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16S rDNA BASED IDENTIFICATION OF BUTACHLOR DEGRADING SOIL BACTERIAL ISOLATES FROM RICE FIELD IN UTTARAKHAND TERAI REGION

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Abstract

Organic pesticide accumulation in the field causes ammonia and nitrite poisning which create water and soil pollution. It becomes essential to do efforts regading removal of such waste. Micro-organisms play important role in removal of such compounds by evolving their metabolisms. In this study culturable bacterial biodiversity of butachlor degrading four bacterial isolates indigenous to soil herbicide was assessed. These soil bacterial isolates were characterized through a variety of phenotypic, morphologic, biochemical and molecular properties. Molecular identification of these bacterial isolates was done through partial 16s *rDNA* based gene sequencing method. Bacterial 16S ribosomal RNA (rRNA) genes contain nine "hyper-variable regions" (V1–V9) that demonstrate considerable sequence diversity among different bacteria. PCR Amplification of 16S *rDNA* of variable regionV4-V5 for four isolates BC1, BC2, BC3 and BC4 were carried out by using universal primers and products were sequenced commercially. These gene sequences were compared with other gene sequences in the GenBank database. A phylogenetic tree was constructed. It was found that isolates belonged to different species of genus *Bacillus*, sharing 92-99% 16S *rDNA* identity, isolates also had close similarities with *Penibacillus* and *Geobacillus* with 97%, 98% 16S *rDNA*. Gene for partial 16s *rRNA* were deposited to GenBank and accession numbers were obtained. Based on this study it can be concluded that, soil having continuous exposure to butachlor is populated with diverse bacterial groups. Partial 16s *rDNA* sequencing can be used to identify the microflora responsible for degradation of hazardous pesticides.

Keyword:- Biodiversity, Characterization; Butachlor, PCR amplification of 16S *rDNA*.

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Introduction

16s *rDNA* based identification of micro-organisms is an attribute of an area and specifically refers to the varieties within and among living organisms, assemblage of the living organisms, biotic communities and biotic processes, whether naturally occurring or modified by humans. Microbial degradation is the main process affecting the environmental persistence of pesticides. In order to study the environmental degradation and dissipation of butachlor after its application in the field, and to seek the safe and more correct use, several investigations about degradation of this herbicide by several soil microbes have been carried out (Chen *et al.*, 1978). Soil contains a variety of microorganism included bacteria that can be found in any natural ecosystem (Aislabie and Jones, 1995). Apart from biological techniques, molecular based techniques are often used to identify specific micro-organisms or

strains those are exploited for commercial production of enzymes and biodegradation of xenobiotics as well. Molecular cloning and sequencing of genes that code ribosomal 16s *rRNA* is known to be important tool in studying bacterial population in environmental sample (Ercolini, 2004).

The 16S *rDNA* sequences those present as multiple copies in the genome of many bacteria are highly conserved (Lan *et al.,* 1998). PCR-ribotyping is based on the amplification of spacer variability in length and number of copies provides means for classification of strains of different bacteria (Jamil *et al*., 2007).

In the present research work we have studied bacterial diversity in the soil treated with herbicide butachlor. Here we carried out morphological, biochemical and partial 16S *rDNA* based molecular characterization of four bacterial isolates having capability to degrade herbicide butachlor as well as bacterial isolates were also been screened for the production of enzymes amylase and protease. No repot is or very little information is available. In the present study effort has been made to examine the biodiversity profiling of bacterial isolates present in soil having butachlor herbicide.

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MATERIAL AND METHODS:-

The soil samples, generally clay loam soil, used to study microbial degradation were collected from four different rice fields of Crop Research Centre (CRC), at G. B. Pant University of Agriculture and Technology (GBPUA&T) Pantnagar, where the butachlor has been in use for several years (Singh *et al*., 2004). The soil samples were stored at sealed plastic containers. The soil sample collected was further treated with single and double dose of butachlor. A control soil sample was maintained without butachlor treatment. All the samples treated and untreated were incubated at 37ºC±1ºC for one week and moisture was maintained by keeping a water filed plate in it.

The butachlor-degrading bacteria were isolated using the enrichment culture technique (Ou and Sharma, 1989). The culture was incubated at 37ºC at 150 rpm for one week. Five milliliters of enrichment culture was subsequently sub-cultured for another one week. The HPLC was carried out to determine the presence of butachlor, and confirm degradation followed by the isolation of the butachlor-degrading microorganisms by spreading the diluted enrichment culture serially on minimal media plates containing butachlor at 0.1g/L as the sole carbon source and purified agar at 2.0% (w/v). In this way, four bacterial isolates were selected for further characterization and also to find out diversity in the soil treated with butachlor.

Biochemical Tests-The catalase activity was determined by the appearance of air bubbles to an overnight grown single bacterial colony after addition of 3% H_2O_2 solution. The starch hydrolysis was determined by adding Lugal's Iodine to inoculated nutrient agar plates containing 1% of soluble starch. Absence or presence of color around the colonies was monitored and the presence of blue color around the colonies indicated positive test. The gelatin hydrolysis was determined by inoculating the isolates in the nutrient broth test tubes containing 1% gelatin. Hydrolyzed gelatin will remain fluid after chilling the tubes in ice water. Casein hydrolysis was determine by 2% skim milk containing plates inoculated with the isolates by making a 'V'-mark after incubation at 37°C for 24 hr. Casein, which was opaque in the plate before incubation, was converted to soluble products upon hydrolysis, resulting in formation of a clear zone around the site of inoculation, which indicated the hydrolysis of casein. For the urease test, urea broth was inoculated with bacterial culture. Urease production influenced by the bacteria was determined by visualization of color change of phenol red. For citrate utilization, the Simmond's agar was prepare, the culture was incubated at 37°C for 48 hr and citrate utilization was identified on visualization of color change of bromothymol blue from green to blue. Indole production test was carried out using trypton broth which was inoculated with all the four present bacterial isolates at 37°C for 48 hr. The indole ring formation was checked and noticed after supplementation of Kovack's reagent.

Identification of bacterial isolates were carried out according to Bergey's manual of determinative bacteriology (Holt *et al.* 1994), and based on sequence analysis of its 16S *rRNA* gene fragments. The genomic DNA was extracted using the method of high-salt concentration precipitation described earlier (Miller *et al.*, 1988). The 16S *rRNA* gene fragment was amplified by PCR using standard procedures (Lane, 1991) using universal primers, the forward, 5′-CAGCAGCCGCGGTAATAC-3′ and reversed 5'-CCGTCAATTCCTTTGAGTTT-3'(synthetic prokaryotic primers) described by Ercolini (2004). The specific primers could amplify a fragment of 407 nt, positioned in 519 to 926 nt the V4-V5 region of 16s *rRNA* gene. The PCR products were purified using PCR Purification Kit, after purification the PCR products were sent for sequencing. Consensus sequences were taken for further analysis.

Multiple sequence alignments were carried out using the program Clustal X (version 1.81). These gene sequences were compared with other gene sequences in the GenBank databases to find the closely related sequences. The maximum likelihood phylogenetic relationships were determined

using the program MEGA4 (Tamura et al., 2007). The present sequences were deposited to GenBank and accession numbers were obtained under the following accession number: BC1 (JF830086), BC2 (JF830087), BC3 (JF830088), BC4 (JF830089) (Fig.2). Distances were calculated using the Kimura two-parameter distance model. Dataset was bootstrapped 1000 times.

RESULTS AND DISCUSSION:-

The four bacterial isolates selected in the present study were characterised based on morphology and biochemical reaction. The bacterial isolates were characterised on the basis of visualisation of colony colour and morphological characters like size, shape and texture (Table 1). All the isolates showed yellow pigmentation in the colonies except BC1 which showed light blur colonies on Nutrient agar medium. The colonies of all the bacterial isolates are smooth in texture. However, the colonies of the present isolates showed round-circular to irregular in shape, small to large in shape and wavy-undulated to entire margin. The colonies of all the isolates were opaque except isolate BC1 which was translucent. Therefore, the present isolates are found to be diverse in their morphological characters and in pigmentation.

Table 1: Identification of the present bacterial isolates based on morphological characterstics

7	Colony morphology						
	Size	Shape	Colour	Surface texture	Elevation	Margin	Light transmission
BC_1	Small	Circular	Light blue	Smooth	Flat	Entire	Opaque
BC_2	Large	Circular	Yellow	Smooth	Flat	Wavy	Translucent
BC_3	Medium	Round	Yellow	Smooth	Convex	Entire	Opaque
BC_4	Large	Irregular	Yellow	Smooth	Undulate	Undulate	Opaque

All the isolates showed positive reactions in catalase test indicating that all are aerobic in nature. The isolates, BC2, BC3 and BC4 gave positive reaction in milk casein hydrolysis test showing formation of clear zone surroundings the place of inoculation on the medium. This results determined presence of the enzyme caseinase in these bacterial isolates. Bacterial isolates BC2 showed positive result in starch hydrolysis test, which indicated presence of enzymes amylase in this isolate. The isolate BC1 showed productions of β-glucosidase, as blue colonies were observed on LB cellobiose agar plates. The isolates BC1, BC2, BC4 showed urease production, as the colour of reagent phenol red was changed to pink-orange from purple. All the isolates showed positive results for indole ring production as indole ring was observed in the medium after adding Kovac's reagent (Table 2).

The protocols for biochemical identification were same as used to identify and characterise the lactic acid bacteria from fish and prawn (Nair and Surendran, 2005).

Table 2: Biochemical analysis of the present bacterial isolates on respective media

SN	Biochemical analysis	BC_1	BC_2	BC_3	BC_4
1	Starch hydrolysis	-	+	-	-
2	Gelatin hydrolysis	-	-	-	-
3	Casein hydrolysis	-	+	+	+
4	Indole production	+	+	+	+
5	Urease	+	+	-	+
6	Catalase test	+	+	+	+
7	β-glucosidase	+	-	-	-

+ activity shown; – no activity;

The identification of present bacterial isolates was determined based on sequencing of a fragment of 16S *rRNA* gene. In PCR, the specific primers amplified the desired size of 407 nt, from all the four present isolates used (Fig.1). This amplicons were sequenced, and analysed with other bacterial isolates. This sequence region 16S *rRNA* gene has been preferred as phylogenetic marker used in bacteria ecology (Normand, 1996). During the past few years, the availability PCR based techniques and sequencing of the nucleotides have rapidly extended RNA data bases (Ludwig and Schleifer, 1999; Normand, 1999; Lv et al 2014).

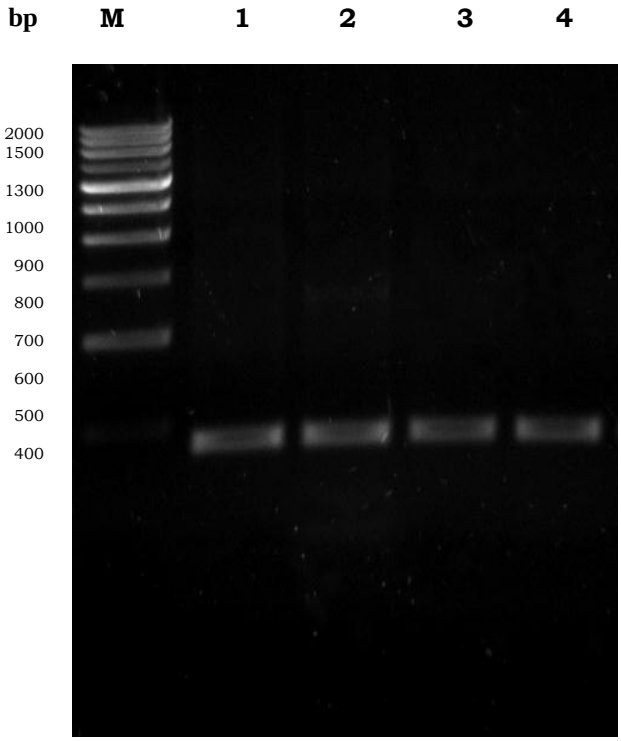

Figure 1. PCR amplified product of bacterial genomic DNA;Lane-M 400bp Ladder, Lane-1 BC1, Lane-2, Lane-3 BC3, Lane-4 BC4.

Preliminary characterization studies depicted great deal of diversity in terms of colonial morphologies and microscopic studies.

In phylogenetic analysis using 16s *rDNA*, it was found that isolate BC1 grouped with 2*Penibacillus sp*., 3*Bacillus sp*. DU52, 4*Bacillus sp.* DB89, 5*Bacillus* SGE20, 6*Penibacillus xylanilticus* strain, 7*Penibacillus pabuli* strain FR142, 8Bacterium RK62, within this group they showed 99% identity among them. The isolate BC2 showed 95% identity with *Bacillus cereus partial*, however, it had 99% identity with isolate BC4. The isolate BC3 showed similarity in sequence identity with 16*Bacillus sp*.DU87, 17*Bacillus megaterium* strain, 18*Bacillus flexus*,19*Marine bacterium*, 20*Endophytic bacterium* BAC-11124, 22*Bacillus sp.2*23, 23 *Bacillus sp.* H41.1 showing 99% similarity within group. The isolate BC4 had 99% identity with 11*Bacillus sp. C2*. (Fig. 2). The present work describes the identification of native bacterial isolates having capability to degrade and mineralize butachlor as a sole carbon source.

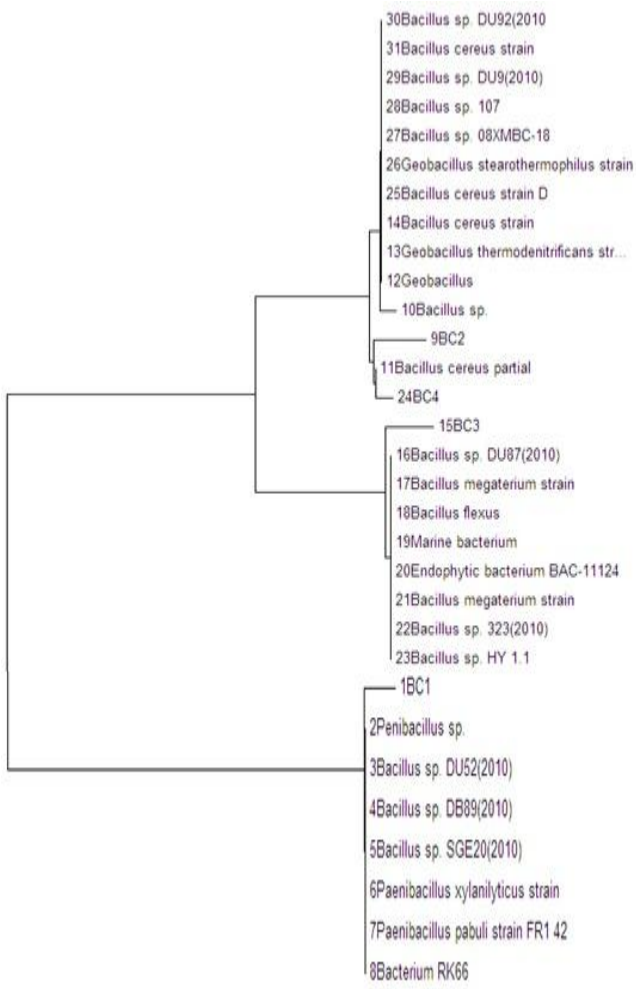

Figure 2. Phylogenetic tree showing the relationships among the selected isolates BC1, and BC2, BC3 and BC4 and other closely related sequences collected from the Gene Bank.

The results presented here further extend the preliminary observations, and indicate considerable phylogenetic diversity within a closed ecological environment.

The phylogenetic analysis using sequence of partial amplified product of 407 bp from 16s *rDNA* ,16s *rDNA* of present bacterial isolates and other bacterial isolates retrieving from the GenBank indicated tentative taxa of the present bacterial isolates. The sequence analysis and comparison of nucleotide sequences in the present study showed that 16s *rRNA* is highly conserved within a species of the same genus (Abd-El-Haleem *et al*., 2000; 2002). The partial 16s *rDNA* gene sequencing and database homology search for different bacterial isolates revealed that present bacterial isolates have tentative close relationship with members of *Bacillus, Penibacillus and Geobacillus.*

On molecular basis, most of the isolates belonged to the *Bacillus, Penibacillus and Geobacillus* genus, indicating that bacteria belonging to this group were more prevalent in soil treated with herbicide butachlor containing environments compared to other microbial groups that may be found under such environments. Enzymes produced by some of these isolates such as proteases, amylases, can extensively used in the industries for the manufacture of pharmaceuticals, foods, beverages and confectioneries as well as in textile and leather processing, paper industry and waste water treatment. The majority of the enzymes used in the industry are microbial in origin because microbial enzymes are relatively more stable than the corresponding enzymes derived from plants and animals. Soil has proven to be a rich source of microorganisms harbouring industrially important enzymes. These bacteria have indicated production of enzymes amylase and protease, which can be use commercially. Culturing microbes and molecular analysis give us an edge to have more and more cultured microorganisms with their taxonomic status from any sort of such environment. Though, traditionally extraction of environmental DNA may enhance the extant of biodiversity contained in these samples, but at the same hand gives us less opportunity to know the exact profile of cultural characteristics of the microorganisms of any such environment. Therefore the strategy to culture microorganisms from any environmental sample and then designing a methodology that delineates biodiversity increases the prospect of having microbial resource in hand which can be further used for other purposes at a later stage.

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