International Standard Serial Number (ISSN): 2249-6807

International Journal of Institutional Pharmacy and Life Sciences 5(2): March-April 2015

INTERNATIONAL JOURNAL OF INSTITUTIONAL PHARMACY AND LIFE SCIENCES

Life Sciences **Research Article……!!!**

Received: 15-02-2015; Revised: 26-02-2015; Accepted: 01-03-2015

ISOLATION AND IDENTIFICATION OF CARTAP HYDROCHLORIDE DEGRADING BACTERIA

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Keywords:

Cartap hydrochloride, *Enterobacter aerogenes*, gene sequence, enrichment technique

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ABSTRACT

Cartap hydrochloride is a thiocarbamate insecticide used to kill insects of Lepidoptera and Coleoptera. It is used for crops such as sugar cane, rice, maize, etc., In this study Cartap hydrochloride degrading bacteria were isolated by using Mineral Salts medium. Among the isolated bacteria, the efficient organism is identified by using Plate assay method. Of the three micro organisms, *Enterobacter aerogenes* was found to be efficient and screened by 16s rRNA sequencing.

INTRODUCTION

In nature, microorganisms have evolved degradative pathways as a result of continuous or repeated exposure to xenobiotic chemicals. However, because of the enhancement of microbial degradation of many chemicals, the efficacy of several pesticides has been reduced[1] .In particular, loss of insecticidal activities has been reported in soils that have received continuous applications, resulting in the enhanced degradation of these compounds by soil microorganisms[2].Isolation of pesticide degrading microorganisms is important to determine the mechanism of process of microbial metabolism, mechanism of enzyme/gene evolution and use of microbes for the detoxification of polluted environment. Many microorganisms that utilize pesticide as a sole source of energy have been isolated[(3,4,5)]. Characteristics of microorganisms such as their small size, ubiquitous distribution, high specificity, surface area, potentially rapid growth rate and unrivaled enzymatic and nutritional versatility cast them as recycling agents. Moreover, the diversity of inorganic and organic materials present on Earth match diversity of habitats whose physical and chemical characteristics span wide ranges of pH, temperature, salinity, oxygen tension, redox potential, water potential, etc. This distribution of resources between environments gave origin to a selective evolutionary diversification of microorganisms, resulting in an evolved microbial world capable of exploiting all the naturally occurring metabolic resources on Earth[6]. Biodegradation is a metabolic process that involves the complete breakdown of an organic compound. When this compound is broken down into its inorganic components, the process is referred to as mineralization. Biodegradability represents the susceptibility of substances to be altered by microbial processes. The alteration may occur by intra- or extracellular enzymatic attack that is essential for growth of the microorganisms. The attacked substances are used as a source of carbon, energy, nitrogen, or other nutrients or as final electron acceptor. The rate of biodegradation depends on environmental factors, numbers and types of microorganisms present, and the chemical structure of the target compound[6].

MATERIALS AND METHODS

Collection of soil sample

Samples of a black loamy soils, collected from sugarcane cultivated fields of Tirupati, Chittoor district of Andhra Pradesh, India, to a depth of 12 cm, were air-dried and sieved through a 2-mm mesh before use. Physico-chemical characteristics of the soil was analyzed using standard methods.

Pesticide

Cartap hydrochloride is one of the main insecticides used in India particularly for the crops of Rice and Sugarcane to control weevil and caterpillars. It acts at very low concentration and its efficacy is very prolonged. It controls all stages of the insect life cycle.

Isolation of Cartap hydrochloride degrading bacteria

10 gm of soil sample was added to the MS medium supplemented with Cartap hydrochloride and incubated at room temperature 28^0C +/- 4^0C for 7 days on a rotary shaker at 150 rpm. 1ml of bacterial inoculums was serially diluted at daily intervals and 0.1 ml of 10^{-6} to 10^{-8} dilutions were plated in duplicate on mineral salts agar plates supplemented with the same concentration of Cartap hydrochloride and incubated at 37^o C for 24 hours.

Identification of bacteria

Identification of the bacterial isolates was carried out by the routine bacteriological methods such as preliminary tests like colony morphology, motility, Indole, Methyl red, Voges-proskauer, Citrate, oxidase and sugar fermentation tests[7]tests were performed to identify the isolates and further characterization was done according to Bergey's Manual of Systemic Bacteriology Vol. I & II[8].

Screening of Cartap hydrochloride degrading bacteria

Isolated strains were screened for potential Cartap hydrochloride utilizing character by performing a plate assay method and Enrichment culture technique.

Molecular characterization of Cartap hydrochloride degrading bacteria

Cartap hydrochloride degradation was characterized by analysis of the 16s rRNA gene partial sequencing and was further used for BLAST analysis from the NCBI database to obtain sequence similarity with related organisms. These sequences were extracted from the database and aligned using CLUSTALW programme and phylogenetic tree was constructed using PHYLIP analysis programme.

RESULTS

Physico-chemical properties of the soil

The **Physico-chemical** properties of black loamy soil were tested in Soil Testing Centre, S.V. Agricultural College, Tirupati, A.P, India. As shown in the (Table 1).

TABLE 1- PHYSICO-CHEMICAL PROPERTIES OF THE SOIL

Properties	Black soil
Sand (%)	49%
Silt (%)	20%
Clay (%)	28%
pH[a]	7.7
Texture	Black loamy sandy soil
Water holding capacity(ml g^{-1} soil)	0.226
Electrical Conductivity (dS m^{-1})	0.18
Organic matter (%)	0.9%
Total nitrogen (%)	0.075kg/ha
Available Phosphorus	22 kg/ha
Available potassium	142 kg/ha

Chemical structure of Cartap hydrochloride

As shown in the (Fig 1), its basic chemical structure is ***S,S***-[2-(dimethylamino)-1,3-propanediyl] dicarbamothioate and is normally used as the hydrochloride (Cartap hydrochloride). Its molecular Weight is 273.80 and the molecular Formula is $C_7H_{15}N_3O_2S_2$ HCl.

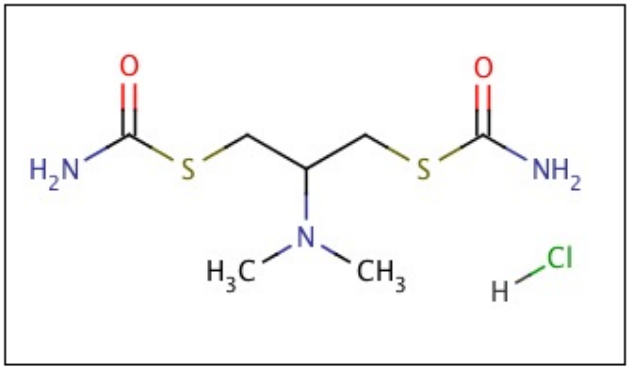


FIGURE 1-CHEMICAL STRUCTURE OF CARTAP HYDROCHLORIDE

Isolation of Cartap hydrochloride degrading bacteria

They were processed by cultivating in mineral salts medium enriched with Cartap hydrochloride to obtain pure cultures of Cartap hydrochloride degrading bacteria. From mineral salts medium, the inoculums was placed on mineral salts medium agar plates supplemented with Cartap hydrochloride and incubated for 24 hours. After incubation, a total of three different bacterial isolates were obtained from soil sample and the colony morphology was recorded for all isolates and were labelled as CHC I, CHC II and CHC III.

Identification of Cartap hydrochloride degrading bacteria

Three different morphologically distinguishable bacterial isolates were selected for further study. The results were shown in the (Table 2).

TABLE 2- MORPHOLOGICAL, PHYSIOLOGICAL AND BIOCHEMICAL TESTS FOR CHARACTERIZATION OF SCREENED ISOLATES

TEST	CHC I	CHC II	CHC III
COLONY MORPHOLOGY			
Configuration	round	Round	Filamentous
Pigment	-	+	White to grey
Gram's reaction	-	-	+
Cell shape	Straight rods	Thin short rods	Rods
Motility	+	+	+
Spores	-	-	+
PHYSIOLOGICAL TESTS			
Growth at temperatures (^oC)		-	+
15	-	-	+
20	+	+	+
37	+	-	-
42	-		
52		-	
Growth at pH	+	+	+
4.5	+	+	+
6.5	+	-	+
8.5	-	-	-
10.5	-		-
12.5		+	
Growth at NaCl (%)	+	+	+
2	+	-	+
4	+	-	+
6	-		-
8		+	
BIOCHEMICAL TESTS	-	+	+
Oxidase	+	-	+
Catalase	-	-	-
Indole	-	-	-
Methyl Red	+	-	-
Voges Proskauer	+	+	+
Citrate utilization	-	+	-
Urease test	+	+	-
Acid from glucose	+	+	-
Acid from lactose	+	+	-
Acid from sucrose	+	-	-
Acid from mannitol	-	+	+
Arginine dihydrolase	+	+	+
Gelatin liquefaction	-	-	-
Starch hydrolysis	-	-	+
Casein hydrolysis		+	
H_2S production	-	-	+
Utilization of sugars as carbon source	+		-
Utilization of sugars as nitrogen source			

Screening of Cartap-hydrochloride degrading bacteria

All the three isolates were subjected to screening to detect the potential Cartap hydrochloride degrading character by using a simple plate assay method based on the formation of clear haloes as a result of hydrolysis of Cartap hydrochloride was developed for rapid screening of all Cartap hydrochloride- degrading bacteria[9] .The bacterial isolates were spot inoculated onto Luria-Bertani plates containing 0.75 mM Cartap hydrochloride and maintained at 37^{o} C for 48 hours. Colonies were selected based on the formation of a large clear zone on the plate as shown in (Fig 2).

FIGURE 2- PLATE ASSAY METHOD

Enrichment culture technique

Cartap hydrochloride application by using Mineral Salts medium enriched with commercial grade insecticide Cartap hydrochloride (90% E.C) gm/L as a sole source of carbon. After successive plating of the enrichment culture, individual colonies were sub cultured onto mineral agar plates containing Cartap hydrochloride until pure cultures were isolated. Stock cultures of those isolates were maintained on nutrient agar slants and preserved under refrigerator (4^{o}C) until further use.

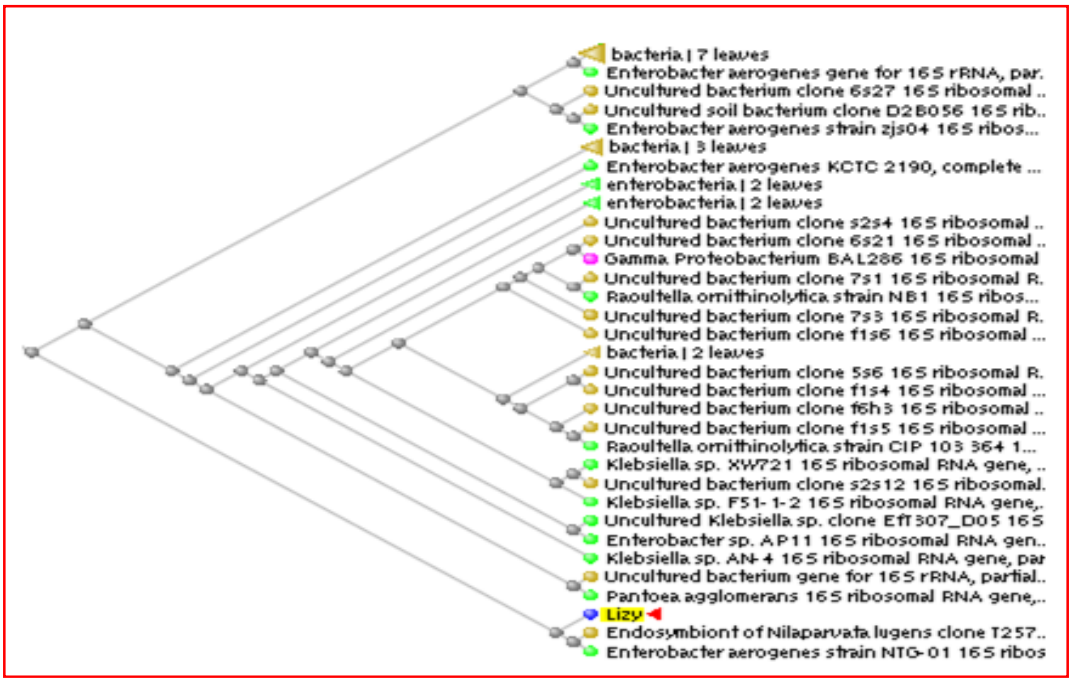
Molecular characterization of Cartap hydrochloride degrading bacteria

Among the three isolates CHC I, potential isolate was selected for further studies based on its rapid luxurious growth indicating relatively maximum Cartap hydrochloride hydrolyzing ability on mineral salts agar plates supplemented with Cartap hydrochloride as nitrogen source. Hence, this isolate was further confirmed and characterized by conducting partial gene sequencing of 16S rRNA. It was confirmed and characterized as *Enterobacter aerogenes* by using partial sequencing of 16S rRNA and BLAST analysis (Fig 3). Its sequence of 16S rRNA gene showed 99% similarity with species of *Enterobacter*. The

sequence was further used for BLAST analysis from NCBI database to obtain 99% sequence similarity of related organisms and aligned by using CLUSTAL W programme. The phylogenetic tree was constructed using PHYLIP analysis programme (Fig 4).

1 cttgttacga cttcacccca gtcatgaatc acaaagtggt aagcgccctc ccgaaggtta
61 agctacctac ttcttttgca acccactccc atggtgtgac gggcggtgtg tacaaggccc
121 gggaacgtat tcaccgtagc attctgatct acgattacta gcgattccga cttcatggag
181 tcgagttgca gactccaatc cggactacga catactttat gaggtccgct tgctctcgcg
241 aggtcgcttc tctttgtata tgccattgta gcacgtgtgt agccctactc gtaagggcca
301 tgatgacttg acgtcatccc caccttcctc cagtttatca ctggcagtct cctttgagtt
361 cccgaccgaa tcgctggcaa caaaggataa gggttgcgct cgttgcggga cttaacccaa
421 catttcacaa cacgagctga cgacagccat gcagcacctg tctcagagtt cccgaaggca
481 ccaaagcatc tctgctaagt tctctggatg tcaagagtag gtaaggttct tcgcgttgca
541 tcgaattaaa ccacatgctc caccgcttgt gcgggccccc gtcaattcat ttgagtttta
601 accttgcggc cgtactcccc aggcggtcga cttaacgcgt tagctccgga agccacgcct
661 caagggcaca acctccaagt cgacatcgtt tacggcgtgg actaccaggg tatctaatcc
721 tgtttgctcc ccacgctttc gcacctgagc gtcagtcttt gtccaggggg ccgccttcgc
781 caccggtatt cctccagatc tctacgcatt tcaccgctac acctggaatt ctacccccct
841 ctacaagact ctagcctgcc agtttcgaat gcagttccca ggttgagccc ggggatttca
901 catccgactt gacagaccgc ctgcgtgcgc tttacgccca gtaattccga ttaacgcttg
961 caccctccgt attaccgcgg ctgctggcac ggagttagcc ggtgcttctt ctgcgagtaa
1021 cgtcaatcgc caaggttatt aaccttaacg ccttcctcct cgctgaaagt actttacaac
1081 ccgaaggcct tcttcataca cgcggcatgg ctgcatcagg cttgcgccca ttgtgcaata
1141 ttccccactg ctgcctcccg taggagtctg gaccgtgtct cagttccagt gtggctggtc
1201 atcctctcag accagctagg gatcgtcgcc taggtgagcc attaccccac ctactagcta
1261 atcccatctg ggcacatctg atggcatgag gcccgaaggt cccccacttt ggtcttgcga
1321 cgttatgcgg tattagctac cgtttccagt agttatcccc ctccatcagg cagtttccca
1381 gacattactc acccgtccgc cgctcgtcac ccgagagcaa gctctctgtg ttaccgctcg
1441 acttgcatgt gttaggcctg ccgccagcgt tcaatctgag ccatgatcaa act

FIGURE 3- 16S RRNA SEQUENCE OF ISOLATED BACTERIA



(The strain *Enterobacter aerogenes* was designated as LIZY in the tree.)

FIGURE 4- PHYLOGENETIC RELATIONSHIP OF *ENTEROBACTER SP.* BASED ON PARTIAL 16S RNA SEQUENCE

DISCUSSION

Three different organisms were isolated from sugarcane cultivated soils and among them two were gram negative bacteria and one was gram positive bacteria, according to *Bergey's Manual of Systemic Bacteriology*, Vol I and II (Krieg, 1984). Screening of soil bacteria capable of utilizing Cartap hydrochloride as sole nitrogen source was done by enrichment technique on mineral salts medium containing Cartap hydrochloride. All the three isolates were subjected to screening to detect the potential Cartap hydrochloride degrading character by using a simple plate assay method on the formation of clear haloes as result of Cartap hydrochloride hydrolysis. The three screened isolates are tentatively identified as *Enterobacter aerogenes*, *Pseudomonas putida* and *Streptococcus pilosus* based on their biochemical characters and relatively larger zone was recorded in *Enterobacter aerogenes* over the other two isolates. The highly potential Cartap hydrochloride utilizing bacterial isolate was further characterized and confirmed as *Enterobacter aerogenes* based on the biochemical and molecular characterization. 16S rRNA gene of the isolated strain deposited in Genbank and Accession number is KF731618[10] was determined. The nucleotide alignment of the strain showed 99% similarity with *Enterobacter aerogenes* KCTC 2190, complete genome.

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