. 8). In the control wells (C0) exposed to L-15 medium alone, the cell monolayer appeared homogeneous (Fig. 8a) maintaining typical fibroblast morphology after 5 days of incubation. In case of the cell monolayers exposed to filtrate from C1 (Fig. 8b), on 5^{th} day post-exposure, slight damage to the cells were observed. These minor changes in cell morphology even in the absence of cyfluthrin could be attributed to the effect of extracellular products released by the bacterial cells of T14 (Olgerts *et al*., 1972). The extent of cell destruction was higher in cells exposed to filtrates containing cyfluthrin (T and C2) with comparatively lesser cell destruction observed in T (Fig. 8c). As evident from Fig. 8d, almost complete destruction of cells and rounding occurred when treated with C2 filtrate which had only cyfluthrin and MM. Cytotoxic effects on cells in T were found to be appreciably low in all the triplicate wells as compared to C2, which clearly indicated biodegradation of cyfluthrin by the bacterial strain *P. ganghwense* T14. The results clearly indicate that the probable biodegraded metabolites of cyfluthrin meditated by *P. ganghwense* T14, in T filtrate are less toxic in nature than the parent compound.

As evidenced from Fig. 8c and d, toxicity of T was found to be comparatively lesser than C2 which implies the lesser toxicity of degradation products than the parent cyfluthrin. On a comparative analysis, the effect of cyfluthrin on fish cell line was found to have reduced when treated with T14. Here fish cell line is used as a

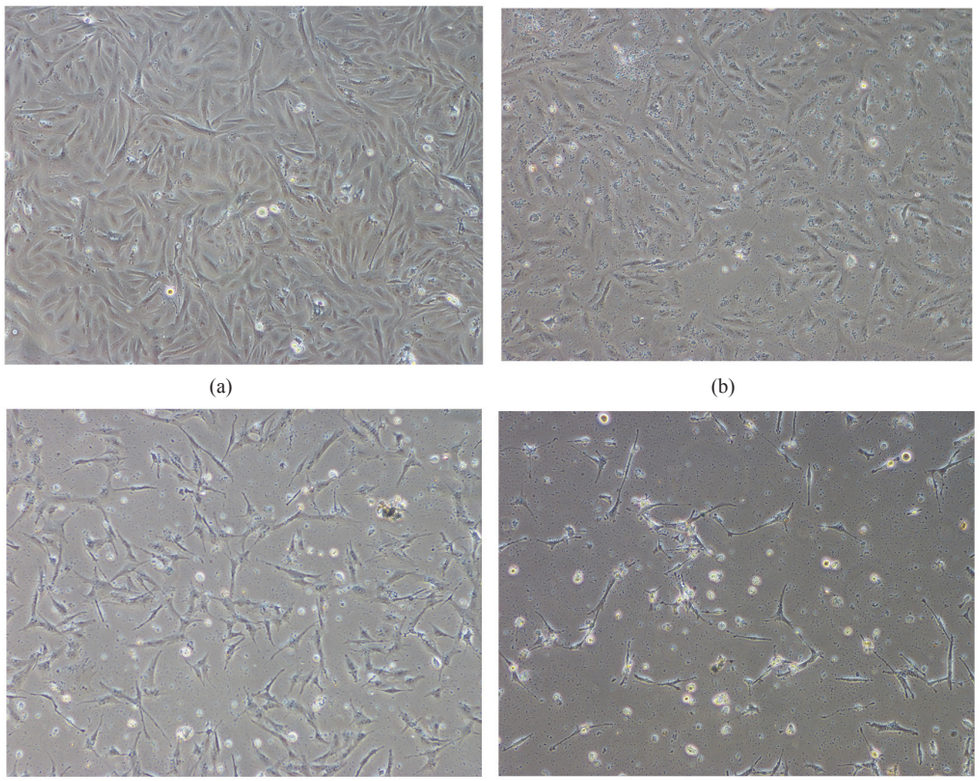

Fig. 8. Phase contrast photomicrograph of fish cell line EM4SpEx on day 5 incubation after exposure to filtrates. (a) Control cells (C0) showing fully confluent monolayer comprising normal cells; (b) Cells exposed to filtrate C1 showing slight disruption of the monolayer and presence of granules in the medium; (c) Cells exposed to filtrate T displaying shrinking of cells and a discernible reduction in the density of the monolayer and (d) Cells exposed to filtrate C2, exhibiting rounding and considerable detachment of cells with majority of the cells found dead

tool to qualitatively analyse the difference in cyfluthrin cytotoxicity post-bacterial treatment. Fish cell lines have been successfully employed as a biological alternative to the use of live animals for ecotoxicology studies, especially to monitor potential toxic effects of contaminants in aquatic ecosystems (Davoren *et al*., 2005). Damages to fishes by pyrethroids in aquatic ecosystems should not be disregarded. Run off of pyrethroids from the agricultural and urban areas into the waterways have become a serious concern. Marine fishes are severely affected by these chemicals and their concentrations have now reached a level which is lethally and sublethally toxic to aquatic organisms (Antwi and Reddy, 2015). Studies on toxic effect of organochlorine and organophosphorus pesticide residues on tilapia fish tissue revealed that continuous exposure to pesticide residues would result in significant haemato-biochemical alterations and DNA damage in vital organs like gills and liver (Elbialy *et al*., 2015). Endocrine disrupting potential of pyrethroids in mammals and fishes adds up the criticality of the situation (Susanne *et al*., 2016).

The bacterial strain *P. ganghwense* T14 isolated and characterised in this study, clearly demonstrated its ability to utilise cyfluthrin as carbon source and is effective in degrading the pesticide at a concentration as high as 100 mg l^{-1}. Further investigations on metabolomics are necessary to provide insights into the microbial processes involved in the pesticide degradation and transformation. Also, the scope extends further to the use of genetic engineering tools to modify the enzyme producing bacterial genes as well as recombinant DNA technology, to engineer bacteria having higher pyrethroid degradation potential. Exploitation of hydrolysing enzymes responsible for the degradative ability in bacterial strains can also be a promising strategy. The pyrethroid degrading organisms are reported to target the ester bond in the pesticide and hydrolyse it by carboxyl esterases (carboxylic-ester hydrolase, EC 3.1.1.1), yielding carboxylate and alcohol (Aranda *et al*., 2014). Because of the broad spectrum activity of these enzymes, esterases can degrade various pyrethroids with similar molecular structure (Wu *et al*.,

2006). However, the rate of hydrolysis may vary. This points towards the possibility for the co-application of other pesticide degrading strains along with *P. ganghwense* T14 for a complete remedial strategy. In addition, microbes having potential to degrade a wide range of pyrethroids can be efficiently utilised as a consortium to overcome the problem of formation and accumulation of toxic metabolites during pesticide degradation. This can be made possible by making use of the varying ability of the members of the consortium to use the intermediate toxic products as their growth substrates. This paper offers a scope on study of co-metabolism of other pyrethroid degrading bacteria with *P. ganghwense* T14 strain. Several bacterial strains belonging to the genera *Bacillus, Brevibacillus, Ochrobactrum, Pseudomonas, Serratia* and *Sphingobium* were found to be highly effective in pyrethroid degradation. *Bacillus* strains were reported to completely degrade cypermethrin and bifenthrin at 100-1200 mg l^{-1} concentrations in less than 7 days (Tiwary and Dubey, 2016). Similarly, *Ochrobactrum* sp. and *Serratia nematodiphila* were found to be capable of directly utilising more than 90% of the initial dose of cypermethrin (100 mg l^{-1}) within 5-10 days. Among fungi, removal of fenvalerate (50-400 mg l^{-1}) by *Cladosporium* sp. and bifenthrin (100-400 mg l^{-1}) by *Candida pelliculosa* were reported (Chen *et al*., 2011a). Some bacterial strains have been reported to degrade a wide spectrum of pyrethroids (Guo *et al*., 2009). Many studies have reported an efficient elimination of pesticides by microbial treatment by simultaneous biostimulation and bioaugmentation (Guo *et al*., 2014) which was explained as due to the increase in the catabolic potential of the soil and by the synergistic ability of the native microorganisms to degrade certain pesticides (Zhang *et al*., 2016). Among the various approaches tried, biological methods were found to be more effective, economic and ecofriendly (Akbar *et al*., 2015). Studies have proved the ability of diverse microorganisms in successfully eliminating pyrethroids from water and soils (Jin and Webster, 1998). When it comes to the field level application for bioremediation, environmental elements comes into role. Since the abiotic factors such as temperature, pH, nutrient level, inoculum size, moisture, organic matter content, the initial pesticide concentration and additional carbon sources (Zhang *et al*., 2010) critically control the onsite degradation efficacy, an elaborate study on the interactions of these microorganisms with the environment is essential, prior to exploration of its field application. The most important among the biotic factors are interaction between autochthonous and inoculated microorganisms including predation, competition for space and food. The survival rate of inoculants in the treatment area is another important factor that determines the success rate of bioremediation (Karpouzas and Walker, 2000). Loss of degradative ability of the inoculum *in situ* or antagonistic inhibition by indigenous microorganisms is detrimental factors in degradation process (Cycon *et al*., 2014). However, repeated inoculation with concomitant progress in degradation as well as use of immobilised microorganisms are possible alternative counteracts. Immobilisation not just extends the abidance and vitality of inoculants but also protect the organism against adverse conditions (Tallur *et al*., 2015).

The detrimental effects of the pesticide use and its effects on the environment have been well studied. Although, pyrethroids have comparatively lesser toxicity towards non-target organisms, its long-term exposure may impair organ functions (Soni *et al*., 2011) and can be carcinogenic as well as anti-androgenic (Zhang *et al*., 2010). Reports have shown persistence of cyfluthrin in crops above its Maximum Residue Limit (CAC, 2004). Therefore the remediation of pesticide contaminated sites and prevention of further accumulation is an urgent concern. The confirmed negative effect of pyrethroids on the ecosystem emphasises the need of a safe, efficient and economical strategy for pesticide removal from the contaminated environments. This paper offers a small contribution to the big scenario of bacterial mediated bioremediative strategies.

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