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Characterization of cypermethrin and profenofos resistant bacteria recovered from an agricultural field of Pantnagar

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Abstract
Agriculture sector contributes significantly in Indian economy where application of different pesticides has become mandatory to protect the quality and quantity of agricultural produce. Indigenous use of toxic chemicals including pesticides gives rise to environmental pollution as percolation of these chemicals in ground water causes contamination which is a matter of great concern for the environmentalists as well as for the health of human beings. In the present study, a Cypermethrin and Profenofos resistant bacteria was isolated from a pesticide contaminated soil of an agriculture field of Pantnagar using enrichment technique. The bacterium was characterized as a species of *Staphylococcus* using standard microbiological, biochemical and molecular methods (based on 16S rRNA sequencing). The bacteria showed maximum sequence homology with *Staphylococcus gallinarum*. Cypermethrin and profenofos are highly toxic, soluble and absorbable pesticides and used to control Boll worm, shoot and fruit borer and some Lepidopteron pests of cotton and tobacco. *Staphylococcus gallinarum* was capable to grow in the presence of Cypermethrin and Profenofos upto 100 ppm concentration. This bacterial strain could also effectively degrade Cypermethrin and Profenofos as it used the pesticides as a primary carbon source in minimal salt medium. Out of various enzymes like laccase, manganese peroxidase and lignin peroxidase tested to characterize the microbe, *S. gallinarum* showed the presence of lignin peroxidase. Present study provides a good opportunity to remediate pesticides-containing soil using a pesticide resistant bacteria.

Keywords: Pesticides, degradation, lignin peroxidise, environment

Introduction
Agriculture sector is under immense pressure to feed the growing population worldwide. Modern agriculture is based on chemicals where different pesticides are used to manage varied groups of pests to maximise crop production and to meet the demand of higher supplies of food globally. Application of synthetic agrochemicals has revolutionized the agricultural production in last few decades because of their efficiency to kill insect pests but at the cost of the environment. Pesticides occupy a central place in the management of crop diseases and pests. They belong to a category of chemicals which are used most commonly as herbicide, insecticide, fungicide, rodenticide, molluscides and nematicides and plant growth regulators to control weed, pests and diseases in crops as well as health of human and animals. Pesticides have played an important role in agriculture sector of India by controlling pests, insects and enhancing cosmetic values of the produce. However, the widespread use of pesticides has resulted in various problems which are caused by their interaction with the biological systems in the environment as residual pesticides persist for long in the soil and affect indigenous microbial population and physicochemical properties of the soil adversely [14]. A large percentage of pesticides applied in agriculture never reaches to their target organisms [10]. They are dispersed through soil, water and air, and detected in food for human consumption. Biodegradation is one of the most important processes which influence the fate of pesticides in the soil. Pesticides also influence the biochemical processes driven by the microbes and their enzymatic machineries. Soil microbes, the basic component of the soil ecosystem are vital for soil fertility and for the degradation of organic pollutants in soil.
Cypermethrin is a photos table synthetic pyrethroid insecticide. Half-life of Cypermethrin ranges from 14.6 to 76.2 days depending upon the physicochemical properties of the soil. Microbial activities of the soil also play vital role in determining the fate and behaviour of Cypermethrin in the soil. Under natural environment, hydrolysis of the ester linkage is a common degradation route of Cypermethrin which leads to the formation of 3-phenoxybenzoic acid (PBA) and cyclopropane carboxylic acid (DCVA).

Cypermethrin displays low water solubility and due to non-polar nature it is readily adsorbed onto the soil surface however, the metabolites of Cypermethrin (PBA and DCVA) are organic acids and mobile in nature. PBA is a potent toxicant because of its antiestrogenic activity. It is classified as an endocrine disrupting chemical. Profenofos, a well-known organophosphate pesticide has been in agricultural use over the last two decades to control Lepidopteron pests of cotton and tobacco. Profenofos has been classified as a moderately hazardous (Toxicity class II) pesticide by World Health Organization (WHO) and it has a moderate order of toxicity following oral and dermal administration. Intensive use of profenofos leads to its contamination in the environment. Acute toxic action of profenofos is related to inhibition of acetyl-cholinesterase activity which also results in toxicity in humans.

Keeping in view the current scenario of the performances of various pesticides in the environment, present study was conducted to isolate and characterize a bacterial species, capable of growing and utilizing two common pesticides in culture broth.

Materials and Methods

Reagents and Chemicals

Standard pesticides (Cypermethrin and Profenofos) were kindly provided by Department of Agricultural Chemicals, IARI, New Delhi. Stock solutions of the pesticide were prepared by dissolving 1mg/ml standard pesticide(s) in hexane. Stock solutions were sterilized using 0.22µm bacterial filter and kept in freeze till use. Other chemicals used for isolation and characterization of the bacteria were purchased from Hi media, Bombay.

Collection of Soil Sample

Soil samples used for the isolation of pesticide resistant microbes were collected from the rhizospheric region of pesticide contaminated soil from the fields of Norman E. Borlaug, Crop Research Centre, G. B. Pant University of Agriculture & Technology, Pantnagar where Cypermethrin and Profenofos are being used for many years. Rhizospheric or subsurface soil was collected in sterile polythene bags, and kept in deep fridge till use.

Isolation of Pesticide degrading microorganisms

The culture enrichment and isolation procedure used to recover pesticide resistant bacteria were as follows. Air dried composite soil sample (50g), collected from the pesticide contaminated site was mixed in the pesticide containing basal salt medium (50ml). Enriched flasks, inoculated with soil samples were incubated on rotary shaker at 100 rpm and 30±2 ºC for 4 weeks. After incubation, soil suspension (10ml) was used as inoculum and inoculated into fresh pesticide containing fresh medium. Enrichment practice was repeated for three times. At last, enriched microbial cultures present in the soil suspension were purified by using spreading and streaking-plate techniques. Inoculated plates were incubated at 30±2 ºC for 2 days. Pure bacterial colonies growing on the pesticides were obtained after several transfers or streaking. Formulation of pesticide containing basal salt medium included KH_2PO_4 (3.0g/L), NH_4Cl (1 g/L), NaCl (0.5 g/L), $MgSO_4$ (0.25 g/L). All the chemicals were sterilized by autoclaving and 2.0% (w/v) agar was added in agar medium preparation on and when required.

Screening of bacterial isolate using biochemical characters

The recovered bacterial isolate was tested for the production of siderophore, lipase, catalase, lignin peroxidase and ammonia along with antibiotic sensitivity test using standard methods.

Lipase activity

For lipase activity, overnight grown bacterial cultures were spot inoculated on the plates containing medium (g/L): peptone, 10; NaCl, 5; $CaCl_2.2H_2O$; agar-agar, 20 and Tween 80 @ 10mL (v/v). Inoculated plates were incubated at 30 ºC for 24-48 h. Appearance of white colored precipitates around the bacterial colonies indicates the presence of lipase enzyme. [8].

Catalase test

To check the presence of catalase enzyme in an organism, few drops of 3% Hydrogen peroxide were added on a drop of overnight grown bacterial culture, taken on a clean glass slide. Immediate evolution of oxygen bubbles confirms a positive catalase test [15].

Siderophore production

Bacteria producesiderphores to sequester ferric iron from the environment [13]. An indicator was developed using Chrome azurol S (CAS) and Hexadecyl methyl ammonium bromide (HDTMA). CAS/HDTMA complex tightly binds with ferric iron in an acidic medium to produce blue colour. On the formation of siderophorecremaceous iron from the dye complex, the colour of the medium in the surrounding of the bacterial colonies changes from blue to yellow/orange. CAS medium was prepared by mixing Chrome Azurol S (60.5 mg) in distilled water (100 ml) to which 10ml $FeCl_3.6H_2O$ (1mM) in HCl (10mM) was added gradually. The mixture was then added to HDTMA solution (72.9 mg in 40 ml distilled water). Dark blue colour solution obtained was autoclaved at 15 lb psi for 20 min. Sterilised nutrient agar (300 ml) was mixed with CAS solution in the ratio of 1:10 (CAS solution: media).

Citrate Utilization test

This test is used to determine the ability of an organism to utilize sodium citrate as sole carbon source and ammonium salt as sole nitrogen source. Bacteria showing positive test turn the medium from acidic to alkaline condition. This is indicated by the change in colour of bromothymol blue indicator from green to blue. Slants of Simmon citrate agar medium were prepared. After streaking of 24 h old culture, tubes were incubated for 24-48 h at 30 ºC. Metabolism of citric acid generates CO_2 which combines with water and sodium to form sodium bicarbonate. Sodium carbonate is an alkaline product and changes the colour of the indicator from green to blue.

Antibiotic Assay

Hundred µL of overnight grown bacterial culture was uniformly spread on nutrient agar plates with the help of a glass spreader. After spreading the culture, antibiotic discs were placed at different corners of the plates. Plates were then incubated for 24-48 hours at 30 ºC. Formation of clear zone(s) around the antibiotic discs shows the susceptibility of bacteria for the antibiotic.

Lignin peroxidase

Mineral salt medium supplemented with 0.002% Azure B dye

and 2mM hydrogen peroxide (3%) was autoclaved and poured into sterile plates. After point inoculation of bacterial culture, plates were incubated at 30 ºC for 48 hours. Appearance of clear zone around bacterial colony confirms positive test for lignin peroxidase enzyme [2].

Ammonia Production

Test tubes containing Peptone water broth (5 ml) were autoclaved and inoculated with active bacterial culture. Inoculated test tubes were incubated for 3-5 days. After 5 days, broth was centrifuged. Obtained supernatant was then treated with Nessler's reagent. Nessler's reagent was prepared by mixing Potassium iodide (5g) in 5ml distilled water to which mercuric chloride (2.2g) was added in 35 ml distilled water and volume was made up to 50ml. Afterwards, supernatant (4ml) was mixed with 1ml of Nessler's reagent. Appearence of brown to yellow/orange colour confirms the presence of ammonia [9].

Characterisation of the test bacteria

Morphological, microscopic, physiological and biochemical tests of the isolate were performed according to the standard methods. Phylogenetic analysis of the bacteria was done using 16SrRNA sequencing according to [3].

Amplification of Genomic DNA with 16S primers

Genomic DNA of the test bacterial isolate was extracted according to the method of [11]. Primers used for the amplification of the gene encoding 16S rRNA were: 27F (AGAGTTTGATCMTGGCTCAG), 1492R (TACGGYTACCTTGTTACGACTTA). Single PCR reaction tube contained 100ng of DNA in a 20µL reaction mixture containing 10X Buffer, each deoxy-nucleoside tri-phosphate at 0.1 mM, each primer at 0.5 µM, and 2U of Taq polymerase (R00IC TaKaRaTaq™). Polymerase chain reactions were subjected to initial denaturation for 3 min. at 95 ºC, 32 cycles (denaturation, 30s at 95 ºC; annealing, 30s at 55 ºC; extension, 1 min at 72 ºC) and 1 final extension cycle at 72 ºC for 20 min. Twenty microliters of the reaction mixtures was run on 1.5% agarose gel using Tris-Cl-sodium acetate-EDTA buffer containing 0.5µg of ethidium bromide per ml. Band appeared were observed under UV light.

Phylogenetic analysis of sequence data

16SrDNA sequences of test bacterial isolate were analysed for homology with known 16SrDNA sequences available in NCBI (National Centre for Biotechnology Information) database using BLAST (Basic Local Alignment Search Tool) [1]. Isolate was identified on the basis of per cent similarity of the query sequences with the available sequence in the database. Sequence was aligned using Clustal W programme. Neighbour joining method [12] was used to infer the evolutionary history of the bacterial isolate on the basis of which distance matrix was calculated from sequence data. Evolutionary analysis of the recovered bacterial isolate was conducted using MEGA 6.0 software [16].

Result and Discussion

Isolation and identification of pesticide degrading bacteria

A bacterial isolate was recovered from the pesticide exposed soil samples using enrichment technique. The isolate was able to tolerate 100 ppm of Cypermethrin and profenofos. Moreover the organism degraded the pesticides in minimal salt medium and used them as sole carbon source. The strain was coccus shaped, gram positive and motile. Results of biochemical tests are presented in Table-1.Molecular analysis of the test bacteria using 16S rRNA gene sequencing revealed its sequence homology with *Staphylococcus*. By evolutionary analysis and comparing it with previously published 16S rRNA gene sequences, the strain was classified as a member of genus *Staphylococcus*. Morphological characteristics of pesticide degrading bacteria have been shown in Table 2.

Purification and maintenance of cultures

Isolated bacterial colonies were purified by streaking on nutrient agar and then purified bacterial culture was maintained on Nutrient agar slants.

Determination of Minimum Inhibitory Concentration of Pesticides in bacterial isolate

Bacterial culture isolated from the soil was screened for its ability to utilize Cypermethrin and profenofos under laboratory conditions. Bacterial culture was grown in 25ml minimal salt broth (supplemented with different concentrations of the pesticides) to test its maximum tolerance level for the pesticides. Highest concentration of the pesticide that supported maximum growth was considered as minimum inhibitory concentration of the pesticide [7]. Bacterial isolate was screened on the basis of their tolerance to maximum pesticide concentration and most potent culture was selected for further study. The selected culture showed tolerance to above mentioned pesticides up to 100 ppm since it was using the pesticides in minimal salt medium as carbon source for its growth.

Morphological and Biochemical Characterisation of Bacterial Isolate

Morphological and cultural characteristics of the bacterial isolates were studied according to [5]. All the tests were performed using actively growing bacterial culture.

Colony morphology

Bacterial cultures were grown on Nutrient agar plates to examine the shape, size, margin, elevation, surface and pigmentation properties (Figure 1b).

Gram staining

A loopful of actively growing culture was taken on a clean slide, smear was prepared and heat fixed. Slides were observed under microscope using immersion oil. However, the bacterial culture was coccus, gram positive and motile (Figure 1c).

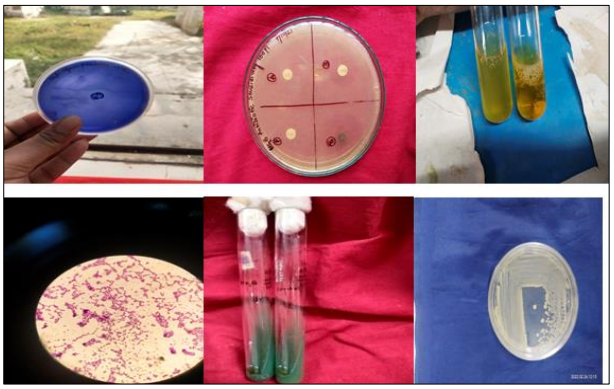

Fig 1: (a) Biochemical tests confirming the activity of *S. gallinarum* (b) Colony morphology of *S. gallinarum* (c) gram staining of *S. gallinarum*

Molecular characterization

Bacterial isolate was identified as *Staphylococcus gallinarum* on the basis of BLAST (Figure 2).

Fig 2: Phylogenetic tree analysis of *S. gallinarum* through 16S rRNA sequencing

Table 1: Biochemical characterization of *S. gallinarum*

	Biochemical Tests	*Staphylococcus gallinarum*
1.	Catalase test	(+)
2.	Citrate UtilizationTest	(-)
3.	Lignin peroxidase	(+)
4.	Ammonia Production	(-)
5.	Streptomycine	(+)
6.	Tertacycline	(+)
7.	Lipase	(+)
8.	Siderophore production	(-)

Table 2: Morphological characteristics of *S. gallinarum*

S no.	*Staphylococcus gallinarum*	Morphological traits
1.	Colony morphology	Coccus shaped
2.	Growth pattern	Slow grower
3.	Staining	Gram positive

Conclusion

There is evidence that degradation of pesticides and tolerance of bacteria to the significant pesticide in simple laboratory systems may differ from that observed in the field trials, but laboratory studies always present positive apprehension. According to some previous studies, microorganisms can use pesticides as carbon and nitrogen sources to promote their own growth [4, 6] and degrade them. The production of pesticides is increasing, and their consumption has become inevitable because of urbanization and tremendously growing world population. Pesticides are usually classified by the target pest, chemical composition, pesticide characteristics, mode of action and entry. However, their widespread, non-regulated and inappropriate use has led to significant threats to all ecosystems and the living beings. However, the bacterial isolate, recovered in the present study was able to tolerate both Cypermethrin and profenofos till 100 ppm by utilizing them as carbon, phosphorus or energy sources. Further research is required to interpret the successful laboratory trial experiments into vigorous application. Additional degradation approaches are still needed to reduce their accumulation probabilities.

Conflict of interest

The authors declare that they have no conflict of interest.

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