*https://doi.org/10.33472/AFJBS.6.4.2024.820-833*

African Journal of Biological Sciences

# Exploring Microbial Isolates From Organophosphate-Contaminated Soil For Chlorpyrifos Biodegradation Potential

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Article History
Volume 6, Issue 4, 2024
Received: 05-04-2024
Accepted: 02-05-2024
Doi: 10.33472/AFJBS.6.4.2024.820-833

**Abstract**

Chlorpyrifos, an organophosphate pesticide to control pest populations for a very long time. because its toxicity to living things and persistence, chlorpyrifos removal from polluted locations has become a critical problem. Hence, the bioremediation process is the ideal option for removing chlorpyrifos from farming soil samples. In this regard, in this present study is aimed to isolate the chlorpyrifos degradation potential of soil microbes from pesticide contaminated agricultural fields. The chlorpyrifos is identified using Gas Chromatography and Mass Spectroscopy analysis and microbial stains such as P aeruginosa and B. huaxiensis and A. niger and F. incarnatum species were identified using 16 S rRNA gene sequencing technique. Samples following a 14-day duration of incubation, the HPLC examination of the remaining chlorpyrifos showed P. aeruginosa, B. huaxiensis, A. niger, and F. incarnatum all had a higher percentage of chlorpyrifos that had been degraded than 47%, 71%, 65.3%, and 40.4%, respectively. Among these, the B. huaxiensis bacteria showed highest degradation against Chlorpyrifos and decrease in the level of pesticides in the mineral salt medium. These results demonstrate the potential for these microbes to be employed in the removal of pesticide waste that has gotten into the environment and also show that the isolate B. huaxiensis is more effective at degrading chlorpyrifos in liquid culture and can also be used in bioremediation of soils that have gotten into the environment contaminated with chlorpyrifos.

**Keywords:** Organophosphate pesticide, Chlorpyrifos, Agricultural soil, Microbes

## Introduction

In order to accommodate the growing global population, it is predicted that global food demand would increase from 80% to 100% over the next few decades (Grafton *et al.*, 2015; Rhodes, 2017). Farmers use a huge number of agrochemicals (over 2 million per year) to meet this need, including organophosphate insecticides (Ali *et al.,* 2021). They are being used to boost productivity by preventing 40% of the crop losses brought on by pest infestation (Sharma *et al.*, 2019; Ali *et al.*, 2021). However, only a small portion (roughly 1.0%) of pesticides are effective at killing the intended pests, and the remaining pesticide residues are left in agricultural soil, where they cause serious harm to both terrestrial and aquatic

ecosystems through leaching and hydrolysis (Sharma *et al*., 2019; Ali *et al*., 2021). One of the most widely used broad spectrum synthetic organophosphate insecticides is chlorpyrifos (Jaiswal *et al*., 2017; Priyadarshini and Dangar, 2017). Food crops, cash crops, oil crops, garden plants, and landscaping plants often use chlorpyrifos to control several kinds of pests.(Rathod and Garg, 2017; Santoiemma *et al*., 2021 It is a non-systemic pesticide penetrating the intestinal tract, skin and pulmonary membranes after coming into contact with or consuming an insect.(Rathod and Garg, 2017; Santoiemma *et al*., 2021).

Chlorpyrifos' insecticidal effects are brought on by the suppression of the enzyme acetylcholinesterase (AChE) (Rathod and Garg, 2017; Santoiemma *et al*., 2021), which causes the nervous system to be overstimulated, resulting in paralysis, convulsions, and ultimately death. Many strategies for the detoxification of chlorpyrifos have been developed recently, including titanium dioxide photocatalysis, biochar adsorption, ultrasonic treatment, and synthetic nanocomposites. (Soltani-Nezhad *et al*., 2020).These procedures, however expensive and technically difficult, are also likely to result in secondary pollutants with increased toxicity (Huang *et al*., 2019; Bhatt *et al*., 2020). According to Birolli *et al*. (2018), using indigenous microbes to remove chlorpyrifos from environmental substrates has been increasing in popularity as a study topic because of its high efficiency, cost effective, and sustainability. Restoration is the process of using microbes to break complex organic substances into small inorganic molecules that are less hazardous or destructive. Alizadeh *et al*. (2017) claim that microbial biodegradation is a very appealing method for eliminating hazardous substances from the environment. Bioremediation is a low-cost, environmentally benign, and easy method for removing the chemical from the environment. (Rayu *et al*., 2017). According to a recent study, Azotobacter sp. decomposed chlorpyrifos at high concentrations without having any negative effects on PGPR (Conde-Avila *et al*. 2021). Additionally, various chlorpyrifos degradations based on bacterial consortia were also described (Elshikh et al. 2022). Feng et *al*. (2017) isolated five endophytic bacterial species from a chlorpyrifos-contaminated agricultural area and created a consortium that included Pseudomonas sp., Bacillus sp., Sphingobacterium sp., Stenotrophomonas sp., and Curtobacterium sp. These bacteria decomposed chlorpyrifos (90%). The ecological remediation of chemical organophosphates such as chlorpyrifos utilizing the microbes lacks much attention yet, but it needs more. Consequently, in this investigation, organophosphate pesticide-degrading bacteria and fungi were isolated from agricultural areas and characterised for their ability to break down the pesticide chlorpyrifos.

## Materials and Methods

### Collection of soil sample

The agricultural region of Oonamancherri, Kancheepuram District, Tamil Nadu (12.8603° N, 80.1045° E), served as the source for the soil samples. A soil auger was used to collect the soil at a depth of 10 cm, and it was then transferred in sterile, airtight plastic bags. The samples were air-dried and crushed prior passing through 2 mm sieves for further analysis.

### Identification of Chlorpyrifos from soil sample

For this study, Gas Chromatograph method (GC-7890A/MS-5975C, In order to identify the chlorpyrifos in a soil sample. High energy electron ionisation at 70 eV is employed for mass spectrometry determination. The 99.95% of pure helium gas and initial temperature (50 to 150 °C) and final temperature (300 °C) with increasing rate 3 °C per min to 10 °C per min are used, respectively. Using the appropriate solvent (ethanol), 1 mL of crude extract was diluted before being

injected. In the GC chromatogram, the different concentrations of the chemicals found in soil extracts were expressed as proportion peaks. The retention period was used to determine the chemicals from NIST databases. After the constituents were determined, the data were tallied and compared to those found in the computer database (NIST and Willey) associated to the GC-MS equipment.

### Isolation of Chlorpyrifos degrading microbes

Chlorpyrifos-degrading microorganisms were isolated from soil samples by using the enrichment culture technique on a medium consisting of mineral salt yeast extract medium with chlorpyrifos as the sole source of carbon. Chlorpyrifos was added at a concentration of 10 mg/L to a volume of 100 ml of the mineral salt yeast extract medium. This was autoclaved at 121°C for 15 minutes before 5g of the soil sample was added. The flasks had been placed in a rotary shaker for 7 days at 30 degrees Celsius and 120 revolutions per minute (rpm). Following two days of incubating at thirty degrees Celsius. 1 ml of the culture was inoculated onto Chlorpyrifos mineral salt yeast extract agar. The Chlorpyrifos mineral salt agar contained 0.5% methanol (a pesticide solvent carrier), 1.5% agar-agar, 1% sodium citrate, and 10 mg/L chlorpyrifos. The fungus was also isolated using Potato Dextrose Agar. Until pure cultures were obtained, the morphologically comparable bacterial and fungal growths on the agar were chosen and subcultured repeatedly. For additional research, the pure cultures were kept on potato dextrose agar slant and chlorpyrifos mineral salt, respectively, 4° Celsius.

### Molecular identification of bacterial and fungal isolate

A bacterial and fungal culture containing 100 to 150 ml was centrifuged at 16,000 rpm for one minute. The pellet was treated to DNA extraction using a DNA extraction kit to identify the bacterial and fungal culture after the supernatant was discarded. The primer sequences for amplifying the 16S rRNA ribosomal subunit were as universal primers 27 5'-GGTTACCTTGTTACGACTT-3' and 1492R 5'-GAGTTTGATCCTGGCTCAG-3''. The amplified products performed further sequencing. BioEdit 7.2.5.0 was used to construct the sequences, and ClustalX 2.1 was used to align them. The phylogenetic analysis tree was produced using the Maximum Likelihood technique in MEGA 6.06 applying the Kimura 2-parameter model. The phylogram was statistically analyzed by bootstrapping 1000 sub-replicates. The bacterial specie sequence and the 16S rRNA phylogenetic trees agreed with each other. The study's sequences were retrieved from NCBI (https://www.ncbi.nlm.nih.gov/nucleotide/) and carefully checked before being oriented by Clustal X's presets.

### Biodegradation of Chlorpyrifos in Liquid Culture

The bacterial and fungal isolates were grown with 50 ml of mineral salt yeast extract medium (MSM) for a period of 24 hours by shaker that rotated at 37 degrees Celsius and 120 rpm. As an inoculum, 1 ml of the 24-hour culture containing roughly 1.1 $\times$ 104 CFU/ml (as determined by the viable count method) was employed. This was used to inoculate triplicate 250 ml flasks with 100 ml MSM and 20 ml/L of chlorpyrifos. The control flask was left uninoculated. For 14 days, the flasks were incubated in a rotating shaker at 120 rpm and 30 degrees Celsius. Five millilitres of the culture were removed from each flask after 14 days of incubation and put in centrifuge tubes. Centrifuging this portion of the culture twice for 20 minutes at 150 rpm while using an equivalent volume of ethyl acetate as the extraction reagent. The filter paper of Whatman No. 1 served to remove residual chlorpyrifos present

in ethyl acetate. A tandem reverse-phase cartridge column (C-18) was used in HPLC to measure the amount of Chlorpyrifos that was still present in the culture. A Tanden reverse phase cartridge column was fitted using a temperature programmed for 170 °C for 30 seconds at an average flow rate of 0.8 ml/min at 25 °C. The detection limit for chlorpyrifos in aqueous phase was 20 g/L, and the retention period was 15 seconds. By taking 1 ml of the filtrate, transferring it into a 15 ml high performance liquid chromatography vial that contained 60% methanol and 40% water (3:2), and the amount of the insecticide chlorpyrifos was estimated after HPLC analysis of the outcomes. For the chlorpyrifos, calibration curves from 0 to 100mg/L were created. With the aid of a pre-standardized curve, the extract peaks visible in the chromatogram were recognised, and the concentration of pesticide residue was calculated.

## Results:

### Identification of Chlorpyrifos from soil sample

In the current investigation, GC-MS technique was performed to determine the presence of the chlorpyrifos in the soil sample. The research confirmed the presence of chlorpyrifos based on peak retention times. The metabolic profile of chlorpyrifos was validated by both mass spectra and by National Institute Standard and Technology's (NIST) library identification programme. Chlorpyrifos was identified in the soil sample dependent on its distinctive component ionization peaks and chemical particle (m/z), which displayed a m/z value of 197 with a retention time of 19.259 (Figure -1).

### Molecular identification of bacterial and fungal isolate

As a result, the locations' soils are suitable ecological niches for isolating microorganisms that can break down the pesticides. In this research, we identified and genetically analysed 2 varieties of bacteria as well as 2 fungus species. The specific type of microorganism is *P aeruginosa* and *B. huaxiensis* and the fugal species is *A. niger* and *F. incarnatum* species is sequenced by 16 S rRNA gene and submitted in the GenBank of NCBI and were assigned suitable accession numbers (OQ927055, OQ826467, OQ924355 and OQ919248, respectively). The phylogenetic tree constructed using 4 sequences of closely related to same species. The sequence is compared to homologous sequences and found to be similar with their respective sequence in GenBank and identification ranged from 91% to 100%. Hence, the present gene sequencing technique is established based on the 18S rRNA sequence data for precise species level identification of soil bacteria.

### Biodegradation of Chlorpyrifos in Liquid Culture

In the current investigation, HPLC was utilised to track the deterioration of chlorpyrifos. The HPLC technique was used to evaluate the rate of Chlorpyrifos breakdown in the purified bacterial and fungal isolates, which were then cultured in a medium enriched with mineral salts (Figures 6 to 9). In this present study, the isolates are able to degrade the Chlorpyrifos. The isolates were able to degrade the Chlorpyrifos and the percentage of degradation is 47% in *P. aeruginosa*, 71% in *B. huaxiensis*, 65.3% in *A. niger* and 40.4% in *F. incarnatum*. Based on our study, all the microbes are showed good Chlorpyrifos degradation properties. Among these, the *B. huaxiensis* bacteria showed highest degradation against Chlorpyrifos.

*Serratia marcescens* and *Pseudomonas aeruginosa* are among the bacteria that have been isolated, those have been demonstrated that these bacterial colonies catabolize and co-metabolize

chlorpyrifos. *Arthrobacter sp*. and *Flavobacterium sp*. were shown to co-metabolize the drug chlorpyrifos.

## DISCUSSION:

Since many years, pesticides, a crucial component of pest control, have protected agriculture from the destructive impacts of pest damage (Furlan & Kreutzweiser, 2015). Due to their toxicity and perseverance, they are essential for managing crop disease and pests, but they also harm living creatures, ecological systems, and agricultural ecosystems. (Maurya and Malik, 2016). Broad-spectrum insecticides known as organophosphates make up about 38% of all pesticides used in agriculture to combat various pests (John *et al*., 2018). Due to the frequent use of such chemical compounds, there are serious environmental problems such as airborne contaminants and waterborne residue in the soil (Özkara *et al*., 2016). Chlorpyrifos serves as a typical wide-range synthetic organophosphate insect-control agent used to eradicate phytopathogens in numerous agricultural systems. (Kumar *et al*., 2021) Due to the fact that current methods of removing chlorpyrifos residues are impractical, expensive, or environmentally hazardous, the microbial detoxification of chlorpyrifos has garnered a lot of research interest. Chlorpyrifos is a toxic substance that has the potential to harm human health because some pesticide residue seeps through water and soil. According to Tale and Ingole (2015), the kind of soil and its physicochemical properties affect how quickly a pesticide degrades. Therefore, it's possible that the soil-isolated organisms won't be able to biodegrade chlorpyrifos in other soils. Several microbial species have been identified from soil that has been polluted with chlorpyrifos, and researchers have examined how well they are able to degrade the substance. However, a variety of microbial communities with various functions degrade chlorpyrifos in natural ecosystems.

The aims of this research were to determine, characterize, and evaluate the ability of microorganisms to metabolize chlorpyrifos. Pesticides have a propensity to flow more slowly through soil (Vymazal and Bezinová, 2015). Overuse of pesticides decreases soil fertility by disrupting the soil's microflora and fauna (Farhan *et al*., 2021). It has frequently been identified as a potential neurotoxin and endocrine disorder around the world. (Farhan *et al*., 2021). Chlorpyrifos must therefore be identified in agricultural soil in order to lessen the harm to public health presented by residues of the pesticide in agricultural goods. Having extensive and trustworthy information about the degree of pollution will also enable one to develop policies that will ensure the public's access to safe food.

Need for novel restoration techniques for polluted soils is great. Because it is more effective, environmentally friendly, selective in its destruction, and inexpensive, bioremediation is becoming more and more popular (Farhan et al., 2021). Agricultural soils, wetlands, sludge, and ground water have all been effectively decontaminated through bioremediation. By injecting particular bacteria, pesticide-contaminated soil can be recovered (Patel et al., 2021). The best place to look for resistant microorganisms is in soils that have been contaminated by pesticides (Morillo and Villaverde, 2017). Over time, native bacteria in contaminated soils or places gain resistance. An ecologically friendly technique of in situ detoxification is developed by the isolation and characterisation of local microbial strains (Megharaj *et al*., 2011). Indigenous species are favoured as they have less of an impact on the soil's microflora (Weidenhamer and Callaway, 2010). Autochthonous bacterial populations have adapted to live in toxic environments, such as the muddy ground from dairies used in the current study. (Asamba *et al*., 2022).

In particular, when it comes to the amount of time required for maximal degradation, the new results are superior than those reported in several earlier investigations. For instance, 30.78% Chlorpyrifos

breakdown showed observed by Hamsavathani *et al*., 2017. Additionally, findings are more noteworthy than those made by Kumar (2011), who found that 77% of chlorpyrifos was destroyed. Results support earlier research that identified bacterial communities with the capacity to break down chlorpyrifos in soil and liquid culture. *Serratia marcescens* and *Pseudomonas aeruginosa* are among the bacteria that have been isolated, according to studies by Singh et al. (2004), Anwar *et al*. (2009), Yadav et al. (2015), Hamsavathani *et al*. (2017), and Anode *et al*. *Arthrobacter sp.* and *Flavobacterium sp.* were shown to co-metabolize the drug chlorpyrifos, according to Yadav *et al*. (2015). The creature does not utilise chlorpyrifos as its energy source, study has revealed. Instead, according to Singh *et al*. (2004), the bacterium hydrolyzes chlorpyrifos into TCP and diethyl thiophosphate (DETP), which it then uses for energy and growth. The primary metabolic byproduct of CP breakdown has been validated by numerous other investigations (Fawzy *et al*., 2014; Briceo *et al*., 2020; Bose *et al*., 2021). It may therefore be claimed that the microbial consortium's capacity to biodegrade chlorpyrifos illustrates the ability of such isolates' ability to break down dangerous compounds in polluted soil. More study is still needed to comprehend the molecular level of the pesticide's biodegradation in varied forms of soil, crops, and circumstances.

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## Statements & Declarations:

### Funding:

The authors declare that no funds, grants, or other support were received during the preparation of this manuscript.

### Competing Interests:

The authors have no relevant financial or non-financial interests to disclose.

**Author Contributions:**

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by [**Ashwini Venkatesan**], [**Murugan Karuvelan**] and [ **Ashok Kumar Krishna Kumar**]. The first draft of the manuscript was written by [**Ashwini Venkatesan**] and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

**Data Availability:**

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request

**Ethics approval:**

Nil

**Consent to participate:**

Nil

**Figure legends:**

**Fig. 1** GC-MS technique screening of chlorpyrifos

**Fig. 2** Phylogenetic tree and Illustrative DNA Barcode of *P. aeruginosa* on 16SrRNA gene-based sequence

**Fig. 3** Phylogenetic tree and Illustrative DNA Barcode of *B. huaxiensis* on 16SrRNA gene-based sequence

**Fig. 4** Phylogenetic tree and Illustrative DNA Barcode of *A. niger* on 16SrRNA gene-based sequence

**Fig. 5** Phylogenetic tree and Illustrative DNA Barcode of *F. incarnatum* on 16SrRNA gene-based sequence

**Fig. 6** HPLC chromatogram of *P. aeruginosa* for the degradation of Chlorpyrifos (A. Prior treatment. B. Post treatment)

**Fig. 7** HPLC chromatogram of *B. huaxiensis* for the degradation of Chlorpyrifos (A. Prior treatment. B. Post treatment)

**Fig. 8** HPLC chromatogram of *A. niger* for the degradation of Chlorpyrifos (A. Prior treatment. B. Post treatment)

**Fig. 9** HPLC chromatogram of *F. incarnatum* for the degradation of Chlorpyrifos (A. Prior treatment B. Post treatment)

**Figures:**

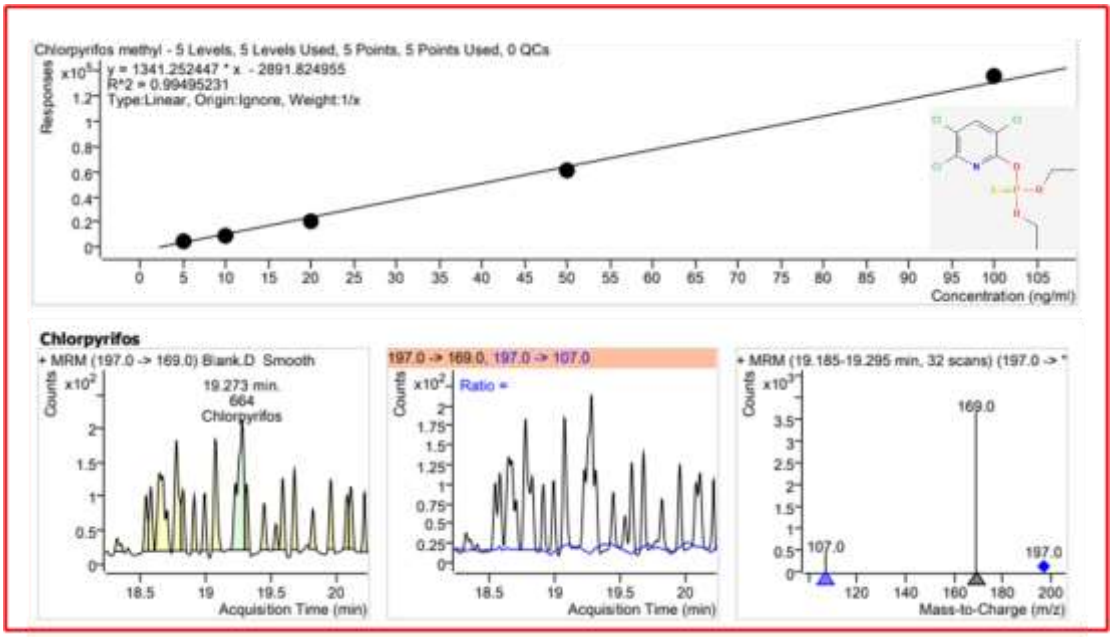
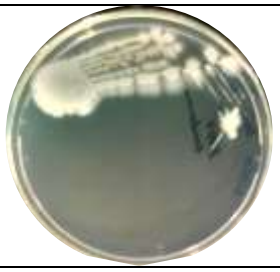
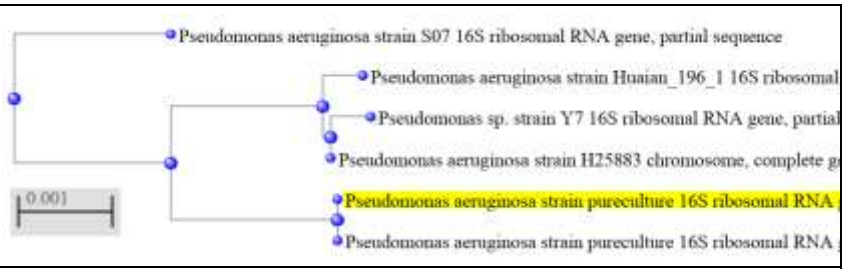



**Fig. 1**





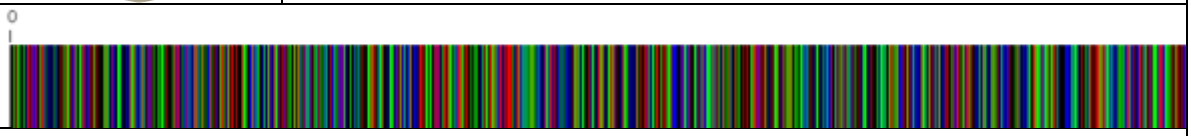



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**Fig. 2**

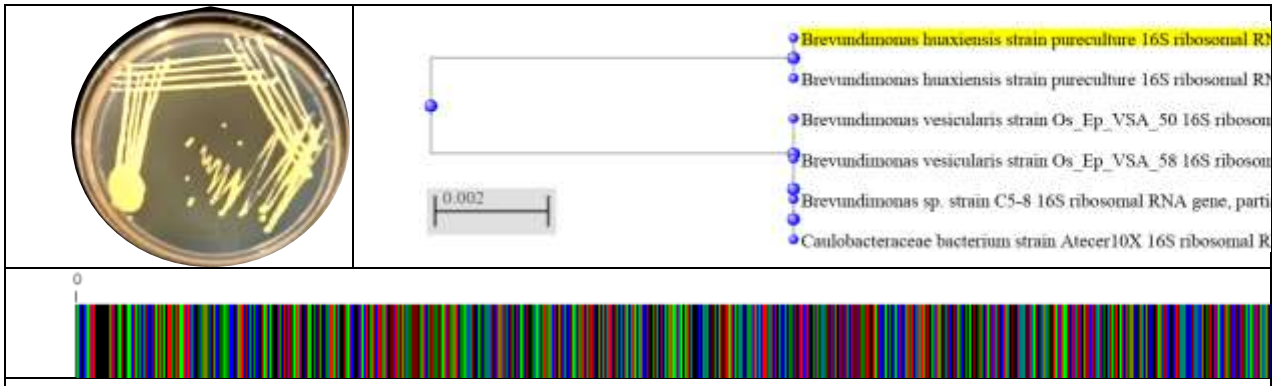


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**Fig. 3**

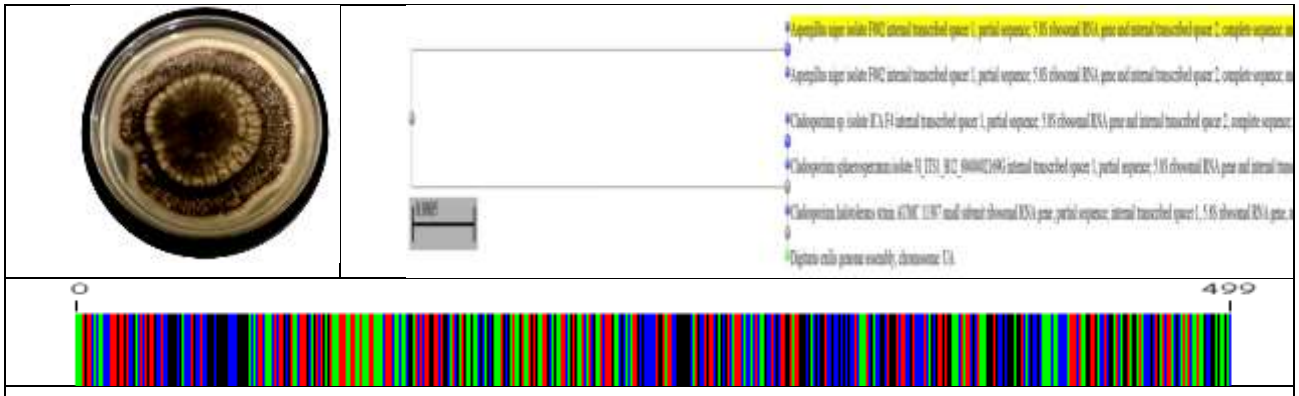


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**Fig. 4**

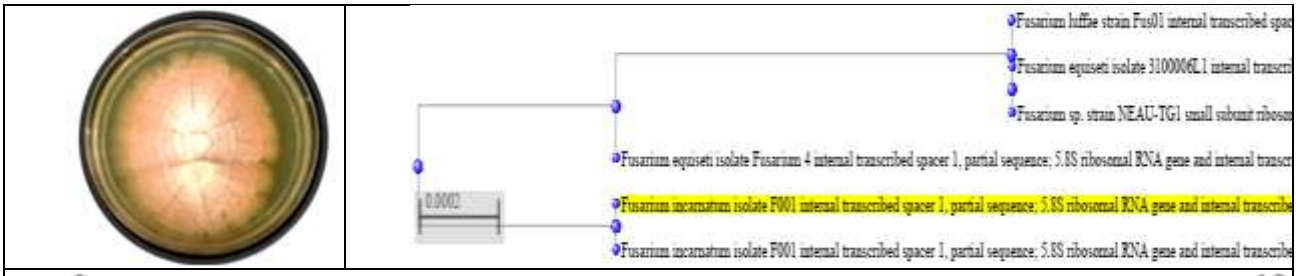


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**Fig. 5**

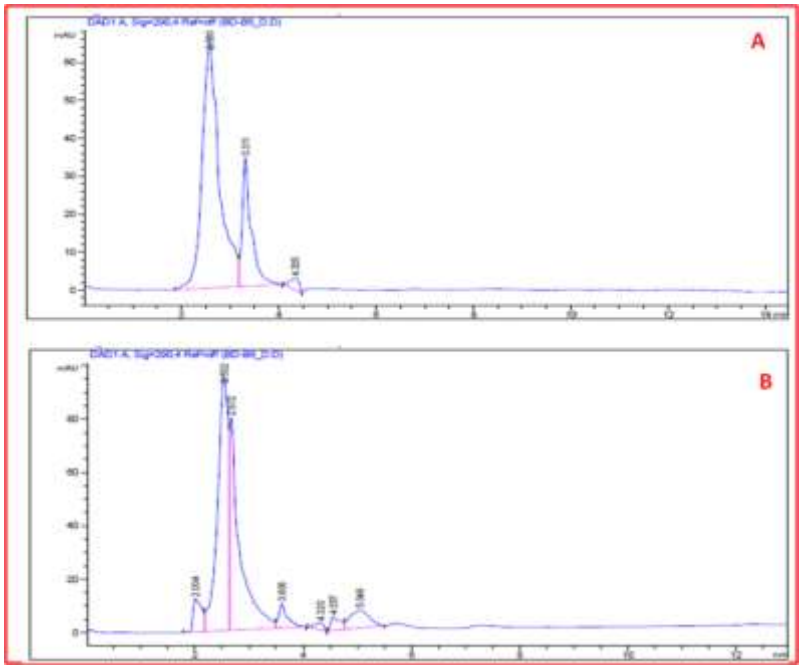


**Fig. 6**

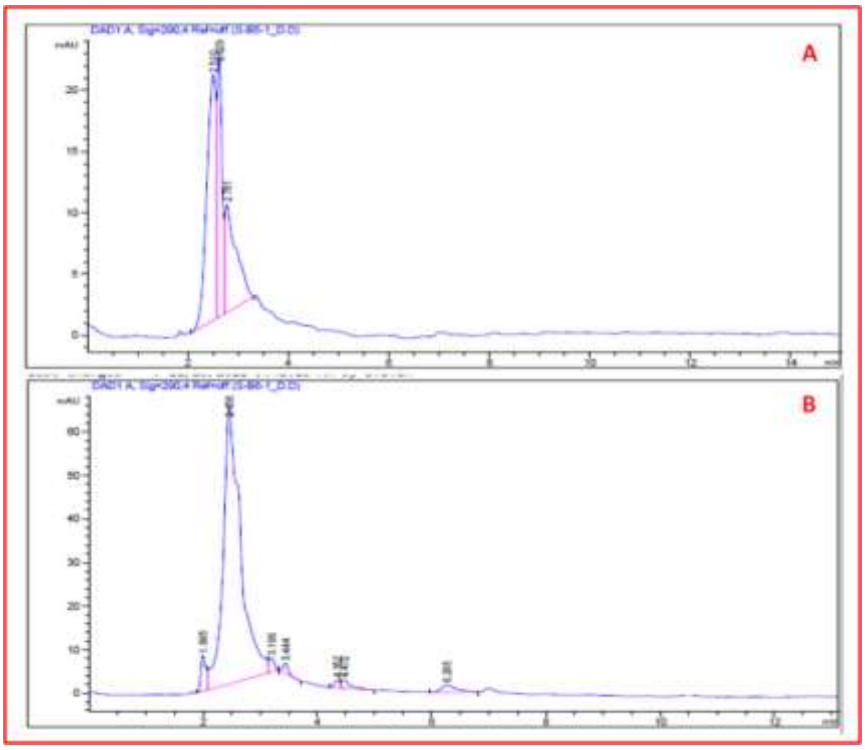


**Fig. 7**

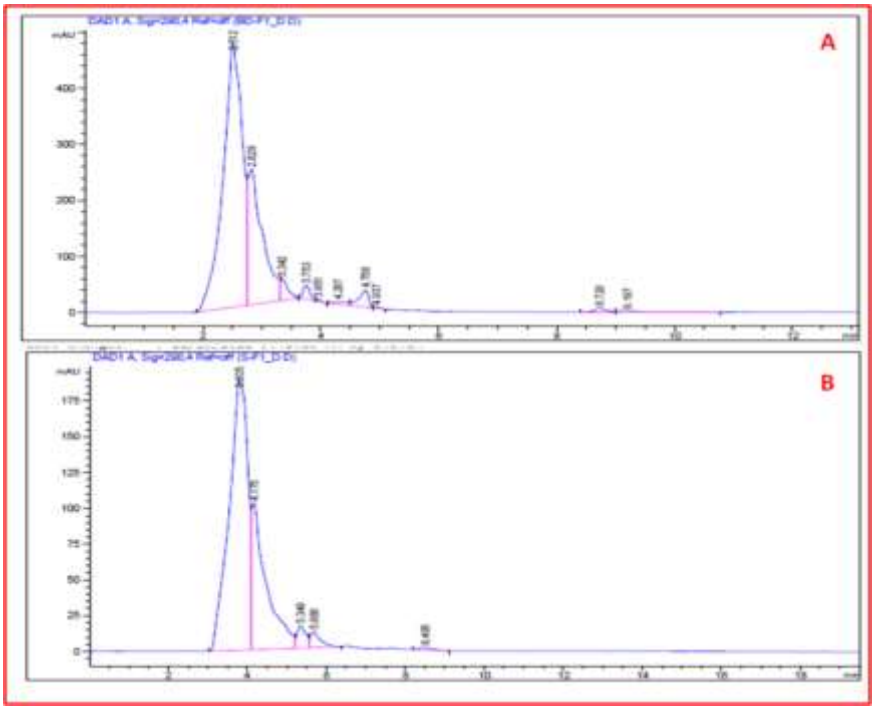


**Fig. 8**

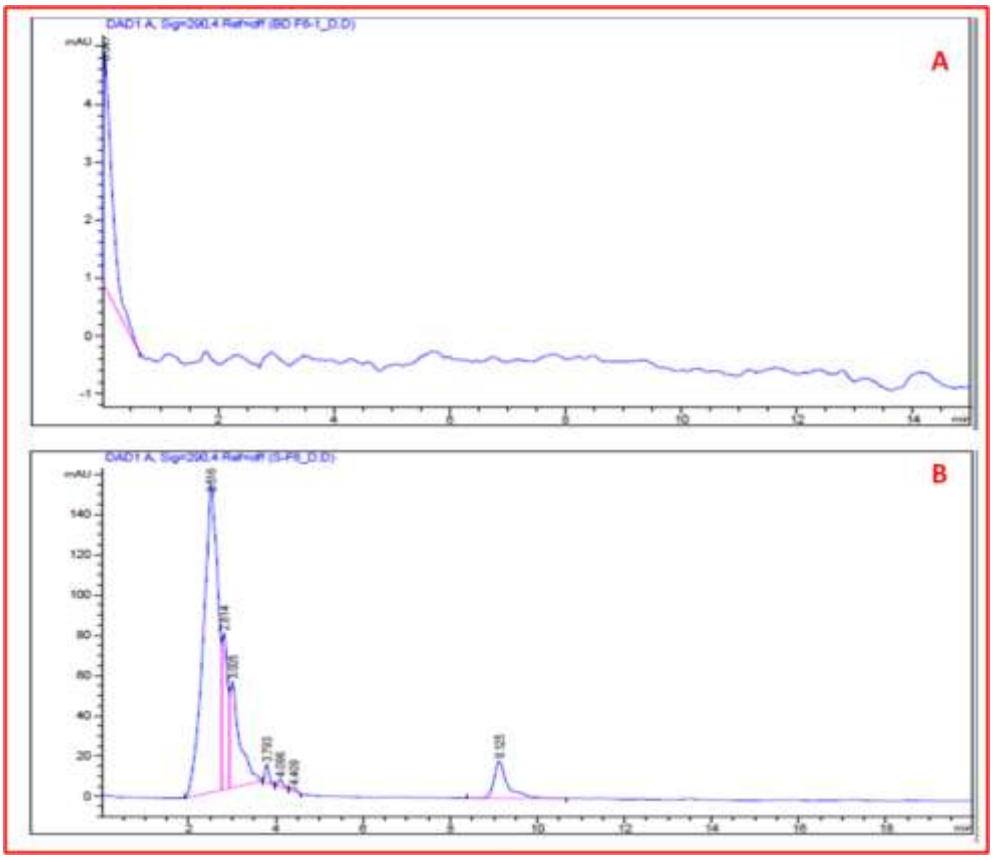


**Fig. 9**