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ISOLATION, IDENTIFICATION AND USE OF CARBOFURAN DEGRADING MICROORGANISMS FOR THE REMOVAL OF CARBOFURAN PESTICIDE FROM CONTAMINATED WATERS

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ABSTRACT
Carbofuran is one of the pesticides which is banned or severely restricted in other countries, but it is still used in India. In this study, microbial strains effective in degrading carbofuran were isolated from the soil samples treated with carbofuran. Isolated bacterial strains were exposed to 10mg/l of technical grade carbofuran to determine the degradation efficiency. Significant degradation of carbofuran *i.e.* 43% by C1, 44% by C2, 35% by C3 and 55% by C4 isolate was observed in 8 days under laboratory conditions. When the bacterial strains were used in combination, the C2-C4 combination was found to be most efficient with a degradation rate of 60% in 8 days. C1, C2, C3, C4 were identified as *Arthrobacter globiformis, Streptomyces sp. Bacillus beijingensis, Bhargavaea indica* using 16S rRNA technique.

KEYWORDS: Pesticides, Carbofuran, Bioremediation, Microbial Consortium, 16S rRNA.

INTRODUCTION

Pesticide is defined as a substance used for destroying, preventing, repelling or mitigating any pest[1]. Pesticides consist of insecticides, herbicides, fungicides, virucides, and others. The use of pesticides in agriculture is increasing to improve crop production. As a consequence, the use of pesticide has caused contamination of soil and water components of the environment[2]. Moreover the exposure of humans to excess amount of pesticides can be harmful for the health. Insecticides impede the normal functioning of the nervous system. Organochlorine and organophosphates stimulate the nervous system [3]. There is destruction of nerve fibers due to chronic exposure to organophosphates. It also leads to muscle tissue damage (myopathy) [4]. Organochlorines accumulate in the adipose tissue.

Physico-chemical methods used to treat pesticides are not efficient. Pesticide residue present in the soil-water environment, enter the food chain[5]. Hence, bioremediation techniques are required to degrade pesticides. Carbofuran is an insecticide which is broad spectrum and has high oral toxicity. In rats the oral LD50 for carbofuran is reported to be 11 mg/kg of body weight. It is used in crops like corn, rice, cotton *etc*. [6]. Carbofuran is highly toxic to wild birds and mammals [7, 8] and moderately toxic by dermal absorption [9, 10]. A study carried out by Goad *et al*. (2004) evaluates its impact on the levels of endocrine hormones in the serum of male rats. The results showed that acute exposure to this pesticide could result in the disruption of the endocrine system. After repeated exposure this may cause reproductive problems [11]. Carbofuran has high potential for groundwater contamination[12]. GR 6% form of carbofuran is mainly use in India in the rice fields (1kg/ha).High environmental contamination with carbofuran in soil (0.010-1.009 mg/kg), in water samples from two rivers (0.005-0.495 mg/L) and in water samples from ponds situated near the farms in Kenya (2.3 mg/ L) were found. 0.04-1.328 mg/kg of carbofuran was found in maize plant [13]. Many methods have been employed for degradation of Carbofuran . A study has been carried out where a novel species of *Pseudomonas* has been isolated from agricultural soil and the efficiency of the strain to degrade Carbofuran was assessed [14]. The degradation rate was reported as 0.035 mg/kg/day. Complete degradation of carbofuran was achieved within 40 days. In a study carried out by Shih *et al*. (2010) advanced Oxidation Processes were implied to carry out the degradation of Carbofuran. A O3/UV combined method was used for the treatment of wastewater containing 100 mg/L carbofuran at pH 2. It was found that within 50 min 90% of the carbofuran was oxidized [15].

The present study aims to isolate efficient microorganisms from soil which are able to degrade Carbofuran, followed by their identification and using them singly or in combination to remove carbofuran maximally with the future objective of using these strains for the removal of carbofuran in agricultural runoff in rural areas before they contaminate the water bodies.

MATERIALS & METHODS

Carbofuran

Carbofuran is a carbamate pesticide. It is marketed by FMC Corporation and Curater under the trade name Furadan. The chemical name is 2, 3-dihydro-2, 2-dimethyl-7-benzofuranyl methylcarbamate (CAS number: 1563-66-2). It is synthesised on reacting methyl isocyanate with 2, 3-dihydro-2, 2-dimethyl-7-hydroxybenzofuran.

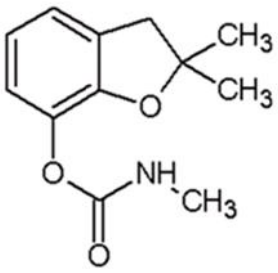

FIGURE 1: Chemical structure of Carbofuran

The fortification of the soil was done with commercial Carbofuran 3% GR insecticide (SUMO). For the degradative studies technical grade carbofuran was used which was obtained from Sigma Aldrich.

Minimal Salt Medium

I. Minimal medium M1 (g/L): $MgSO_4$, 0.01;Na_2HPO_4, 2.1; $CaCl_2$.$2H_2O$, 0.1; $CuSO_4$, 0.04; $FeSO_4$.$7H_2O$, 0.001; Na_2MoO_4,0.002; $(NH_4)_2SO_4$, 1.0 and 1% glucose., Agar agar- 15 g/L.
II. The M2 minimal medium was prepared in a similar manner as M1, but without $(NH_4)_2SO_4$ and glucose. Carbofuran 3% GR (0.1 g/L) was added as the source of carbon and nitrogen.
III. The M3 minimal medium was prepared in a similar manner as M1, but without $(NH_4)_2SO_4$, Carbofuran 3%GR (0.1 g/L) was added as the source of nitrogen
IV. M2 broth (g/L): $MgSO_4$, 0.01; Na_2HPO_4, 2.1; $CaCl_2$.$2H_2O$, 0.1; $CuSO_4$, 0.04; $FeSO_4$.$7H_2O$, 0.001; Na_2MoO_4, 0.002; carbofuran,1mM.

Isolation of Microorganism

Soil was collected from SSK Ltd. Jawhar, Kolhapur, India. (pH- 7.10, Electrical conductivity- 696 µs/cm). Soil samples were treated with 100mg/kg carbofuran. Treated soil samples were incubated at 30^0C for 25 days. 5.0 g of soil samples were added to 45 mL of a sterile solution (0.85% NaCl (w/v) and serial dilutions were made. Surface plating was done in minimal mineral salt media: M1 and M2 for carbofuran. The pH was adjusted to 7.0. For bacterial growth the inoculated plates were incubated at 30°C for eight days in darkness. Each bacterial isolate grown on M2 was purified by streaking it on agar in minimal medium M2. Each isolate was then plated in nutrient agar medium and NAC (nutrient agar medium containing Carbofuran)

Total four microorganisms were isolated from soil samples capable of degrading Carbofuran. Identification of the bacterial species and growth curve 16S rRNA sequencing was done for microbial identification.

16S rRNA sequencing: C2 and C4 are shown here as their combination was found to be the most efficient.

C2:

1. C2-F.abi: Data obtained with Forward primer

2. C2+R.abi: Data obtained with Reverse primer

> C2-F (bp)

GGCCGCAAGGCTAAAACTCAAAGGAATTNGANCGGGG
GCCCNGCACNAANGCAGNCGGAGCATGTGGCTTAATTC
GACNNGCAACGNCGAANGAACCTTACCAAGGCTTGAC
ATATACCGGAAAGCATTAGAGATNAGTGCCCCCCTTGT
GGTCGNGTATACAGGTGGTGCANTGGCTGTCGTNCAGC
TCNGTGTCNNGNNNTGNAGATNGTTGGGTNTAAGTCCC
GCAACGAGCGCAACCCTTGTCCTGTGTTGCCAGNCATG
CNCCTTCGGGGTGATGGGGACTCACAGGAGACCGCCG
GNGGTCAACTCGGAGGAAGGTGGGGACGACGTCAAGT
CATCATGCCCCTTATGTCTTGGGCTGCACACGTGCTACA
ATGGCCGGTACAAAGAGCTGNCGATGCCGCGNAGGCG
GAGCNGANATCTCAAAAAGCCGGTCTCAGTTCGGATTG
GGGTCTGCANACTCGACCCCATGAAGTCGGAGTTGCTA
NGTAATCGCAGATCAGCATTGCTGCGGTGAATACGTTC
CNCNNNGGGCCTTGTACACACCGCCCGTCACGTCANCG
AAAGTCGNGTNANNNACNACCCGAAGCCNNNGGNTGG
CCCAACCCCTTGTGGGNAGGGANGCTGTCGAAGGTGN
NGGNACNTGGCGATTGGGACGAAGTCGTAACAAGGGT
AAC

> C2+R (994bp)

TTTGATCCTGGNCTCAGGACGAACGCTGGCGGNGCNNG
TGNCTTAANNCNACATGCAANGTCGACGANNNTNNNG
AAGNCNNNNCCTNTNCNGGNNNGGNTNNGNGATTAGT
GGCGAACNNGNGGTNGAGTAAACACGTGGNGCAATNN
CTGCCCTTCANCTCTGGGACAAGCCCTGGAAAACGNNG
GGTCTAANTACCGGANNTANCGNANCNNTNGNCNNGN
GNNGNNAGGCATNCTCCTGCGGNNTGNNGNNNNNNNA
NANNAGNCTNCCGGCGGTGAANNNGGATGANGCCCNN
GNCGNNGNCCNNTNATCANGNNCNTNNTGNNTTGGNN
TGGNNNNGNGNTNNAATGGCCNNTACCAAGNGCNGAC
GANNCGGNGTAGCCGGCNCTGAGAGGGCGNNNANCCG
GNCNCANNNCNNACTGGGANCTGNAGACACGGCCCNA
NGANNNCNNNNTCNCTACGGGANNGNNGNNCAGCANN
GNTGNGGNGAATANNTTGCACAATGGGCNNNGNANAN
ANNGCCTGNNATGNCAGCGACNGCCGCGTGAGGGATG
ACNNGNNGCCTTCGGGTTGTANAACCTCTTNTCAGCAG
GGAAGAAGNCGAAAGTGACGGTACCTGNCNANNGNNA
AGAAGNCGNNNCCNGGCTAACTACGTGCCAGCAGCCG
CGGTAATACGTAGGGCGCAAGCGTTGTCCGGAATTATT
GGGCGTAAAGAGCTCGTAGGCGGCTTGTCACGTCGGGT
GTGAAAGCCCGGGGCTTAACCCCGGGTCTGCATCCGAT
ACGGGCAGGCTAGAGTGTGGTAGGGGAGATCGGAATT
CCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGA
ACACCGGTGGCGAAGGCGGATCTCTGGGCCATTACTGA
CGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTA
GATACCCTGGTAGTCCACGCCGTAAACGTTGGGAACTA
GGTGTTGGCGACATTCCACGTCG

Consensus Sequence Sample (1020bp)

>Contig-0

GGCCGCAAGGCTAAAACTCAAAGGAATTTGATCCGGG
GCCCAGCACGAACGCAGGCGGAGCATGTGGCTTAATTC
GACATGCAACGTCGAACGAACCTTACCAAGGCTTGACA
TATACCGGAAAGCATTGAGATTAGTGCCCAACTTGTGG
TCGAGTAAACACGTGGTGCAATGGCTGCCCTTCAGCTC
TGGGACAAGCCCTGGAAAACGTTGGGTCTAAGTACCGC
AACGAGCGCAACCCTTGTCCTGTGTTGCCAGGCATGCT
CCTGCGGGTGATGGGGACTCACAGGAGACCGCCGGCG
GTCAACTCGGAGGAAGCCCGGGACGACGTCAAGTCAT
CATGCCCCTTATGTCTTGGGCTGCACACGTGCTACAAT
GGCCGGTACAAAGAGCTGACGATGCCGCGTGCCGGAG
CTGAGAGCGCAAAAAGCCGGTCTCAGTTCGGACTGGG
ATCTGCAGACACGACCCCATGAAGTCGGAGTCGCTACG
GAATCGCAGATCAGCATTGCTGCGGTGAATACGTTCCA

CAATGGGCCTTGTACACACCGCCCGTCACGTCAGCGAA
AGCCGCGTGAGGGACGACCCGAAGCCTTCGGGTGGCA
CAACCCCTTGTCAGCAGGGAAGAAGTCGAAAGTGACG
GTACCTGGCGATTGGGAAGAAGTCGTAACAAGGCTAA
CTACGTGCCAGCAGCCGCGGTAATACGTAGGGCGCAA
GCGTTGTCCGGAATTATTGGGCGTAAAGAGCTCGTAGG
CGGCTTGTCACGTCGGGTGTGAAAGCCCGGGGCTTAAC
CCCGGGTCTGCATCCGATACGGGCAGGCTAGAGTGTGG
TAGGGGAGATCGGAATTCCTGGTGTAGCGGTGAAATGC
GCAGATATCAGGAGGAACACCGGTGGCGAAGGCGGAT
CTCTGGGCCATTACTGACGCTGAGGAGCGAAAGCGTGG
GGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCG
TAAACGTTGGGAACTAGGTGTTGGCGACATTCCACGTC
G

C4:

1. C4-F.abi: Data obtained with Forward primer

> C4-F (754bp)

TGACGCTGAGGCGCGAAAGCGTGGGGAGCAAACAGGA
TTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTG
CTAAGTGTTAGGGGGTTTCCGCCCCTTAGTGCTGCAGC
TAACGCATTAAGCACTCCGCCTGGGGAGTACGGCCGCA
AGGCTGAAACTCAAAGGAATTGACGGGGACCCGCACA
AGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAA
GAACCTTACCAGGTCTTGACATCCTCTGACCACCCTGG
AGACAGGGCTTTCCCCTTCGGGGGACAGAGTGACAGG
KGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTT
GGGTTAAGTCCCGCAACGAGCGCAACCCTTGATCTTAG
TTGCCAGCATTCAGTTGGGCACTCTAAGGTGACTGCCG
GTGACAAACCGGAGGAAGGKGGGGATGACGTCAAATC
ATCATGCCCCTTATGACCTGGGCTACACACGTGCTACA
ATGGACGGTACAGAGGGCTGCAAACCCGCGAGGGGGA
GCCAATCCCACAAAACCGTTCCCAGTTCGGATTGCAGG
CTGCAACTCGCCTGCATGAAGCCGGAATCGCTAGTAAT
CGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGTC
TTGTACACACCGCCCGTCACACCACGAGAGTTTGCAAC
ACCCGAAGTCGGTGGGGTAACCCTTACGGGAGCCAGCC
GCCGAAGGTGGGGCAGATGATTGGGGTGAAGTCGTAA

Consensus Sequence Sample (754bp)

>Contig-0

TGACGCTGAGGCGCGAAAGCGTGGGGAGCAAACAGGA
TTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTG
CTAAGTGTTAGGGGGTTTCCGCCCCTTAGTGCTGCAGC
TAACGCATTAAGCACTCCGCCTGGGGAGTACGGCCGCA
AGGCTGAAACTCAAAGGAATTGACGGGGACCCGCACA
AGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAA
GAACCTTACCAGGTCTTGACATCCTCTGACCACCCTGG
AGACAGGGCTTTCCCCTTCGGGGGACAGAGTGACAGG
NGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTT
GGGTTAAGTCCCGCAACGAGCGCAACCCTTGATCTTAG
TTGCCAGCATTCAGTTGGGCACTCTAAGGTGACTGCCG
GTGACAAACCGGAGGAAGGNGGGGATGACGTCAAATC
ATCATGCCCCTTATGACCTGGGCTACACACGTGCTACA
ATGGACGGTACAGAGGGCTGCAAACCCGCGAGGGGGA
GCCAATCCCACAAAACCGTTCCCAGTTCGGATTGCAGG
CTGCAACTCGCCTGCATGAAGCCGGAATCGCTAGTAAT
CGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGTC
TTGTACACACCGCCCGTCACACCACGAGAGTTTGCAAC
ACCCGAAGTCGGTGGGGTAACCCTTACGGGAGCCAGCC
GCCGAAGGTGGGGCAGATGATTGGGGTGAAGTCGTAA

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Growth Studies of cultures

Growth studies of isolated bacterial cultures were performed to check the growth rate of each bacterial culture. A loop full of microbial culture capable of degrading carbofuran was inoculated in 150ml nutrient broth containing 10mg/l carbofuran in side-arm conical flask. It was then incubated in shaking conditions at 120 rpm for 24 hours at 30 ± 2°C. Optical density was measured at 540nm at the interval of 30 minutes. Absorbance was checked till constant optical density was reached. Optical density versus time graph was plotted.

Carbofuran degradation curve:

Isolated bacterial colonies were inoculated in conical flasks containing 100ml M2 broth having 10mg/L of carbofuran. It was then incubated in shaking conditions at 120rpm for 8 days at 30 ± 2°C. An aliquot (2ml) of each culture was aseptically transferred to a cuvette and its optical density at 660nm was determined after regular intervals of incubation. The loss of carbofuran from the media was monitored by the spectrophotometric method [16]. The bacterial isolate most efficient in degrading the pesticide was selected.

Degradation rate (%) = $(C_2 – C_1) / C_2 \times 100$

Where C_1 is the quantity of carbofuran in the treated sample and C_2 is the quantity of carbofuran in the control.

Developing microbial consortium

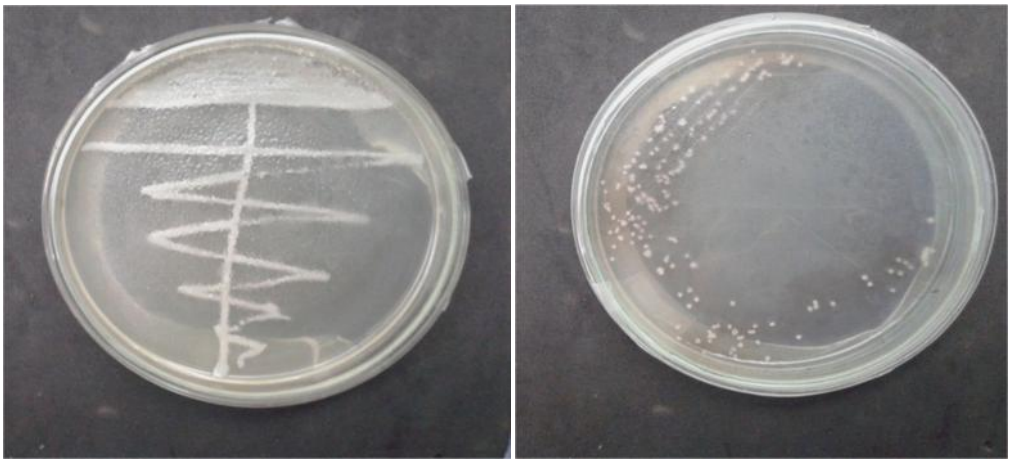
Isolated bacterial colonies were inoculated in combination of C1-C4, C2-C4 and C3-C4 in conical flasks containing 100ml M2 broth having 10mg/L of carbofuran. It was then incubated in shaking conditions at 120rpm for 8 days at 30 ± 2°C. An aliquot (2ml) of each culture was aseptically transferred to a cuvette and its optical density at 660nm was determined after regular intervals of incubation. This was done to check the bacterial growth in the media. Preparation of standard curve and Carbofuran concentration were estimated by spectrophotometric method[16]. The degradation rate was calculated.

RESULTS & DISCUSSION

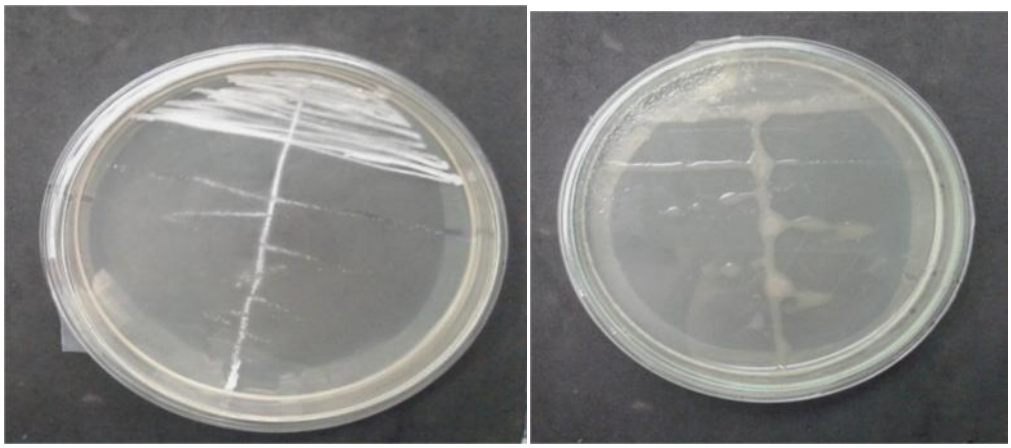
Isolation of pesticide degrading bacterial strains

On treating the soil with carbofuran (100 mg/kg), the soil bacterial count decreased. The colony count fell from 9.24 x 10^5 in control soil to 6.25 x 10^5 in carbofuran treated soil. The decrease in population of fungal colonies on application of pesticides has been reported in the past. There was significant decrease in fungal colonies on application of carbofuran and pendimethalin [17].

Four colonies were isolated which were capable of degrading pesticide Carbofuran. Carbofuran degrading isolates were named as C1, C2, C3 and C4. These isolates were able to grow in NA medium with carbofuran. These isolates were capable of using carbofuran as a carbon and nitrogen as they were grown on M2 medium (with Agar). The isolates C1, C2, C3 and C4 were identified to be *Arthrobacter globiformis, Streptomyces sp. Bacillus beijingensis, Bhargavaea indica* respectively.



(*Arthrobacter globiformis*) (Streptomyces sp.)



(*Bacillus beijingensis*) (*Bhargavaea indica*)

FIGURE 2: Bacterial pure cultures capable of degrading carbofuran

The organisms were characterized using Gram staining test, Ureas test, Indoletest , Methyl red test, citrate test and catalase test. The characteristics of the organisms are as below.

TABLE 1: Gram characterization of isolates

Strain	Gram-nature	Shape	Size	Arrangement
C1	Negative	Spherical	Pin- point	Cluster
C2	Positive	Rod	Small	Chain
C3	Negative	Rod	Small	Single
C4	Positive	Rod	Small	Chain

TABLE 2: Biochemical characteristics of isolates

Strain	Catalase	MR/VP	Urea	Citrate	Indole	Motility
C1	+	-/+	+	+	-	-
C2	+	-/+	+	-	+	-
C3	+	-/+	+	+	-	+
C4	+	-/+	+	+	+	+

Growth Curve Analysis

Growth curve analysis of 4 strains was performed as they were morphologically different. A loop full of 24 hour old culture was inoculated in 50ml nutrient broth. Growth curve analysis was performed in nutrient broth medium and nutrient broth medium containing 10 mg/L of pesticide.

A delay in the onset of logarithmic phase was observed when the medium was nutrient broth with the pesticide in case of bacterial strains *Arthrobacter globiformis, Bacillus beijingensis* and *Bhargavaea indica*. Fig. 4 show growth curves of isolated bacteria capable of degrading carbofuran in NB medium.

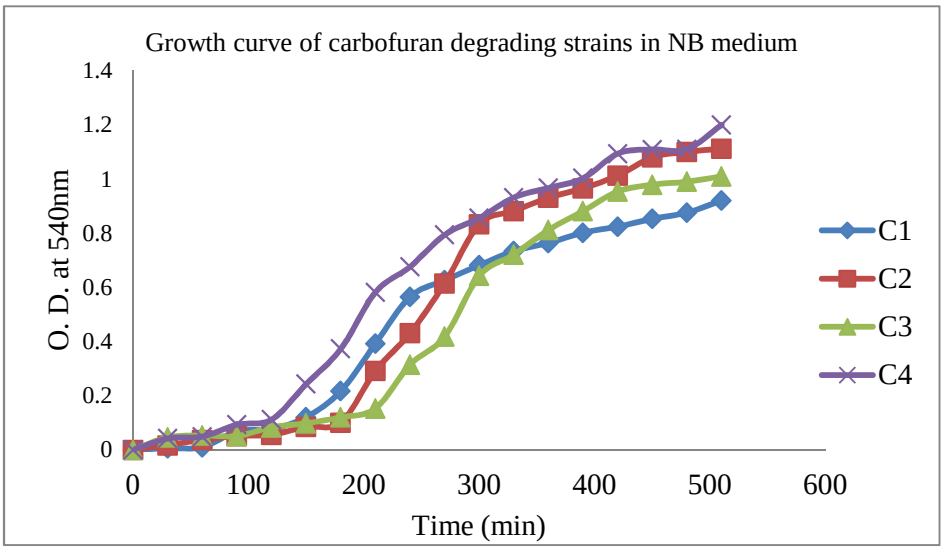


FIGURE 3: Growth curve analysis of Carbofuran degrading strains in nutrient Broth medium

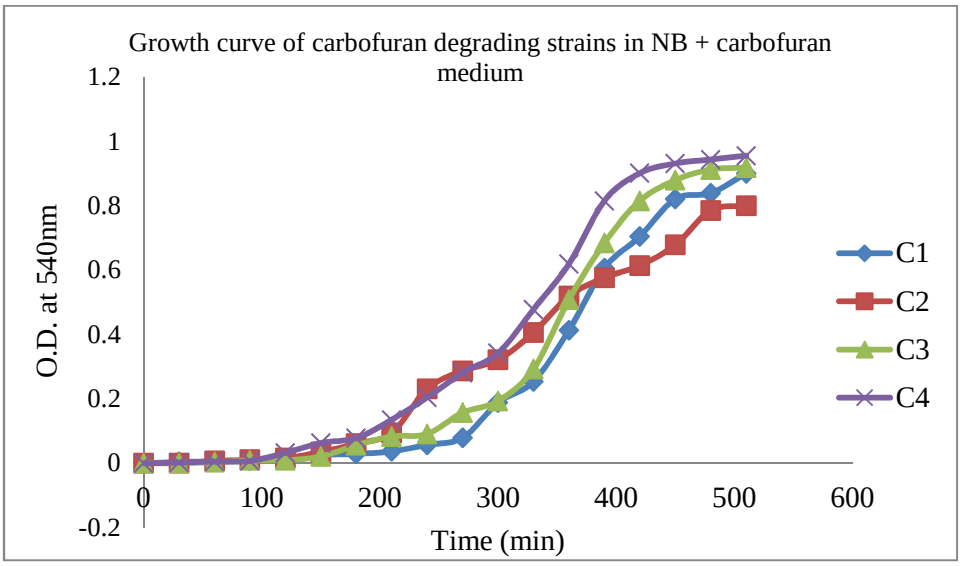


FIGURE 4. Growth curve analysis of carbofuran degrading strains in nutrient broth medium containing 10 mg/l carbofuran

Carbofuran degradation by the isolates

Four microorganisms were isolated from the M2 agar medium with Carbofuran (source of nitrogen and carbon). Isolated microorganisms had slight difference in the rate of growth in M2 broth medium. The degradation rate of the isolates is shown below:

TABLE 3: Degradation rate of Carbofuran using isolated bacterial strains

Isolates	Degradation rate
Arthrobacter globiformis	43%
Streptomyces sp	44%
Bacillus beijingensis	35%
Bhargavaea indica	55%

Based on the results obtained *Bhargavaea indica* was found to be the most efficient microorganism in degrading Carbofuran. *Bhargavaea indica* has not been used for the degradation of the pesticide in earlier studies. Hence the further research is needed to carry out more detailed study using this organism. *Arthrobacter globiformis* have been used for in the past for herbicide degradation**.** A number of *Arthrobacter globiformis* group isolates have been reported to utilize organic chemicals [18]. A study carried out by Fuentes *et al*. (2013) uses *Streptomyces* species for degradation of pentachlorophenol and chlorpyrifos. The efficiency of the organisms was checked in free cell form and immobilized form. It was found that the efficiency of pesticide removal was more in immobilized form. Pure culture of *Streptomyces* sp. A5 exhibited highest pentachlorophenol removal (10.6%), while *Streptomyces* sp. M7 had the best chlorpyrifos removal results [19].

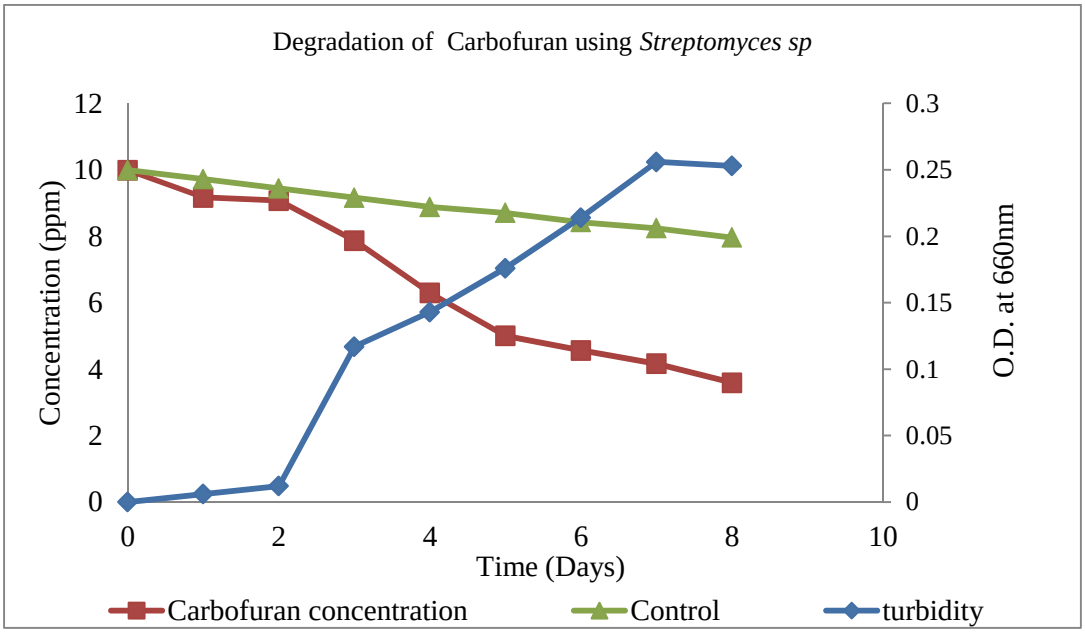


FIGURE 5: Degradation of Carbofuran using Streptomyces sp.

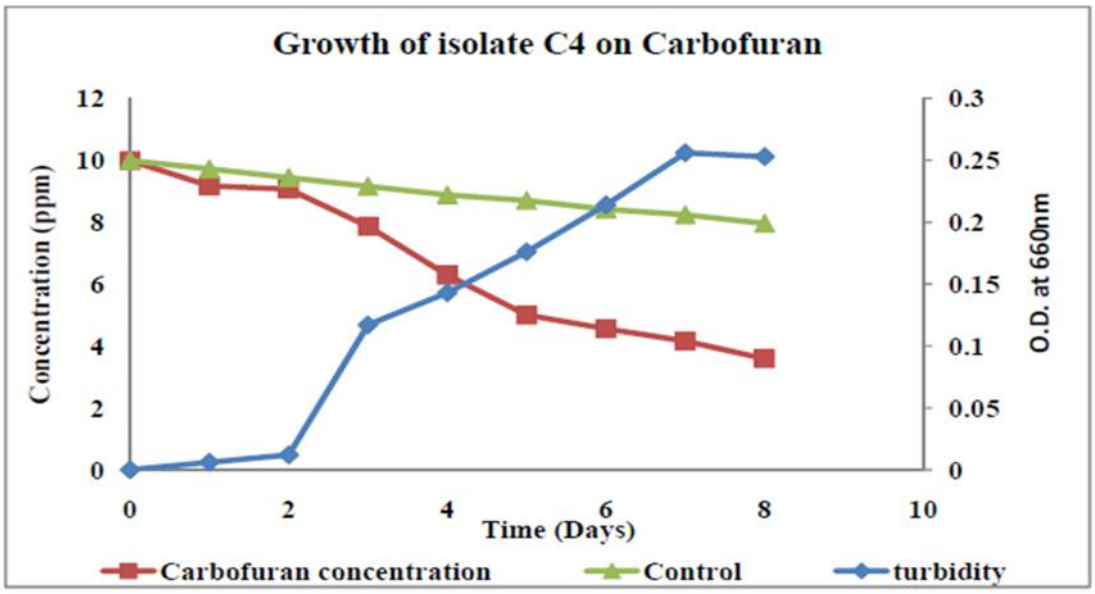


FIGURE 6: Degradation of Carbofuran using *Bhargavae indica*

CONCLUSION

Carbofuran has an adverse effect on soil bacterial population as indicated by a lower count of soil bacteria upon treatment of the soil with carbofuran. Four efficient bacterial strains capable of degrading carbofuran were identified as *Arthrobacter globiformis, Streptomyces sp. Bacillus beijingensis, Bhargavaea indica.* Most efficient microbial strain capable of degrading carbofuran was *Bhargavaea indica* .When the bacterial strains of *Streptomyces sp and Bhargavaea indica* were used in combination there was a significant increase in the degradation of carbofuran. The bacterial combination was able to reduce 10 mg/L of carbofuran by 60 % in 8 days. It implies that carbofuran removal is 0.6 mg/L per day. Such concentrations are found in river water samples and a little higher amount (1 mg/L) in the soil. Hence, these bacterial species can be used as a bioremediation tool for the removal of the pesticide from farmland runoffs. Further, they can be combined with a phytoremediation system for further depolluting rural waters.

ACKNOWLEDGEMENT: KRC No.: CSIR-NEERI/ KRC/ 2016/OCT/MZL/2

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