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Isolation, screening and identification of organophosphate pesticide degrading bacterium, *kocuria* sp.

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Abstract

Chlorpyrifos is an insecticide that has been one of the pesticides most used worldwide since 1965. A soil bacterium capable of utilizing chlorpyrifos as sole carbon source was isolated by selective enrichment on mineral medium containing chlorpyrifos. The objective of this study was to identify the Organophosphate pesticides degrading bacterium. Dilution plating was used to quantify the numbers of *Kocuria* propagules, and DNA sequence analysis was used to identify *Kocuria* species. *Kocuria* species was identified basing on the microscopic observations and Biochemical characterization. It was ultimately confirmed based on DNA sequence (16S rRNA sequence) analysis and finally concluded by BLAST analysis by constructing a phylogenetic tree.

INTRODUCTION

Organophosphate pesticides are a group of highly toxic agricultural chemicals widely used in plant protection. Their usage has become an indispensable tool in agriculture for the control of weeds, insects and rodent pests. They are poisonous but play an important role in generating plenty of food to the world population [1-2]. Compounds of this family are spontaneously hydrolyzed and cause neurotoxicity in mammals [3]. Excessive pesticide usage resulted in accumulation of pesticide residues in crops, soils, and biosphere creating an ecological stress [4]. Chlorpyrifos is a broad spectrum systemic phosphorothioate ester insecticide patented and introduced by Dow Chemical Company in United States of America in 1965 [5]. Chlorpyrifos is available in granules, wettable powder, dustable powder, emulsifiable concentrate [6] and used for the control of a wide range of pests such as cutworms, corn rootworms, cockroaches, grubs, flea beetles, flies, termites, fire ants, aphids, lice, leptinotarsa and other insects. It is applied to different crops including cotton, nuts, corn, fruits, vegetables, ornamental plants and is highly persistent in foliar application. Chlorpyrifos causes hazardous effects to the environment and also toxic to human beings resulting in headache, nausea, muscle twitching, convulsions, birth defects and even death. It is toxic to a variety of beneficial arthropods including bees, beetles and parasitic wasps. It kills fishes and birds in minute concentrations. Plants are affected by delayed seedling emergence, fruit deformities and abnormal cell division [7-12]. It has antimicrobial property, hence prevents the proliferation of chlorpyrifos degrading microorganisms in soil [13].

In light of its importance in agriculture and a need to degrade it in the environment, the present study has been taken up to isolate, screen and identify a soil bacterium efficient to degrade chlorpyrifos till the molecular level.

MATERIALS AND METHODS

Isolation of chlorpyrifos degrading bacteria from enriched soil sample by serial dilution technique

Soil samples were collected from agricultural fields where commercial crops like tobacco and cotton were extensively grown and chlorpyrifos pesticide was used intensively, by contemplating such soil would contain pesticide contamination and natural micro-flora experiencing pesticide stress. The samples were pooled together and collected into a sterile polythene bag to avoid external contamination. The polythene bag containing soil sample was brought to the laboratory and stored at 4°C to maintain the biological activity of the soil microbes. 100 grams of collected soil sample was taken in a conical flask and enriched by adding 1ml of chlorpyrifos. 5 ml of water is added to maintain the moisture and incubated at 37°C with pH 7 on a temperature regulated shaking incubator [14]. The enriched soil sample was subjected to serial dilution technique and the samples were inoculated on enriched nutrient agar plates for obtaining pure cultures.

Maintenance of chlorpyrifos degrading bacterial isolates in nutrient agar medium containing 5 ppm chlorpyrifos

Pure cultures of bacteria used in the present investigation were sub cultured on enriched nutrient agar medium plates by cross streak method and stored at 4°C. Every time purity of the cultures was ascertained by microscopic observation of bacteria followed by Gram's staining.

Chlorpyrifos degrading ability of bacterial isolates in minimal salt medium

The ***Minimal Salt Medium*** and chlorpyrifos were dissolved in distilled water and adjusted to pH 7. Separate test tubes each containing 20 ml of medium and 200 µL glucose were taken. 5 µL to 80 µL of chlorpyrifos was mixed to the medium to get different concentrations of chlorpyrifos such as 50 mg/l to 800 mg/l and poured in separate conical flasks. 1 µL of screened culture was inoculated into the medium and incubated at 37°C for 24 hrs.

Microbial and Biochemical characterization of the isolate

The isolated bacteria was analyzed using different staining techniques such as Gram's staining, motility test, capsule staining and different biochemical techniques such as Indole production test, Methyl red and Voges-Proskauer test, Citrate utilization test, Carbohydrate catabolism test, Gelatin hydrolysis test, Hydrogen sulphide production test, Urease Hydrolysis test, Oxidase test,

Caesin Hydrolysis test and catalase test.

Molecular identification

Primer design

The primer sequence representing 16s RNA were retrieved from GenBank (National Center for Biotechnology information; http://www.ncbi.nlm.nih.gov) aligned using Claustal X [15], and the specific primers (forward: 5'- GAGTTTGAT CCTG GCTCAG-3' and reverse: 5'-AGAAAGGAGGTGATCCAGCC-3') were designed based on the homologous regions specific to *Kocuria* genus.

DNA isolation and amplification of 16s RNA gene of *Kocuria sp.* by Polymerase Chain Reaction (PCR)

The template genomic DNA from *Kocuria* species was isolated following the protocol described [16-17]. In Polymerase Chain Reaction, the specific primers Forward and Reverse (Institute of Biological Sciences, Vijayawada) were used to amplify the genomic sequence of the open reading frame (ORF) of the gene. PCR conditions were 94^0C for 2 min, and then 94^0C for 1 min, 60^0C for 1 min, 72^0C for 3 min for a total of 30 cycles, with the extension at 72^0C for 10 min.

Agarose gel electrophoresis

Required amount of agarose (w/v) was weighed and melted in 1X TAE) buffer (0.9M Tris-acetate, 0.002 M EDTA, pH 8.2). Then, 1-2 µL ethidium bromide was added from the stock (10 mg/ml). After cooling, the mixture was poured into a casting tray with an appropriate comb. The comb was removed after solidification and the gel was placed in an electrophoresis chamber containing 1X TAE buffer. The products were mixed with 6X loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF, 30% glycerol in water) at 5:1 ratios and loaded into the well. Electrophoresis was carried out at 60V [18].

Eluting DNA from agarose gel fragments

Ethidium bromide stained agarose gel was visualized under a transilluminator. The fragment of interest was excised with a clean razor blade. After removing the excess liquid, the agarose fragment was placed in the spin column. The tube was centrifuged at 5500 rpm for not more than 45 seconds for the elution of DNA. The eluent was checked by running on an agarose gel and observed on a transilluminator for the presence of ethidium bromide stained DNA. The eluted DNA was used directly in manipulation reactions. This DNA fraction was subjected for sequencing (Institute of Biological Sciences, Vijayawada).

Sequencing and chimera checking

The eluted PCR product was directly sequenced. Sequencing reactions were carried out with ABI PRISM Dye Terminator Cycle Sequence Ready Reaction Kit (Applied Biosystems Inc., USA). All sequences exhibiting less than 95% sequence similarity to existing sequences in GenBank were checked using CHIMERA-CHECK program at the Ribosomal Database Project (RDP) using default settings [19].

Phylogenetic placement

The environmental sequences were compared to the sequences in GenBank using the BLAST algorithm [20] and RDP database [19] to search for close evolutionary relatives.

GenBank accession numbers

The representative sequence of the soil *Kocuria* species was deposited in GenBank of National Centre for Biotechnology Information (NCBI). The Gen Bank accession number is Jf816257.

RESULTS AND DISCUSSION

Isolation and Screening of Isolate for Chloropyrifos Degradation

The organism was isolated by plating the serially diluted sample on minimal salt media enriched with Chloropyrifos at 10^{-6} dilution. The bacterial colony was yellow in colour and transparent in appearance that showed maximal growth on the media enriched with Chloropyrifos.

Microbial characterization of the isolate for Chloropyrifos Degradation

The colonies are round in shape and slightly embossed (Fig.1). Simple staining and gram staining of the isolate revealed that the bacteria capable to degrade Chloropyrifos was spherical in shape (cocci) and were gram positive. The bacterium was non-motile and there was no capsule which can be inferred that it is not pathogenic.

Biochemical characterization of the isolate for Chloropyrifos Degradation

The results of the isolated bacterium with reference to biochemical characterization was given in Table 1.

Molecular identification

Amplification of DNA coding for 16s r RNA

The genomic DNA of the organism screened was subjected for the isolation of the DNA coding for 16s rRNA by using Polymerase chain reaction (Fig. 2). The bands were cut and eluted and the DNA so obtained was subjected for sequencing.

Sequence analysis

The sequence analysis demonstrated that all the corresponding bands on agarose gel belonged to *Kocuria* species.

Fig. 1. Streak plate of the Isolate

Table 1. Biochemical characterization of the isolate

S.No	Biochemical test	Result
1	Indole production test	Negative
2	Methyl Red test	Negative
3	Voges-Proskauer test	Negative
4	Citrate utilization test	Negative
5	Carbohydrate catabolism test	Negative
6	Gelatin hydrolysis test	Positive
7	Hydrogen sulfide production test	Negative
8	Urease Hydrolysis test	Negative
9	Starch hydrolysis test	Positive
10	Oxidase production test	Positive
11	Caesin Hydrolysis test	Positive
12	Catalase test	Positive

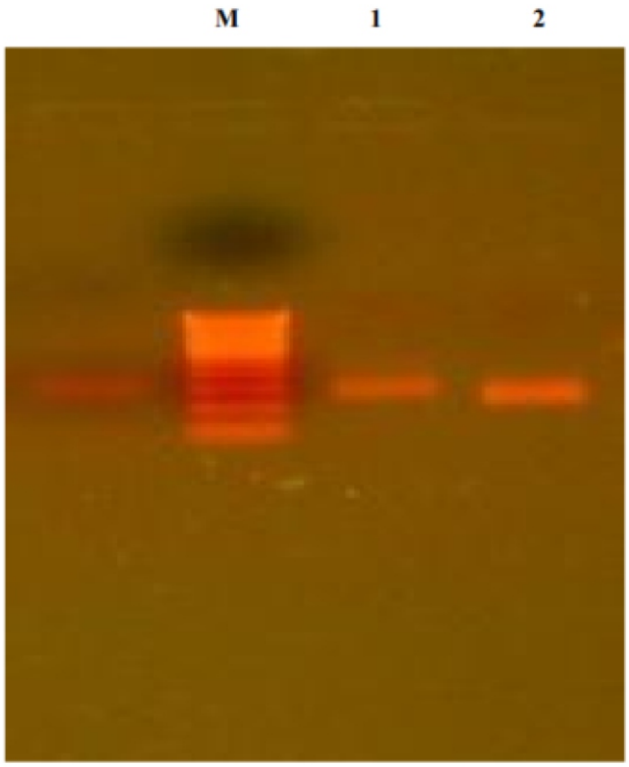

Fig.2: Amplified 16s rRNA of bacterial isolate
M-Marker;
1-Amplified 16s RNA of Bacterial isolate
2- Amplified 16s RNA of Bacterial isolate

The amplified 16S rRNA of bacterial isolate was sequenced and the data obtained correspond to 328 base pairs. The sequence so obtained was as follows.

GTGCTTACCATGCAGTCGAACGCTGAAGCTCCAGCTTGCT
GGGGTGGATGAGTGGCGAACGGGTGAGTAATACGTGAGTAAC
CTGCCCTTGACTCTGGGATAAGCCTGGGAAACCGGGTCTAAT
ACTGGATACGACTCCTCATCGCATGGTGGGGTGTGGAAAGTG
TTTTACTGGTTTTGGATGGGCTCACGCCCTATCACCTTGTTG
ATGTGGTAATGGACTACCAAGACGACCACTGGGTAGCCAAAA
CTGAAAAGGGGACCGTGCACACTGGGACTGATACGCATTGTC
CCACTCCTAGCGGGAGGACAG AATGGGGGAGTATTG

Phylogenetic affiliation of the *Kocuria* based on the ITS sequences

Sequences of the dominant DGGE bands revealed that *Kocuria* species in tested soil was *Kocuria* with the accession no. JF816257 [21]. This sequence can be found in NCBI Genome Databank. The phylogenetic tree was shown in Fig 3.

Indigenous *Kocuria* species are known to have greater tolerance for Organophosphates and like compounds than many other soil microorganisms. It was found that the ITS sequences of the saved colonies from soil dilution plating generally matched with those of the excised bands. The above account on morphological observations, biochemical tests and 16S rRNA sequence analysis carried out on the isolate led to identify it as *Kocuria* sp. which was reported as first chlorpyrifos degrading bacterium. This was correlated with the findings [22] and the organism identified was *providencia stuartii* as one of the bacterial species that degrade chlorpyrifos. In the present study, also the isolation and identification of the bacterium with similar properties that supports its significant role in bioremediation of chlorpyrifos contamination in soils.

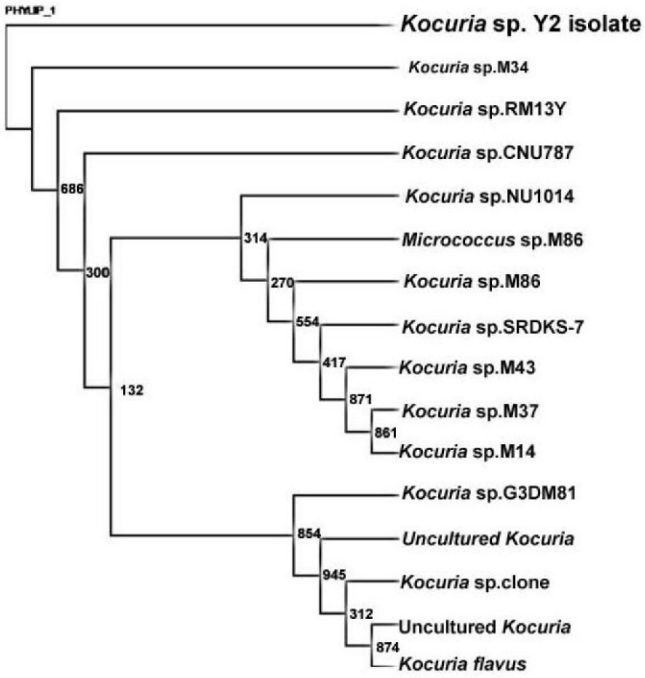

Fig. 3: Phylogenetic tree of Kocuria sp Y2 isolate based on 16S rRNA gene sequences: Bootstrap values obtained with 1000 repetitions were indicated at all branches

CONCLUSION

The present study reports the identification of a bacterium,

Kocuria species, which is capable of utilizing chlorpyrifos as a source of carbon. Utilization of xenobiotic compounds by soil microorganisms is a crucial phenomenon by which these compounds are removed from the environment, thus preventing environmental pollution. Results from the present study suggest that the isolated *Kocuria* species is able to grow in medium in the presence of added pesticide and may therefore be used for bioremediation of pesticide-contaminated soil. In addition, our work suggest that the combination of soil dilution plating, DGGE and DNA sequence analysis are effective approaches to facilitate extensive examinations of the propagule numbers of *Kocuria* species, to reliably identify *Kocuria* species in soils.

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