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Article

Isolation of Carbosulfan Degrading *Lysinibacillus fusiformis* from Paddy (*Oryza sativa* L.) Soil

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ABSTRACT

A branded generic insecticide with a long history of usage in agriculture is carbosulfan. However, research indicated that carbosulfan may be very hazardous to aquatic life. Using the enrichment culture approach, a bacterial culture was isolated from soil samples that had received the insecticide carbosulfan 5 times at a concentration of 5 kg/ha. This bacterial culture was then examined to see how well the pesticide broke down in a mineral salts medium. Based on the 16S rRNA gene sequence, the isolate was recognized as a *Lysinibacillus fusiformis*. In terms of phylogenetic analysis and nucleotide homology, the bacteria were quite similar. In conditions with increased amounts of carbosulfan, the bacteria acquired the ability to thrive. The creation of multi-resistant microorganisms that may be used to return the altered environment to its previous condition may, therefore, benefit from these findings. To eliminate the carbosulfan residues from the medium, an equivalent quantity of dichloromethane was utilized. After the required intervals, the culture filtrate was redissolved in about 10 mL of acetonitrile for the high performance liquid chromatography analysis in the present investigation (0, 1st, 2nd, 3rd, and 4th day).

Key words: Carbosulfan, Paddy soil, *Lysinibacillus fusiformis*, High-performance liquid chromatography.

1. INTRODUCTION

The presence of pesticide residues in food has become a significant problem, and much study has been done on the possible harm; these chemicals may cause to human health. Pesticides have long-lasting detrimental consequences, ranging from short-term illnesses like cancer to acute symptoms such as itching, headaches, and nausea. Several entry points, including the skin, mouth, respiratory system, and eyes, are available for them to enter the body [1]. The body's ability to govern insulin secretion and glucose metabolism may be interfered with by several non-persistent insecticides, exacerbating metabolic problems, and obesity [2]. Pesticide residues in food continue to occur even if the appropriate national authorities have tightened their control of the use of pesticides [3]. In particular, pesticides based on organophosphates and carbamates that block acetylcholinesterase, which are often present in food, are employed to eradicate insects [4,5]. Despite the fact that they leave behind residues in food that are challenging to breakdown, these pesticides are nonetheless frequently employed in agriculture. Therefore, finding reliable techniques to degrade these residues are essential. This study made use of the systemic carbamate insecticide carbosulfan. Their structural equations are shown in Figure 1. They are commonly used in agricultural production, because they may ensure the constant growth of crops. A broad-spectrum carbamate insecticide having the capacity to kill by touch and ingestion, carbosulfan is also known as 2,3-dihydro2,2-dimethyl-7-benzofuranyl[(dibutylamino) thio]-methylcarbamate. Effectiveness and rapid degradation of carbosulfan are thought to contribute to its low toxicity [6]. According to IUPAC data, it is still poisonous to many aquatic creatures and may be genotoxic and mutagenic at low doses [7]. The carp fry 96-h LC50 is 0.6 mg/L [8]. The measurement of carbosulfan and its primary metabolites in rice, potato, and orange might also be done using high performance liquid chromatography (HPLC) combined with a quadruple mass spectrometer [9]. According to Wu *et al.* [10], several carbamate pesticides were detected in water using HPLC and photodiode array (PDA) detection. Residues of carbosulfan, another carbamate insecticide, may also be detected using HPLC-PDA. There is virtually little research on the residue analysis of carbosulfan in fruits, especially mango in India. The present study's objective was to use HPLC-PDA to examine the persistence behavior of carbosulfan in paddy after foliar treatments. However, there is a significant risk that residue from their use will linger in the raised crops. They are suitable for our investigation as a result.

2. MATERIALS AND METHODS

2.1. Bacterial Degradation of Chlorpyrifos

2.1.1. Using an enrichment culture system, bacteria that degrade carbosulfan was isolated from soil sample

The enrichment culture approach was used to identify the soil bacteria that can break down certain pesticides, including carbosulfan and chlorpyrifos.

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Bacteria capable of degrading the chlorpyrifos were extracted using enrichment culture [11]. To obtain a final concentration (50 g/g) of soil, commercial pesticide formulations were applied to 50 g portions of soil samples, temperature (28 ± 4°C) and maintained at 60% WHC. Following five such administrations of chlorpyrifos spaced 10 days apart, triplicate soil samples were collected to isolate soil bacteria using the serial dilution agar plate method. Axenic cultures were, then, used to grow the bacterium.

2.1.2. Carbosulfan degradation by bacterial culture

It was determined that certain isolated bacterial strains have the ability to break down chlorpyrifos. Aliquots from stock solutions of the professional grade chosen pesticides were added to 250 mL sterile Erlenmeyer flasks to have a final concentration of 50 g/g soil, taking into account their hazardous levels in the bacteria. Fifty mL portions of the steam-sterilized mineral salts medium with the following composition were added to each flask under aseptic conditions, after the specified carrier solvent had completely dried. The mineral salt medium composition is as follows: NH_4NO_3 1.5 g, K_2HPO_4 1.5 g, KH_2PO_4 0.5 g, $MgSO_4$ 0.2 g, NaCI 0.5 g, H_2O 1 lit, and pH 7.0. After a day of equilibration to create aqueous solutions of the pesticide residues, inoculum densities of 1.0 OD cells assessed at 600 nm using a U.V. Visible Spectrophotometer (Thermo Scientific) Evolution 201 were incubated at 37°C under continuous shake culture conditions (150 rev/min). Triplicate samples were taken after 0, 1, 2, 3, and 4 days of incubation for solvent extraction and HPLC analysis to identify the parent compound and pesticide residue.

2.1.3. Microbial identification using 16S rRNA based molecular method

Bacterial quality was evaluated on 1.0% agarose gel, a single band of high-molecular weight DNA has been observed. Fragment of 16S rRNA gene was amplified by 16SrRNA-F and 16SrRNA-R primers. A single discrete PCR amplicon band of 1500 bp was observed when resolved on agarose gel. The PCR amplicon was purified to remove contaminants. Forward and reverse DNA sequencing reaction of PCR amplicon was carried out with 16S rRNA-F and 16S rRNA-R primers using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer. Consensus sequence of 16S rRNA gene was generated from forward and reverse sequence data using aligner software. The 16S rRNA gene sequence was used to carry out BLAST with the "nr" database of NCBI GenBank database. Based on maximum identity score, first ten sequences were selected and aligned using multiple alignment software program Clustal W. Distance matrix and phylogenetic tree was constructed using MEGA 10.

2.1.4. HPLC-based chlorpyrifos extraction

With an equivalent volume of dichloromethane, the chlorpyrifos residues from the medium were removed. The culture filtrate was redissolved in about 10 mL of acetonitrile for the HPLC analysis at the necessary intervals (0, 1st, 2nd, 3rd, and 4th day). In the HPLC, chlorpyrifos was found at 254 nm, respectively. HPLC settings utilized for the current study are represented in Table 1.

3. STATISTICAL ANALYSIS

All data were expressed on an air-dry soil basis and were averages of three replicates. The data were analyzed for significant differences ($P \leq 0.05$) between pesticide treated and untreated soil samples using Duncan's multiple range test [12,13].

4. RESULTS AND DISCUSSION

4.1. Biodegradation of Carbosulfan

Carbosulfan is carbamate insecticide that acts by inhibiting the activity of acetylcholinesterase developed by Dhanuka Agritech Pvt. Ltd., and

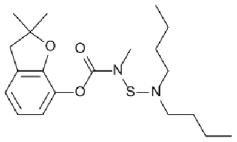

Figure 1: Carbosulfan chemical structure.

Table 1: HPLC conditions that were used in the present study

S. No.	HPLC	Carbosulfan
1	Company	Shimadzu
2	Column	C 18, Qualisil gold
3	Model	SCL-10 AVP
4	Pump	Binary pump
5	Detector	PDA
6	Wavelength	272 nm
7	Retention	6.379 mt
8	Florate	1.0 mL/mt

HPLC: High-performance liquid chromatography, PDA: Photodiode array

can delivers control of soil dwelling and foliar pests. The bacterial culture was isolated by enrichment culture technique, after treating the soil samples 5 times at 5 kg/ha level with carbosulfan, and the culture was tested for its ability to degrade the carbosulfan in mineral salts medium. Based on some of the physiological characteristics and 16S rRNA gene sequence analysis, the isolate was identified as sample *Lysinibacillus fusiformis.* The 16S rRNA gene sequence (Sanger Seq Chromatogram data file Data), gDNA 16SrRNA amplicon Figure 2, phylogenetic tree Figure 3, sequences producing significant alignments Table 2, and estimates of evolutionary divergence between sequences of the bacteria are represented in Table 3. The recovery, with complex extraction and analytical methods employed, of carbosulfan, immediately after application to the culture medium was 99.9%. There was an appreciable decrease in the levels of carbosulfan recovery during the incubation period [Figure 4]. Thus, by the end of 4th day, about 87% of added carbosulfan was lost from the inoculated medium, this observation reveals that biotic degradation of carbosulfan occurred. The HPLC standard curve of carbosulfan is shown in Figure 5. Nearly 18.72%, 35.37%, 48.20%, and 67.01% of carbosulfan had been broken down and added to the mineral salts medium by *L. fusiformis* at the end of the 1st, 2nd, 3rd, and 4th days, respectively [Figure 5]. In the inoculation medium, the recovery percentages of carbosulfan at the first, second, third, and fourth passes were roughly 81.27%, 64.62%, 51.79%, and 32.98%, respectively [Figures 6-10]. The carbosulfan degradation was reduced by JBM 3 by 33.33%, whereas KBM 1 was able to diminish carbosulfan recovery by up to 40.47% [14].

4.2. Sanger Seq Chromatogram Data File Data

4.2.1. Consensus data

GCTCAGGACGAACGCCGGCGGCGTGCCTAATACATGCAAGT
CGAGCGAACAGAGAAGGAGCTTGCTCCTTCGACGTTAGCG
GCGGACGGGTGAGTAACACGTGGGCAACCTACCTTATAGTT
TGGGATAACTCCGGGAAACCGGGGCTAATACCGAATAAT
CTGTCACCTCATGGTGAAACACTGAAAGACGGTTTCGG
CTGTCGCTATAGGATGGGCCCGCGGCGCATTAGCTAGT
TGGTGAGGTAACGCTCACCAAGGCGACGATGCGTAGC
CGACCTGAGAGGGTGATCGGCCACACTGGGACTGAG
ACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAA
TCTTCCACAATGGGCGAAAGCCTGATGGAGCAACGCC

Table 2: Sequences producing significant alignments

Description	Max score	Total score	Query cover	E value	Per. Ident	Accession
Lysinibacillus fusiformis strain DSM 2898	2719	2719	100%	0	99.80%	NR_042072.1
Lysinibacillus fusiformis strain NBRC 15717	2715	2715	99%	0	99.86%	NR_112628.1
Lysinibacillus fusiformis strain NBRC15717	2712	2712	99%	0	99.86%	NR_112569.1
Lysinibacillus sphaericus strain DSM 28	2671	2671	100%	0	99.19%	NR_042073.1
Lysinibacillus sphaericus strain NBRC 15095	2660	2660	99%	0	99.19%	NR_112627.1
Lysinibacillus sphaericus strain IAM 13420	2614	2614	99%	0	98.65%	NR_115529.1
Lysinibacillus macroides strain LMG 18474	2612	2612	99%	0	98.58%	NR_114920.1
Lysinibacillus pakistanensis strain NCCP-54	2593	2593	99%	0	98.44%	NR_113166.1
Lysinibacillus parviboronicapiens strain NBRC 103144	2591	2591	99%	0	98.31%	NR_114213.1
Lysinibacillus boronitolerans strain NBRC 103108	2588	2588	99%	0	98.31%	NR_114207.1

Table 3: Estimates of evolutionary divergence between sequences

2		0.001	0.001	0.001	0.003	0.003	0.004	0.004	0.004	0.005	0.004
NR_042072.1	0.001		0.000	0.000	0.003	0.003	0.004	0.004	0.004	0.004	0.004
NR_112628.1	0.001	0.000		0.000	0.003	0.003	0.004	0.004	0.004	0.004	0.004
NR_112569.1	0.001	0.000	0.000		0.003	0.003	0.004	0.004	0.004	0.004	0.004
NR_042073.1	0.010	0.010	0.010	0.010		0.001	0.002	0.004	0.005	0.005	0.005
NR_112627.1	0.010	0.010	0.010	0.010	0.001		0.002	0.005	0.005	0.005	0.005
NR_115529.1	0.014	0.014	0.014	0.014	0.006	0.005		0.005	0.006	0.006	0.006
NR_114920.1	0.014	0.012	0.012	0.012	0.016	0.017	0.021		0.003	0.006	0.002
NR_113166.1	0.014	0.013	0.013	0.013	0.018	0.018	0.024	0.008		0.007	0.003
NR_114213.1	0.017	0.016	0.016	0.016	0.018	0.019	0.024	0.022	0.027		0.006
NR_114207.1	0.016	0.015	0.015	0.015	0.017	0.018	0.023	0.007	0.011	0.025	

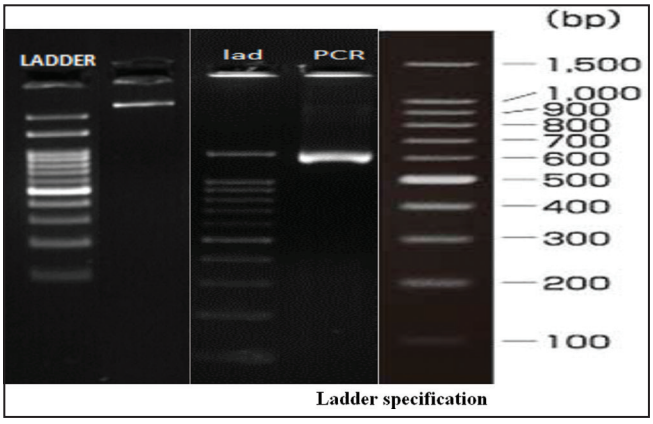

Figure 2: gDNA and 16s rRNAamplicon QC data.

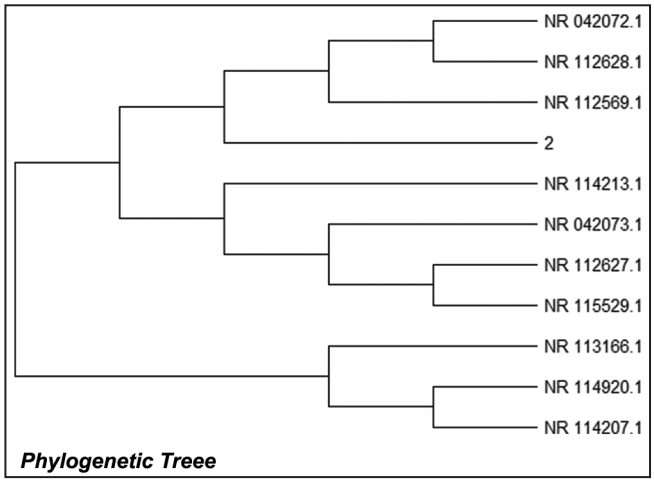

Figure 3: Evolutionary analysis by maximum likelihood method.

GCGTGAGTGAAGAAGGATTTCGGTTCGTAAAACTCTGTT
GTAAGGGAAGAACAAGTACAGTAGTAACTGGCTGT
ACCTTGACGGTACCTTATTAGAAAGCCACGGCTAA
CTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAG
CGTTGTCCGGAATTATTGGGCGTAAAGCGCGCG
CAGGTGGTTTCTTAAGTCTGATGTGAAAGCCCA
CGGCTCAACCGTGGAGGGTCATTGGAAA
CTGGGAGACTTGAGTGCAGAAGAGGATAGTGGAA
TTCCAAGTGTAGCGGTGAAATGCGTAGAG ATTTGGAGGAAC
ACCAGTGGCGAAGGCGACTATCTGGTCTGTAACTGACACT
GAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATA

CCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAA
GTGTTAGGGGGTTTCCGCCCCTTAGTGCTGCAGCTAACGCAT
TAAGCACTCCGCCTGGGGAGTACGGTCGCAAGA
CTGAAACTCAAAGGAATTGACGGGGGCCCGCAC
AAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACG
CGAAGAACCTTACCAGGTCTTGACATCCCGTTGACCACT
GTAGAGATATGGTTTCCCCTTCGGGGGCAACGGTGACAG
GTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTG
GGTTAAGTCCCGCAACGAGCGCAACCCTTGATCTTAGTTGC

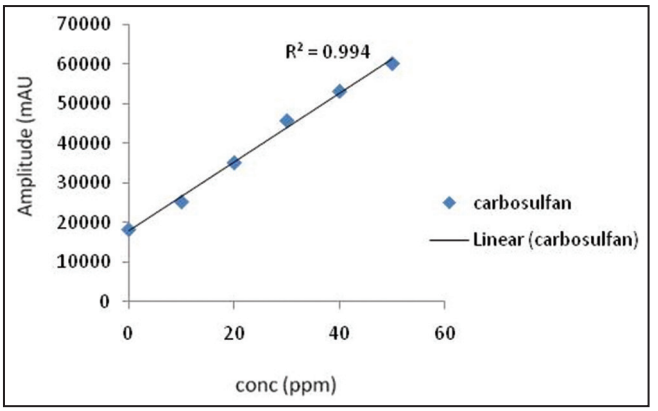

Figure 4: Standard curve of carbosulfan. Retention time = 6.816 min, X = Concentration of carbosulfan, Y = Milli absorbance units (mAU).

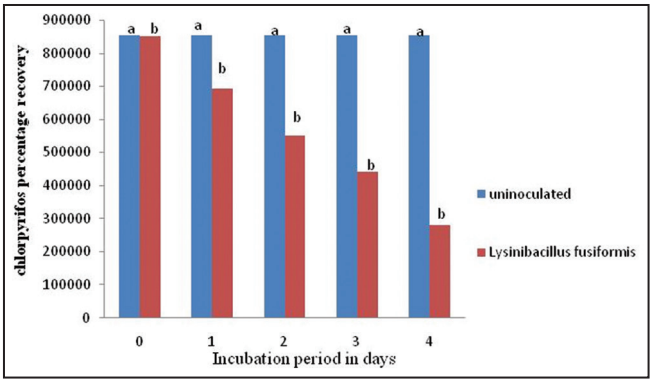

Figure 5: Degradation of carbosulfan by *Lysinibacillus fusiformis*, *L. fusiformis* is isolated by enrichment culture technique from treated with insecticide and carbosulfan and is identified by 16S rRNA gene sequence analysis. Insecticide and carbosulfan (50 ppm) added to 50 mL of mineral salts medium. Means, in each column, followed by the same letter or not significantly different ($P \leq 0.05$) from each other according to Duncan's multiple range test.

CATCATTTAGTTGGGCACTCTAAGGTGACTGCCGGTGACA
AACCGGAGG AAGGTGGGGATGACGTCAAATCATCATGCCCC
TTATGACCTGGGCTACACACGTGCTACAATGGACGATACA
AACGGTTGCCAACTCGCGGAGGGAGCTAATCCGATAAAGT
CGTTCTCAGTTCGGATTGTAGGCTGCAACTCGCCTAC
ATGAAGCCGGAATCGCTAGTAATCGCGGATCAGCAT
GCCGCGGTGAATACGTTCCCGGGCCTTGTACA
CACCGCCCGTCACACCACGAGAGTTTGTAACACCCG
AAGTCGGTGAGGTAACCTTTTGGAGCCAGCCGCCG
AAGGTGGGATAGATGATTGGGGTGAA

The evolutionary history was inferred using the Maximum Likelihood method and Tamura-Neimodel [15]. The tree with the highest log likelihood (−2560.00) is shown. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and Bio NJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 11 nucleotide sequences. Codon positions included were $1^{st}+2^{nd}+3^{rd}$+non-coding. There were a total of 148 positions in the final dataset. Evolutionary analyses were conducted in MEGAX [16].

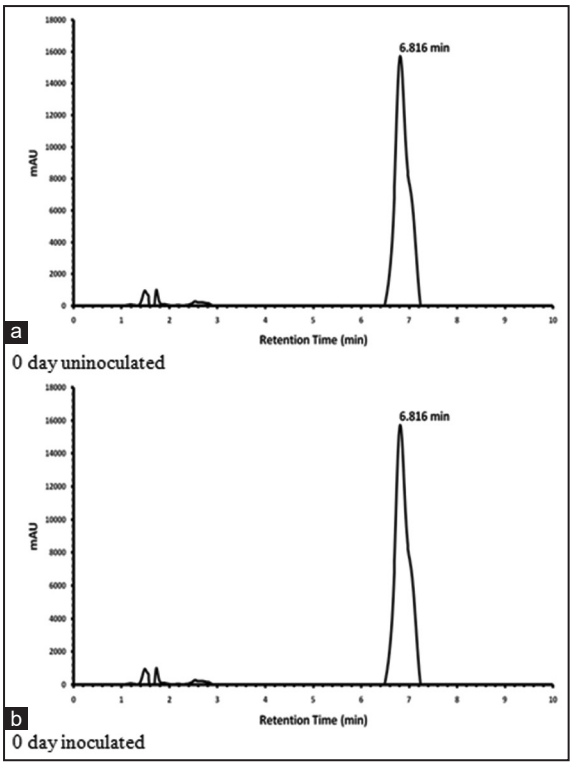

Figure 6: (a and b) High-performance liquid chromatography chromatograms of carbosulfan residues, obtained by solvent extraction from culture after 0 day incubation. X = Retention time, 6.816 min, Y = Milli absorbance units (mAU). Values plotted in the figure are means of triplicates.

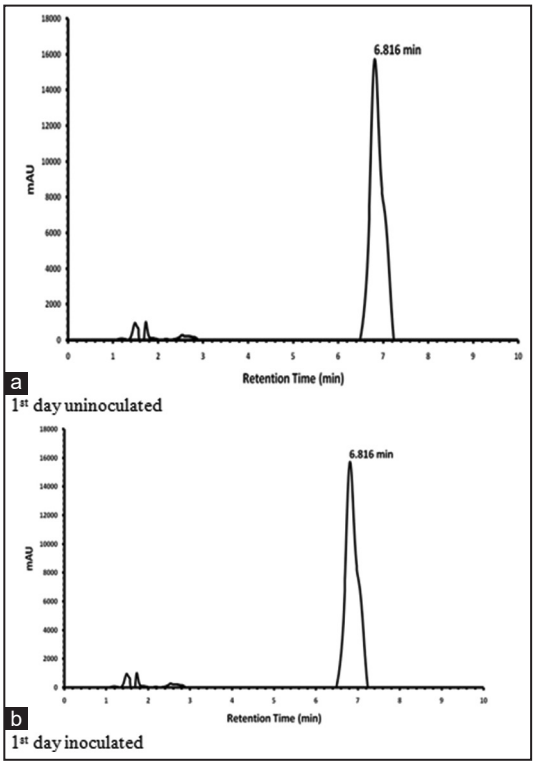

Figure 7: (a and b) High-performance liquid chromatography chromatograms of carbosulfan residues, obtained by solvent extraction from culture after 1 day incubation. X = Retention time (RT), 6.816 min, Y = Milli absorbance units (mAU). Values plotted in the figure are means of triplicates.

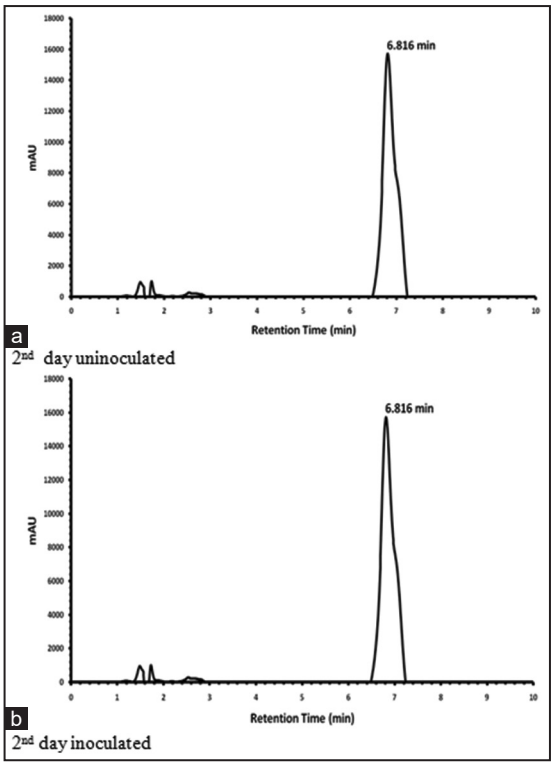

Figure 8: (a and b) High-performance liquid chromatography chromatograms of carbosulfan residues, obtained by solvent extraction from culture after 2 days incubation. X = Retention time (RT), 6.816 min, Y = Milli absorbance units (mAU). Values plotted in the figure are means of triplicates.

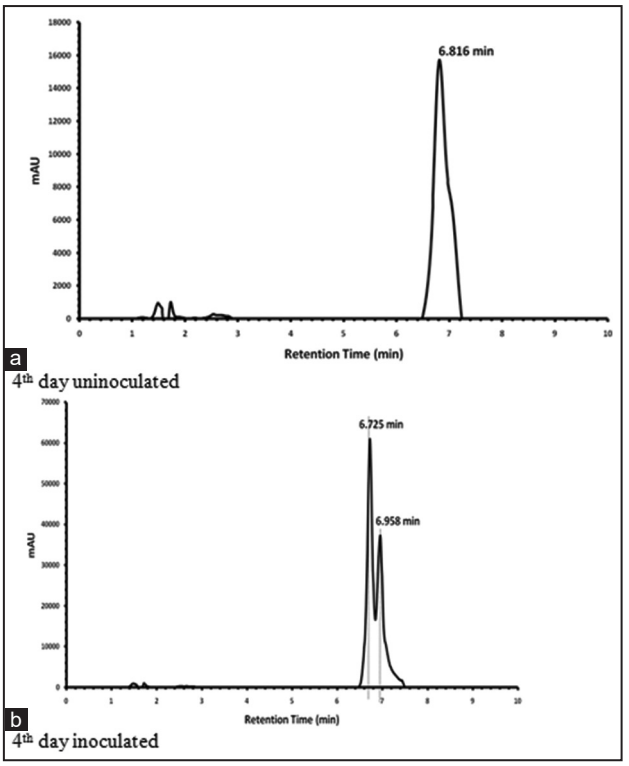

Figure 10: (a and b) High-performance liquid chromatography chromatograms of carbosulfan residues, obtained by solvent extraction from culture after 4 days incubation. X = Retention time (RT), 6.725 and 6.958 min, Y = Milli absorbance units (mAU). Values plotted in the figure are means of triplicates.

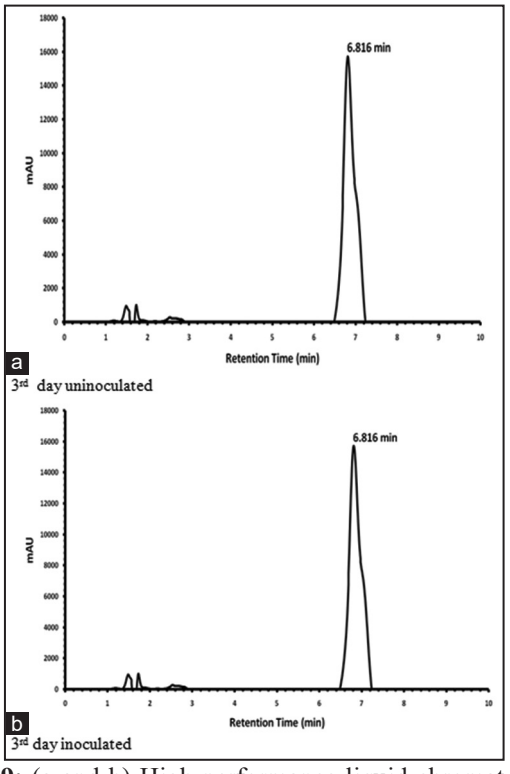

Figure 9: (a and b) High-performance liquid chromatography chromatograms of carbosulfan residues, obtained by solvent extraction from culture after 3 days incubation. X = Retention time (RT), 6.816 min, Y = Milli absorbance units (mAU). Values plotted in the figure are means of triplicates.

4.2.2. Distance matrix

The number of base substitutions per site from between sequences is shown. Standard error estimate(s) are shown above the diagonal and were obtained by a bootstrap procedure (500 replicates). Analyses were conducted using the Maximum Composite Likelihood model [15]. This analysis involved 11 nucleotide sequences. Codon positions included were 1^{st} + 2^{nd} + 3^{rd}+ non-coding. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 1483 positions in the final dataset. Evolutionary analyses were conducted in MEGA X [16].

5. CONCLUSION

The fast rise of pesticides that has resulted is associated with microbial community alterations, decreased soil fertility, and ecological disruption. To counter the negative impacts of pesticides on ecosystems, natural and environmentally friendly methods are becoming more and more popular. Based on the examination of the 16S rRNA gene, the isolate was classified as the strain *L. fusiformis*, which shown strong closeness in nucleotide homology and phylogenetic analysis. After 4 days of incubation, carbosulfan was more effectively degraded by a single pesticide degrading bacterial culture, which was capable of eliminating up to 50 ppm of carbosulfan. This isolate is a top contender for application in the bioremediation of carbosulfan due to its enhanced breakdown and ability to survive at high concentrations of the chemical.

6. CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest in relation to the publication of this article.

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