ChemTech

International Journal of ChemTech Research
CODEN(USA): IJCRGG ISSN : 0974-4290
Vol.5, No.1, pp 06-14, Jan-Mar 2013

Enrichment And Isolation Of Endosulfan Degrading Microorganisms In Cashew Plantations Of Kasargod District, Kerala

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Abstract: Organochlorine pesticides are one of the major groups of chemicals responsible for environmental contamination. Endosulfan a pesticide used mainly in cashew plantation is a highly toxic endocrine disrupter and a contact stomach poison. To study the endosulfan degrading microorganisms, 20 soil samples were collected from five different cashew plantations having a history of endosulfan applications. The bacterial strains were isolated using enrichment techniques and growth kinetic studies were conducted using different concentrations of endosulfan. The bacterial strain showed maximum growth at 100µl per 100ml of distilled water. Based on GC-MS analysis it was observed that there was no residual endosulfan in the soil, therefore complete mineralization has occurred leading to non toxic low molecular weight compounds. The strains were identified using biochemical characterization and by the 16s rDNA gene amplification. DNA sequence of the isolated strains showed 100% similarity with *Pseudomonas* sp 2C and *Alcaligenes* sp. clone XDC22.
***Keywords** : Endosulfan, microbial degradation, 16S rDNA analysis, phylogenetic analysis, GC-MS analysis.*

INTRODUCTION

The widespread use of potentially harmful pesticides has recently come under scrutiny in many parts of the world. The Organochlorine pesticides are one of the major chemicals responsible for the contamination and deterioration of soil and water environments especially in agricultural fields[1]. Endosulfan is an Organochlorine pesticide which was widely being used in agricultural fields like cashew, tea, coffee, cotton plantations to control pest like stem borers, tea mosquitoes and thrips. It is a toxic endocrine disruptor and a major stomach contact poison. Even though the use of endosulfan is being banned in many of the countries its effect is still a major problem as it is highly persistent in soil. Once applied to crops, it can either persist in soil as a sorbed phase or be removed through physical, chemical and biological processes and persist more in acidic conditions.

Over the past twenty to thirty years, the pesticide endosulfan has been aerially sprayed on cashew plantation covering several villages in Kasargod district, Kerala. People residing in these areas were found to have congenital birth defects, reproductive health problems, loss of immunity, etc. In February 2001, a team from the Kerala Agricultural University (KAU) conducted their own study and showed that there is no Endosulfan residue in water, pepper berries, and betal leaf but found high levels of Endosulfan in soil and cashew leaf samples from inside plantations[2].

These health and environmental concerns have led to an interest in detoxification of endosulfan in the environment. Endosulfan can be degraded by attacking the sulphide group by oxidation or by hydrolysis to form toxic metabolite, endosulfan sulphate and the less toxic endosulfan diol, respectively[3]. Many reports have come on the degradation of endosulfan by various bacterial co-cultures[4] and mixed cultures[5]. The biodegradation of and endosulfan in broth medium and soil microcosm by bacterial strain *Bordetella* sp B9 was also reported [6]. *Bacillus* sp SK320 was isolated from endosulfan sprayed cashew plantations of Kerala[7]. *Kleibsiella pneumonia* biologically degraded 8.72μg of endosulfan $ml^{-1}day^{-1}$ when incubated with $93.9\mu gml^{-1}$ endosulfan for 10 days without formation of toxic endosulfan sulphate [8].

Due to the persistence of Endosulfan and its metabolites in soil environment, biological means of detoxification is receiving serious attention as an alternative to existing methods such as incineration and landfill[9]. The objective of the present study was to find out Endosulfan degrading bacteria and to study its phylogenetic relationships. The study also focused on finding out the residual Endosulfan in the soil sample.

MATERIALS AND METHODS

Commercial grade alpha-Endosulfan (35% pure) was purchased from Dhanuka Agritech Limited, Haryana, India. Solvents including chloroform and methanol were of HPLC grade.

SOURCE OF ENVIRONMENTAL SAMPLE

Twenty soil samples collected from four different cashew plantations located in Kasargod district, having a history of endosulfan application were used for the isolation of Endosulfan-degrading microorganisms. All the sites have a sandy and laterite type of soil and are acidic in nature. Pesticides was applied twice in a year ie. during fleshing time (January/ February) and during flowering time (November/ December). For every 1L of water 1.5ml of endosulfan was used and no watering was done thereafter. The soil samples were collected during rainy season ie. in the month of July. Surface soil (0-15cm) was removed and placed in polythene zipped bags and stored in 4ºc in cold room until use. The sampling locations are Rajapuram estate, Panathoor division (647.3hec); Rajapuram estate, Painikara division (285.72hec); Kasargod estate, Periye division (290hec) and Kasargod estate, Muliyar division(200hec). **Table1** describes the sampling point and physical characteristics of the soil.

Table1: Soil Charecterization

SAMPLE CODE	SAMPLING POINT	SOIL TYPE	pH	ORGANIC MATTER
S1	Stream side	Coarse to fine	4.67	0.0517
S2	Road side	“	4.94	0.0525
S3	Road side	“	5.02	0.0517
S4	Hilly area	“	4.98	0.0521
S5	Stream side	“	4.82	0.0517
A1	Road side	“	4.57	0.0465
A2	Road side	“	4.63	0.0470
A3	River side	“	4.40	0.0453
A4	River side	“	4.74	0.0448
A5	Starting point	“	4.68	0.0433
B1	Starting point	“	5.38	0.0459
B2	Stream side	“	5.60	0.0460
B3	Road side	“	5.63	0.0448
B4	Road side	“	5.79	0.0448
B5	Road side	“	5.40	0.0459
C1	Road side	“	5.95	0.0508
C2	Stream side	“	6.20	0.0518
C3	Road side	“	6.13	0.0518
C4	Road side	“	5.79	0.0517
C5	Starting point	“	5.88	0.0499

ENRICHMENT IN M9 MEDIA FOR BACTERIAL ISOLATION

Soil suspensions (1g/100ml of phosphate buffer) were prepared and serial dilutions were made from it. M9 medium(Na_2HPO_4,6g;KH_2PO_4,3g;NaCl, 0.5g;NH_4Cl,1g,$MgSO_4$(1M), 2ml; Glucose, 20%; $CaCl_2$,0.1ml, pH 7.2-7.4) was prepared in 1L of distilled water. About 1ml of the serially diluted soil sample was added to 100ml of the medium spiked with 200µl of endosulfan. The contents were incubated at 29ºc for one week. Thereafter 1ml of sample was drawn from this flask and reinoculated into fresh media with increased concentration of endosulfan and the process was repeated 2 to 3 times. Finally a loopful of this inoculum was streaked onto agar plates made with LB medium and incubated at 29ºc for 24hrs.

IDENTIFICATION OF BACTERIAL STRAINS

To obtain the endosulfan degrading bacteria repeated subculturing was done. Four bacterial strains were initially isolated. These bacterial strains were identified on the basis of various biochemical characterizations like morphology, gram staining, motility, catalase test, H2S production test, voges proskauer test and starch hydrolysis test, methyl red test, growth on Macconkey agar[10]

GROWTH KINETICS STUDIES

Based on the growth in the endosulfan containing broth medium, three strains of bacteria were selected for growth kinetic studies to find out the optimum concentration of endosulfan at which the bacteria shows maximum growth. For this endosulfan concentrations 20µl, 50µl and 100µl per 100ml of distilled water was prepared and added to 100ml of LB media taken in 250ml conical flask inoculated with bacterial culture. The flasks were shaken at 150rpm at 30ºc. The samples were taken at intervals of 0, 17, 26, 41 and 50 hrs. Growth was measured spectrophotometrically at 600nm.

EXTRACTION OF RESIDUAL ENDOSULFAN FROM SOIL SAMPLE

About 10g of soil aliquots were shaken with 20ml of methanol-water (4:1) mixture for 1hr. Supernatant was discarded after the mixture was centrifuged at 10,000rpm for 15min. The procedure was repeated two or more times and the volume of the combined were decreased to approximately 15ml using a rotary evaporator. The aqueous sample was then acidified to pH 1 with HCl and portioned three consecutive times with chloroform (30ml) using a separating funnel. The combined organic phases were again evaporated to near dryness and then redissolved in 10ml of methanol[11].

GC-MS ANALYSIS

A Jeol GC Mate gas chromatograph equipped with Electron Capture Detector (ECD) connected with MS detector and a HP5 column was used to analyse the residual Endosulfan and its metabolites in soil sample. The carrier gas used was helium and the flow rate was 10ml/min. The temperature program was as follows: Initially the temperature was held at 70ºc and then increased to 280ºc at 10ºc/min. The front inlet temperature was maintained at 220ºc.

MOLECULAR IDENTIFICATION OF 16S rDNA SEQUENCE

Amplification of the DNA sample was done by using colony PCR[12]. To each cold eppendorf tube was added a small amount of the culture colony. To do this, a fine yellow pipette tip attached to a pipette was used just to touch the colony. The amount of cells should be small just to fill the end of the opening was sufficient and was properly mixed with 50µl of 1X TE buffer and vortexed it for 2-10min for the lysis of cells. The primers used were 16S rDNA universal primers, forward primer (8F):5'-GAG TTT GAT CAT GGC TCA G-3' and reverse primer(1495R):5'-CTA CCG CTA CCT TGT TAC G-3'. The PCR reaction condition: 1cycle 5 min at 95ºc (initial denaturation), 1min at 95ºc (denaturation); 30cycles 1.5min at 54ºc (annealing), 1min at 72ºc (extension) and 1 cycle 5 min at 72ºc (final extension). To each of the cold PCR tube 2µl of this sample was added. PCR was performed using a thermal cycler in a total volume of 22µl containing 8µl of PCR master mix, 8µl of nuclease free water, 2µl each of forward and reverse primer[13].The PCR products were purified using Biobased columns. Purity of DNA was checked by running on agarose gels (0.8%) prepared in 1X TAE buffer and stained with ethidium bromide. About 4µl of the sample and 1Kbp DNA marker was loaded on to the gel which was resolved at 50V for 1hr. The amplified fragments were visualized and photographed under UV transilluminator and Gel documentation system. To determine the purity of the sample absorbance ratio 260/280 was measured. Sequence data was analyzed using BLAST program for gene homology search with a standard program default. Phylogenetic tree was constructed by neighbour Joining method with the gene sequences obtained.

Table 2: Biochemical characterizations of isolated strains

TEST	N1	N2	N3	N4
Mophology	Round, single	Circular convex	Large, flat	Single, round
Motility	+	-	+	-
Grams staining	-rods	-rods	+rods	-rods
Catalase test	+	+	+	+
H_2S production test	+	-	-	+
Growth on macconkey agar	+	+	+	+
Methy red test	-	-	+	-
Indole production test	-	+	-	-
Voges proskauer test	+	+	-	-
Starch hydrolysis test	+	+	+	+

RESULTS AND DISCUSSION

Enrichment of the culture was obtained by providing endosulfan as the sole source of carbon. Positive growth was determined by an increase in the turbidity of the flasks containing endosulfan as the sole source of carbon. These were plated on to LB agar medium with endosulfan, enabling selection of colonies based on their morphology. Four morphologically different strains were identified based on biochemical characterizations given in table 2. The organisms were identified to be of *Pseudomonas* sp, *Alcaligenes* sp, *Klebsiella* sp, and *Bacillus* sp. Soil enrichment technique was used for the isolation of endosulfan degrading bacteria from cotton growing soil and isolated bacteria that effectively degraded endosulfan into endosulfan sulphate[14]. In a similar study, two strains of fungi were isolated by enrichment in nutrient media[9]. Another enrichment method study in nutrient media reported that endosulfan degradation was effective with bacterial consortium and pure isolates[15]. The biodegradation of Endosulfan by *Achromobacter xylosoxidans* strain C5 using enrichment in TYC media was also reported[16].

KINETICS OF ENDOSULFAN DEGRADING BACTERIA

Among the four morphologically different strains best three isolates viz, N1, N4 and N2 were selected for growth kinetic studies to study the optimum concentration at which the bacteria shows maximum growth. At 20µl concentration of endosulfan, the maximum growth was shown by N1 followed by N4 and N2 as shown in **figure 1.** There was a sudden increase in growth with a short lag phase up to 40th hr (OD 0.8±0.4). This is the period during which the strain has fully adapted itself to the medium. However, growth was stationary after 50th hr.

At 50µl concentration of endosulfan, maximum growth was shown by both N1 as well as N4. There was a sudden increase in growth after 20th hr (OD 1.2±0.4). The growth was stationary after that where the bacteria have utilized most of the source available shown in **figure 2.** At 100µl concentration of endosulfan, maximum growth again showed by all the three strains shown in **figure 3.** The increase in growth was seen at 20th hr (OD 1.0±0.5). The optimum concentration at which three strains showed maximum growth was at 100µl/100ml of dist water.Similar kinds of growth kinetics studies was reported with different concentration of endosulfan with simultaneous increase in bacterial cell mass[5,6,15]. Uninoculated control remains the same without any change in the OD which indicates there is no other means of degradation of endosulfan in the flasks.

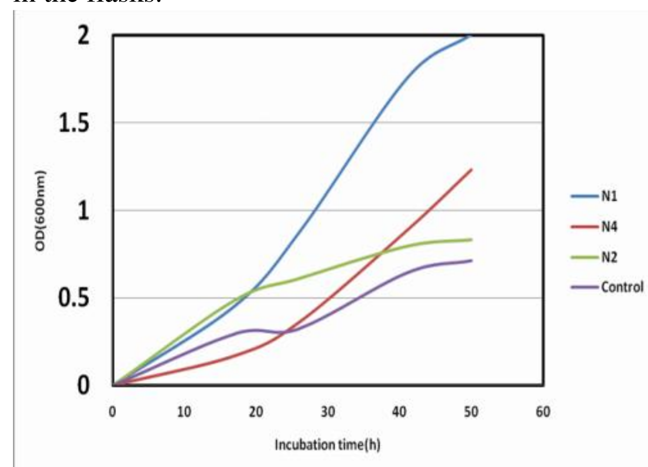


Figure1: Growth kinetic studies, concentration: 20µl

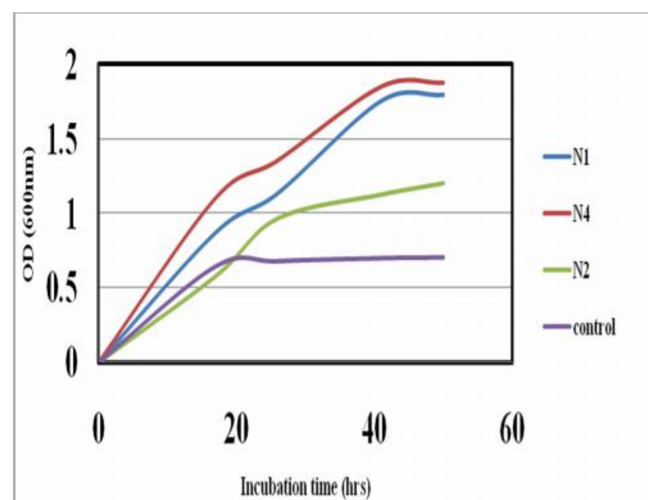


Figure2: Growth kinetic studies, concentration: 50µl

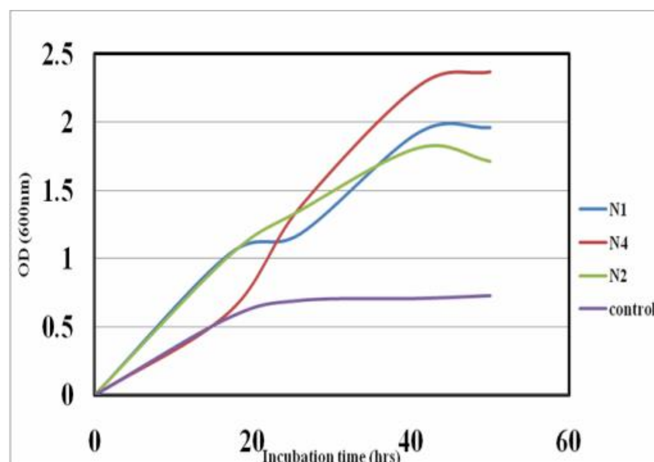


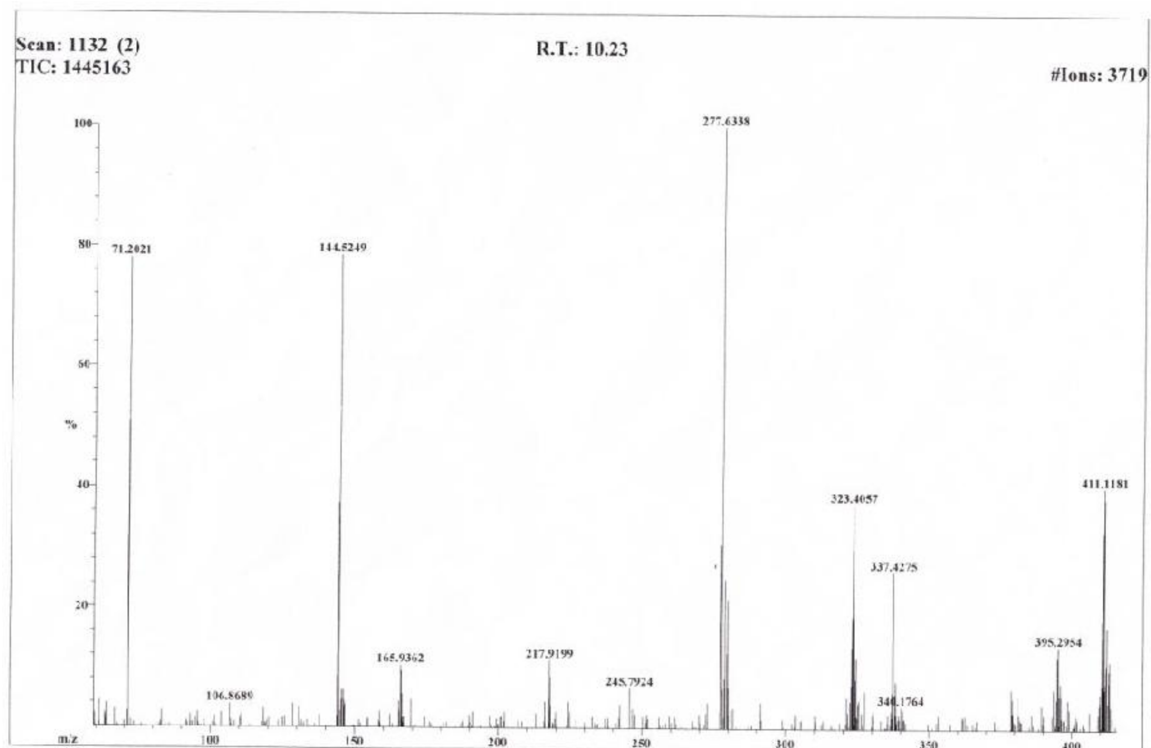
Figure 3: Growth kinetic studies concentration: 100µl

CHARACTERIZATION OF RESIDUAL ENDOSULFAN METABOLITES

Residual Endosulfan or its metabolites were analyzed through GC-MS studies. The results show that Endosulfan has completely mineralized into low molecular weight compounds. At retention time of 10.23, the positive ionization mass spectrum detected two intermediate compounds at m/z 217.8, Trichloromethane sulfonyl chloride (CCl_4O_2S) and at m/z 277.5, 2,4,6,-Tris (1-Chloroethyl)-1,3,5,-trioxane($C_9H_{15}Cl_3O_3$). At retention time 22.48, only one intermediate compound was shown which was at m/z 132.6. At this molecular weight nine different molecules were possible; one was 2-cyclopentyl ethyl chloride ($C_7H_{13}Cl$). At retention time of 20.78, two compounds were again detected, a low peak at m/z 277.5 was shown as well as another peak at m/z 351 ($C_7H_3Cl_7O$) were seen. At retention time of 12.28, four intermediate compounds were detected, two small peaks at m/z 277.5 and at m/z 351 and another at m/z 217.9, Trichloromethyl sulfonyl chloride (CCl_4O_2S) and one at m/z 235.1, Ethyl 3,5-dichloro-4-hydroxybenzoate ($C_9H8Cl_2O_3$). The results suggest that since there is no use of endosulfan for long years the residual endosulfan might have completely mineralized into low molecular weight compounds because the persistence of endosulfan in soil is around 800 days. Endosulfan was degraded via oxidation and hydrolysis pathway leading to the formation of toxic endosulfan sulphate and less toxic endosulfan monoaldehyde[5, 17]. But in the current work none of the reported intermediates were formed. The results did not give any clue to which extent the compound has got degraded so it was not possible to find a specific pathway by which the endosulfan has got mineralized into metabolites. The mass spectrum of residual endosulfan and its metabolites are given in **figure 4.**

16S rDNA GENE AMPLIFICATION

The amplified DNA from all the samples yielded fragments of molecular size in the range of 1500bp. Visualization of DNA on agarose electrophoresis showed discernible quantities, **figure 5** and good reproducibility in replicate samples. The DNA samples gave a yield of 66µg/ml, 57µg/ml and 64µg/ml at an absorbance ratio of 260/280nm. The PCR results were compared with a similar work in which the yield of the DNA obtained was in the range 0.2µg and 2.5µg[18]. DNA was isolated from forest samples and the amplified product showed a single band of 250bp and with a DNA yield of 1.9µg-2.18µg[19].



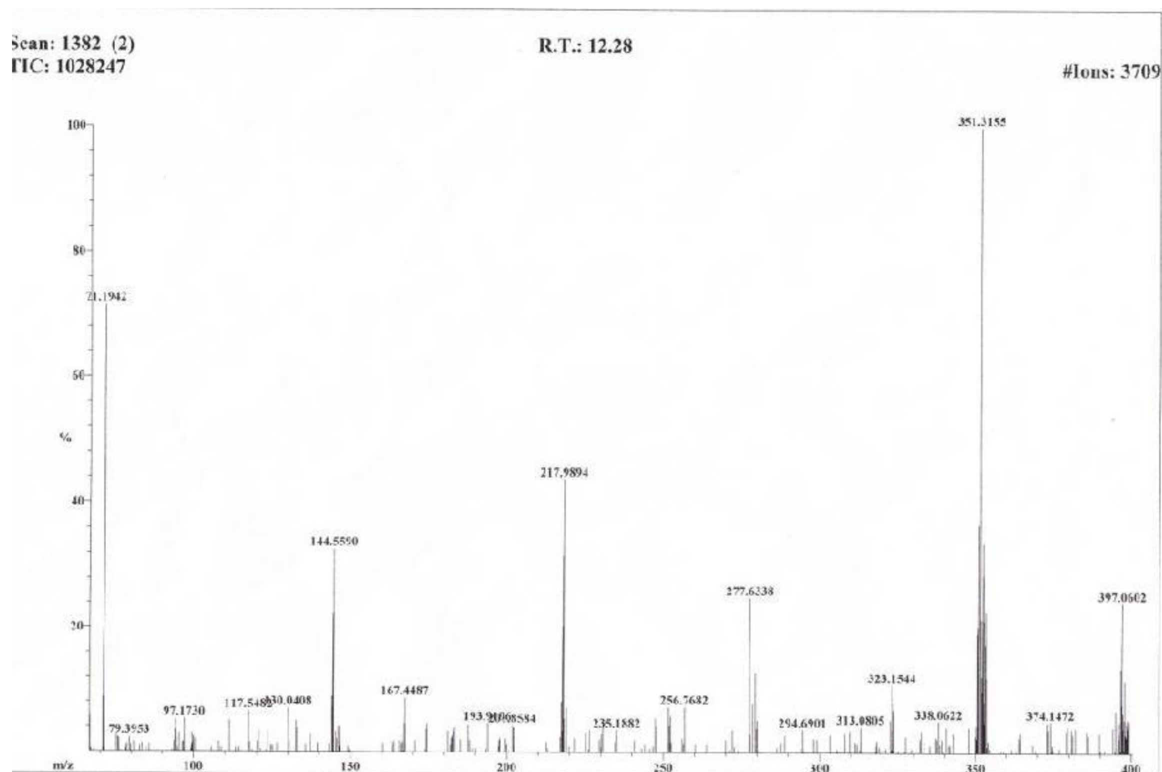


Figure 4: Mass spectra of key intermediates

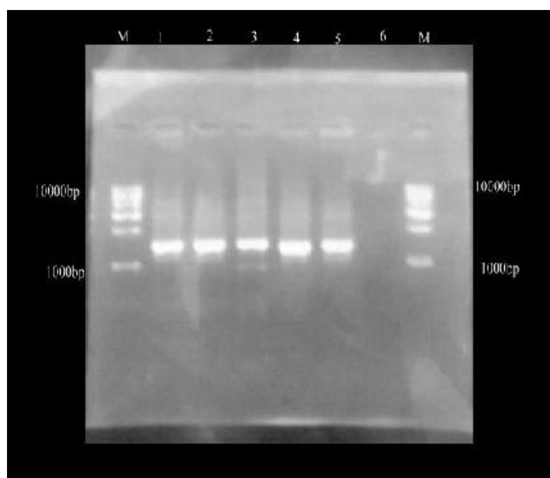


Figure 5: Agarose gel (0.8%) electrophoresis of amplified sample. Lane1 and 8-1Kbp DNA marker. Lane 2, 3, 4, 5, 6 DNA sample.

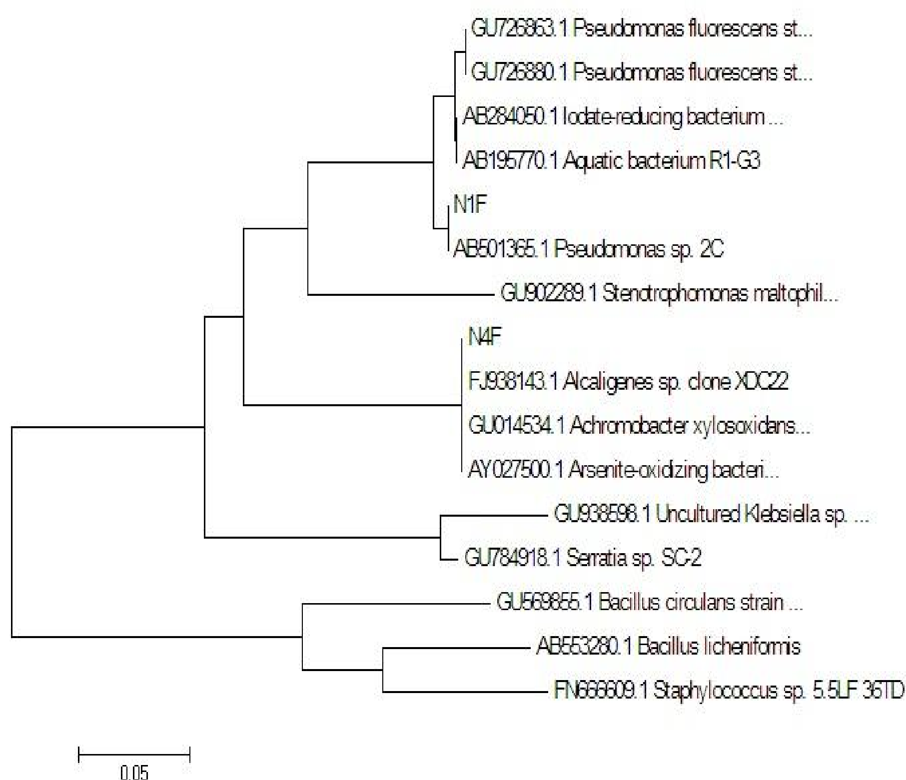


Figure 6: Neighbour joining phylogenetic tree

PHYLOGENETIC IDENTITY OF BACTERIAL STRAIN

Two best bacterial isolate viz N1 and N4 were taken for sequence analysis using BLAST programme. N1 showed 100% identity to *Pseudomonas* sp.2C (Accession number HQ141384) which belongs to the Phylum proteobacteria, Class Proteobactera and Order Pseudomonadales and N4 showed 100% identity to *Alcaligenes* sp clone XD22 (Accession number HM224562) which belongs to the Phylum Proteobacteria, Class Proteobacteria and Order Burkholderiales. The tree was constructed using MEGA4: Molecular Evolutionary Genetics Analysis Software version 4.0. The phylogenetic neighbouring trees of both the strains are shown in figure6. An endosulfan degrading fungus, *Fusarium ventricosum* by LSU rRNA gene sequence analysis was also reported[9]. Based on the first 300bp of the sequence it showed 99.6% similarity to *Fusarium solani* and 98.75% to *Fusarium eumartii.* A phylogenetic tree was constructed with top 10 alignment matches. Soil sustains an immense diversity of microbes, which to a large extent remains unexplored. A range of novel methods,

which are based on rDNA or rRNA analysis have uncovered part of the soil microbial diversity[20]. [21]studied on the distribution of 16S r DNA sequences among five different bacterial divisions to search for relationships between the abundance of microbial groups and soil nutritional status. The results suggested that the soil with a high content of readily available nutrients showed positive selection for and *Proteobacteria.*

SUMMARY

Ongoing research and monitoring has raised concerns about possible environmental and human health risks that endosulfan presents. New research, for example has identified that endosulfan is capable of long range environmental transport because of its volatility and relative persistence in the atmosphere. The residues of endosulfan are major environmental contaminants in several countries of the world. They are potentially hazardous to the living system because of their inclination to bio accumulate in the lipid component of biological species and their resistance to degradation. Even though the use of endosulfan is being banned, it is still used in large especially in developing countries like India. Owing to the physical and chemical properties of endosulfan it is important to develop a strategy for bioremediation of such pesticide in the environment. Bioremediation is a process that uses microorganisms to mediate the transformation of hazardous chemicals to less toxic compounds. This research work aims at the enrichment and isolation of endosulfan degrading microorganisms and to find out the residual endosulfan present in cashew plantations of Kasargod district, Kerala. The major works involved was the enrichment and isolation of Endosulfan degrading bacteria. Around four morphologically different bacteria were used for the study. Growth kinetic studies were carried out to find out the optimum concentration at which the bacterium shows maximum growth by utilizing endosulfan as the sole source of carbon and sulphur. 16S rDNA sequence was amplified by colony PCR and good reproducible results were obtained. The organisms were identified to be *Pseudomonas* sp. 2C (HQ141384) and *Alcaligenes* sp. clone XDC22 (HM224562). Based on the results I conclude that the isolated strains can be used for the bioremediation of contaminated sites. Molecular approaches developed for the identification of microorganisms such as 16S rDNA gene amplification using colony PCR can be used as a valid method for investigating diversity in microbial environment. The results also suggest that there was no residual amount of endosulfan in the soils of these cashew plantations and it has completely got mineralized into nontoxic low molecular weight compounds. Further studies can be done to find out the residual endosulfan in plants and water sources in these plantations thereby we can find out measures to eliminate this toxic completely from our environment.

ACKNOWLEDGMENT

We would like to acknowledge Sophisticated Analytical Instrument Facility in IIT Madras who helped us in carrying out GC-MS analysis of our soil samples.

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