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Original Article

Prevalence and Identification of Carbofuran Degrading Bacteria from Agricultural Fields and Associated Water Bodies

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The extensive use of pesticides in agricultural areas is done to assure protection against insect invasion, to raise crop production, to improve fertility and soil quality, and therefore to efficiently contribute to an agriculture-based economy. However, the unfortunate reality is that the long-term presence of these pesticides in the environment as a result of their complex and xenobiotic chemical structures leads to bioaccumulation as well as bio-magnification, which in turn causes toxicity, chronic disabilities, hormonal imbalance, reproductive and endocrine dysfunctionalities, and many other health issues in humans as well as other vertebrates and causes ecosystem derangement. Similar results are produced by one of the most widely used N-methylcarbamate-based pesticides, carbofuran. There is no better solution to this issue than breakdown of leftover pesticide in the environment to harmless, native components through biological agents, ideally indigenous bacteria. Carbofuran, one of the most widely used N-methylcarbamate-based insecticides, has comparable effects. In light of this, a study was conducted to identify and characterize bacteria that have the innate ability to not only survive pesticide applications to rice and vegetable fields, but also to use the pesticides as a source of energy and break them down into harmless, environmentally friendly components. As a result, samples were taken from a Savar rice and tomato field shortly after a pesticide application, and Carbofuran 5G was the insecticide selected to check for degradability given to its widespread use there. Bushnell Haas broth and Bushnell Haas agar media were employed for enrichment, plating, and subsequent cultures to generate pure culture, with supplemented carbofuran of different concentrations serving as the only carbon source. The identities of the 17 isolates that could grow there were verified by sequencing after PCR and product purification. The evolutionary relatedness was determined using phylogenetic analysis with MEGAX. *Acinetobacter* spp., *Rahnella* spp., *Chitinophaga* spp., *Serratia* spp., *Stenotrophomonas* spp., *Achromobacter* spp., *Pseudoxanthomonas* spp., and *Klebsiella* spp. were the bacteria that were found.

Keywords: Carbofuran, bioremediation, bacterial degraders

Introduction

Agriculture is undoubtedly one of the topmost fields of interest because it contributes significantly to the economy and development of the nation in addition to being a source of food and animal feed. Although all sorts of crops are included in agriculture, rice fields and vegetable fields are held in higher regard in our nation due to their unparalleled demand in the food market and their function as cash crops. Invasion of pests is one of the main obstacles to growing these crops. Insect pests target all parts of the rice plant, all growth stages, and even stored rice grains. Leaf miners, Beetles, Earworms, Hornworms, Cutworms, Armyworms, Cabbage Loopers, Aphids, and Thrips and among the most frequent pests seen in vegetable fields are stem borers, spider mites, and green vegetable bugs[1,6]. The most widely used and effective method to protect the crops from pests and to increase their yield is application of pesticides[14].

Pesticides contain a wide spectrum of substances that comprise of insecticides, fungicides, rodenticides, molluscicides, and nematocides[9]. Additionally, they are designed to increase agricultural yield, soil productivity, product quality, reduce agricultural product loss, and control insect vectors to stop the spread of human and animal diseases[15]. Organophosphates, carbamates, and organochlorines are three prominent pesticide families. Among these, carbamates are frequently utilized in fields of vegetables and grains. Acetylcholinesterase is carbamylated by carbamates, which are N-methyl carbamates generated from a carbamic acid, in neuronal synapses and neuromuscular junctions. They reversibly bind to acetylcholinesterase[21].

Carbofuran 5G is a Carbamate pesticide, widely used all over the world to control a wide variety of insects and nematodes (roundworms) on a variety of agricultural crops. The technical or chemical name of carbofuran is 2,3-dihydro-2,2-dimethyl-7-benzofuranyl methylcarbamate. Carbofuran was chosen because of its excellent efficacy and quick action, which ensures high output. Carbofuran, however, has toxicological effects on humans as well as other vertebrates, just like other pesticides. Alarming

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concerns are raised by bioaccumulation and less prone environmental damage. Of all the common insecticides used on field crops, carbofuran has one of the highest acute toxicity levels for humans. It is regarded as a neurotoxic pesticide since its harmful effects result from its activity as a cholinesterase inhibitor. It mimics the neurohormone melatonin and has been associated to a higher risk of developing diabetes because it can directly bind to the MT2 melatonin receptor and disrupt melatonin signaling[7],[22]. Carbofuran is extremely hazardous to both birds and vertebrates. A bird can be killed by a single grain of carbofuran. Humans can die from I ml (a quarter teaspoon). Carbofuran is an endocrine disruptor and perhaps a drug that impairs reproduction and development. Carbofuran may temporarily change the hormone content at modest exposure levels. Upon repeated exposure, these modifications may consequently cause major reproductive issues[19].

There are laws and agencies to regulate the usage and dosage of pesticide exists such as: Pesticide Laws and Regulations - Environmental Protection Agency (EPA),Federal Insecticide, Fungicide and Rodenticide Act (FIFRA) - Cornell University, Food Quality Protection Act (FQPA) of 1996 - Environmental Protection Agency (EPA),Federal Food, Drug and Cosmetic Act (FFDCA) - Cornell University, Code of Federal Regulations (CFR) Title 40 - Pesticide Programs - Government Printing Office (GPO) and many more, yet even so, they are insufficient since residual pesticide bioaccumulation and pesticide dispersion into nearby ecosystems still play significant roles in pesticide toxicity (National Pesticide Information Center, 2020). Approximately 90% of agricultural pesticide application never reaches its target organisms, instead, is dispersed through the air, soil, and water[13,17]. As a consequence, they are routinely detected in air, surface and ground water, sediment, soil, vegetable, and to some extent in foods. Moreover, numerous soil-applied pesticides used to control soil-borne pests and diseases cause the buildup of their residues and metabolites at unacceptable high levels in soil [2,3]. In addition, water runoff and soil erosion can contaminate the aquatic systems with these xenobiotic substances.

Bioremediation is a solution that combines both agricultural advantages and the avoidance of hazardous pesticide bioaccumulation. Although other techniques have been developed to lessen the effects of pesticides on the environment and human health, these include physical treatments like adsorption and percolator filters; chemical treatments like advanced oxidation that use potent transient species, primarily the hydroxyl radical[10,12],[15] but, they are vulnerable to problems like high costs, time demands, unpredictable outcomes, and others. Hence, it is becoming clear that the best course of action is to make use of the natural capacity of local microorganisms, particularly bacteria, to convert pesticides into safe, environmentally beneficial, and non-toxic residues. Our research focuses on identifying isolates from the native niche that have the potential to act as bioremediating agents, and one of our goals is to shed light on and take into account some lesser-known bacteria.

Materials and Methods

Sampling

The present study's soil samples were collected from paddy and tomato fields in Savar, and the study's water samples came from canals through which the fields' drained water flows and from the neighboring Bangshai River. The date was chosen to be one week after the pesticide was applied so that it could be guaranteed that the isolates obtained were degrading the pesticide in its original form and not one that had been photodegraded, chemically altered, or physically corroded. The carbamate group pesticide Carbofuran 5G was the one evaluated in the study. It was purchased at the Nazma Bazar neighborhood market in Savar.

Sample processing and enrichment

The microorganism from the sample were enriched using Bushnell Haas broth. Because there is no carbon source in the media composition other than the added pesticide with a variety of concentrations, the medium is suitable for enriching the necessary microorganisms. The ability of the microorganisms living in the media to breakdown pesticide and use it as a carbon source may therefore be verified. In order to enrich microorganisms that can degrade the various pesticide concentrations, the collected soil and water samples were added to Bushnell Haas broth in conical flasks containing carbofuran concentrations of 0.025%, 0.05%, 0.075%, 0.1%, 0.125%, and 0.15%. The flasks were shaken for 24 hours at 120 rpm. Hundred ml of medium were inoculated with 5gm of soil and 5ml of water for the water sample. Both paddy field and tomato field soil and water were subjected to enrichment in a similar manner.

Isolation of Bacteria

The next step after enrichment was to isolate the bacteria. Bushnell Haas agar media was created for this, and using the 4 quadrant streak method, one loopful of an enhanced culture was streaked on agar plates with carbofuran concentrations of 0.025%, 0.05%, 0.075%, 0.1%, 0.125%, and 0.15%, respectively. The initial colonies obtained were mixed. Repeated subculturing on Bushnell Haas agar media was used to obtain pure isolates so that, if pesticide degradation was plasmid mediated, it would not be lost as a result of lack of stress.

Assessment of Carbofuran degradation ability

The isolates' capacity to develop on Bushnell Haas Agar supplemented with carbofuran as the only source of carbon alone demonstrates their ability to digest carbofuran in the absence of any other available alternative energy source in the media. Bacterial growth was examined at pH 7 and 37^0C with various carbofuran concentrations (0.025%, 0.05%, 0.075%, and 0.1%).

Identification and characterization of isolates

The isolated organisms were characterized in several ways,

including by looking at colony traits, microscopic inspection (using gram staining and negative staining), biochemical profiling, and last but not least, sophisticated molecular analysis. The biochemical assays included Oxidase Test, Catalase Test, Motility, Urease, Indole Test, MR-VP Test, Citrate Utilization Test, Kligler's Iron Agar Test.

Molecular characterization

The DNA extraction was done using Boiling method (Medici *et al*. 2003). Polymerase chain reaction was carried out using 16S rDNA as primer for the identification of the isolates. Two primers-forward (AGAGTTTGAGCCTGGCTCAG) and reverse (TACCTTGTTTTACGACTT) along with the template DNA and nuclease free water were mixed with the commercial master mix to obtain a final reaction concentration of 350µl. 25µl of the reaction mixture was then dispensed into each the microcentrifuge tubes. These tubes were then brought into the Applied Biosystem Veriti thermal cycler and polymerase chain reaction was carried out. The annealing temperature was 46^0 C and extension temperature was 72^0 C for 40 cycles of reaction. ThermoFisher DNA purification kit was used to purify the PCR product, and an agarose gel run verified the bands. The purified PCR products and primers were forwarded to BioTech Concern for sequencing.

Phylogenetic Analysis

The MEGAX program and the BLAST tool were used to perform this. The acquired sequences were entered into BLAST as query inputs to discover similar bacteria. Other settings were left at their defaults. Five to ten sequences with the best query coverage, percentage identity, and Max score were chosen from the collected BLAST results.

Using MEGAX, the sequences obtained by sequencing and the BLAST results were aligned. The alignment used ClustalW. Following alignment, the sequences were utilized for phylogenetic analysis, and a Maximum likelihood tree was created using the Kimura 2 model while maintaining the other default dialog box settings. The bootstrap value used was 1000.

Results

Isolation of pesticide-degrading bacteria

Several native isolates were produced after enrichment during plating that could use carbofuran as their exclusive source of carbon and thrive at different carbofuran concentrations (Fig 1). They were divided into 17 isolates, which used promising carbofuran concentrations at 37^0C and pH 7.0 and were morphologically distinct (9 from soil samples and 8 from water samples). Most colonies were tiny pinpoints that were transparent, spherical, some were dry, and some were mucoid.

Identification and characterization of isolates

Under a microscope, the majority of the acquired isolates were discovered to be Gram negative and very few to be Gram positive. Negative staining made it easier to see the shape and organization under the microscope. Many of them were short rods with some cocci and were clustered or organized in small chains (Figure 2). The supplemental tables contain information on the biochemical profile of each isolate.

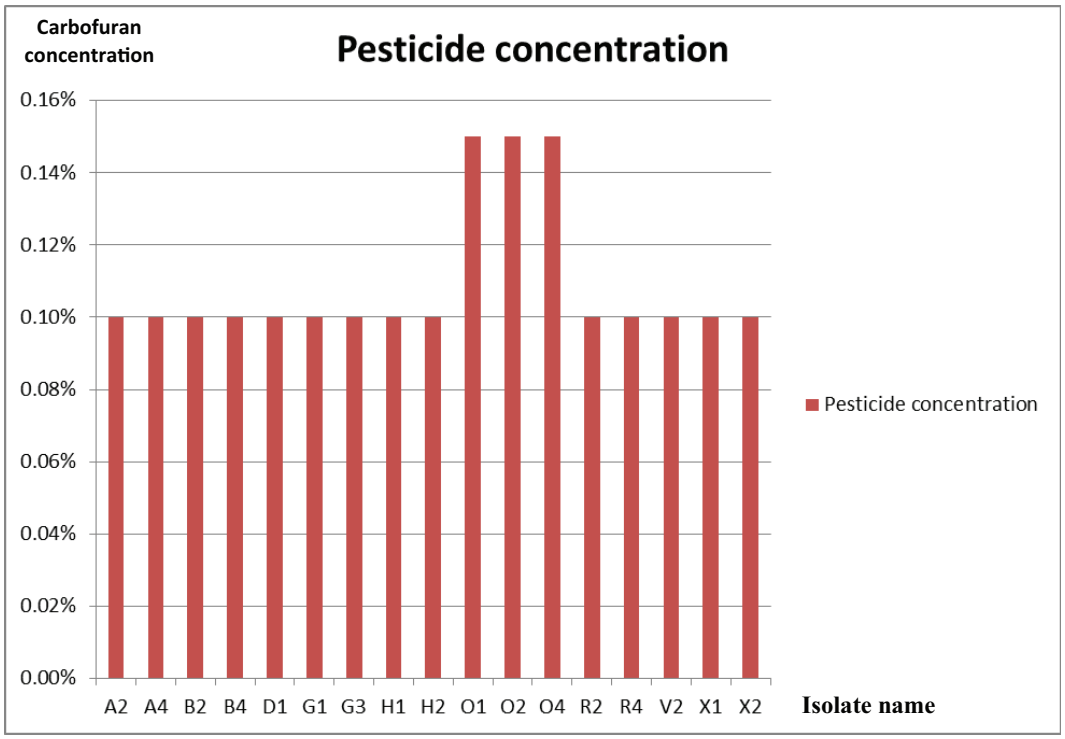

Fig. 1: *Carbofuran degradation extent of each isolate*

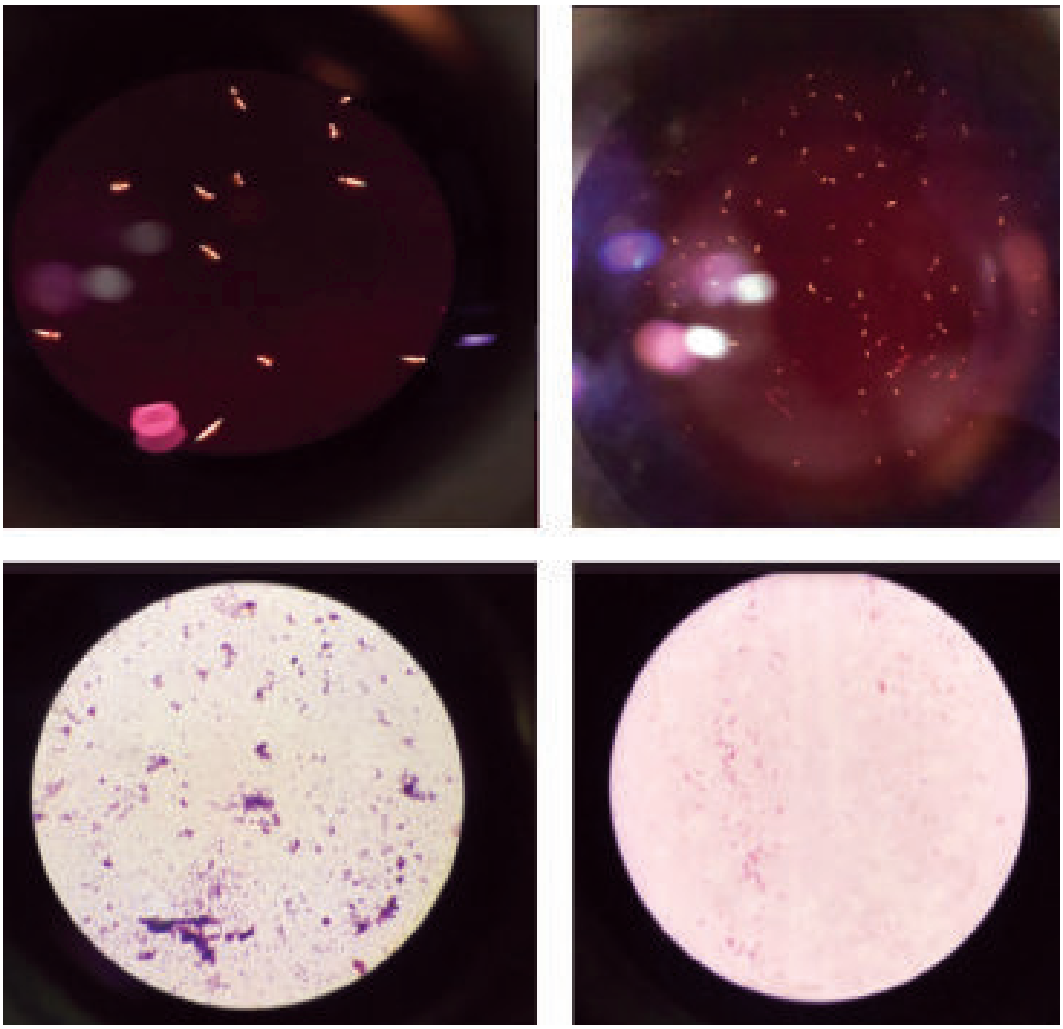

Fig. 2: *Gram staining of the isolates*

For molecular identification, polymerase chain reaction was carried out. Purified products were sequenced using the broad identification range of the 16S rDNA primer that was employed in this instance.

By using the FASTA file of sequencing as input in BLAST search and examining the maximum score, query coverage, and percent identity, the identities of isolates were determined.

The isolates had a very high degree of identity with several bacteria, allowing for their accurate identification. In addition, the microbes included several common soil bacteria including *Acinetobacter*, *Serratia*, and *Stenotrophomonas* as well as some unusual or less well-studied bacteria like *Chitinophaga* and *Rahnella* (Table 1). *Acinetobacter*, *Klebsiella*, and *Pseudoxanthomonas* were found in water samples (Table 2).

Table 1: *The identity of isolates from soil through16S rRNA sequencing*

Isolate name	Query coverage in BLAST search	Percent Identity in BLAST search	Organism with highest similarity in the BLAST database
A2	99%	99.55%	*Chitinophaga spp*
A4	99%	98.37%	*Achromobacter spp*
G1	99%	99.72%	*Stenotrophomonas*
G3	99%	99.19%	*Serratia marcesens*
V2	98%	93.52%	*Rahnella aquatilis*
X1	99%	99.64%	*Acinetobacter*
X2	99%	99.62%	*Acinetobacter*
H1	99%	99.81%	*Acinetobacter*
H2	97%	79.54%	*Bacterium_A1*

Table 2. *The identity of isolates from water through16S rRNA sequencing*

Isolate name	Query coverage in BLAST search	Percent Identity in BLAST search	Organism with highest similarity in the BLAST database
B2	99%	99.18%	*Acinetobacter*
B4	99%	99.29%	*Acinetobacter*
D1	98%	99.63%	*Pseudoxanthomonas spp*
O1	96%	97.58%	*Klebsiella spp*
O2	98%	98.96%	*Klebsiella spp*
O4	97%	99.80%	*Acinetobacter*
R2	96%	92.92%	*Acinetobacter*
R4	98%	99.90%	*Acinetobacter*

However, none of their role in pesticide degradation has been widely studied yet and so the discovery was quite enlightening.

Phylogenetic Analysis

ClustalW of MEGAX was used for alignment. Subsequently, in order to better see and comprehend the evolutionary relationship, two trees were created: one to display the phylogenetic link among the isolates exclusively, and the other to demonstrate the phylogenetic relation of isolates with other comparable microorganisms.

Using a tree analysis of soil samples, it was shown that *Rahnella* and *Serratia* were more closely linked to the Enterobacteriaceae while *Acinetobacter*, *Achromobacter*, *Stenotrophomonas*, and *Chitinophaga* were evolutionarily more related and had a closer relation with *Pseudomonas* (Figure 3).

Phylogeny re-construction of water samples indicated that the isolates were mostly of *Acinetobacter spp.* (Figure 4). The molecular characterization matched with the biochemical and microscopic profiles.

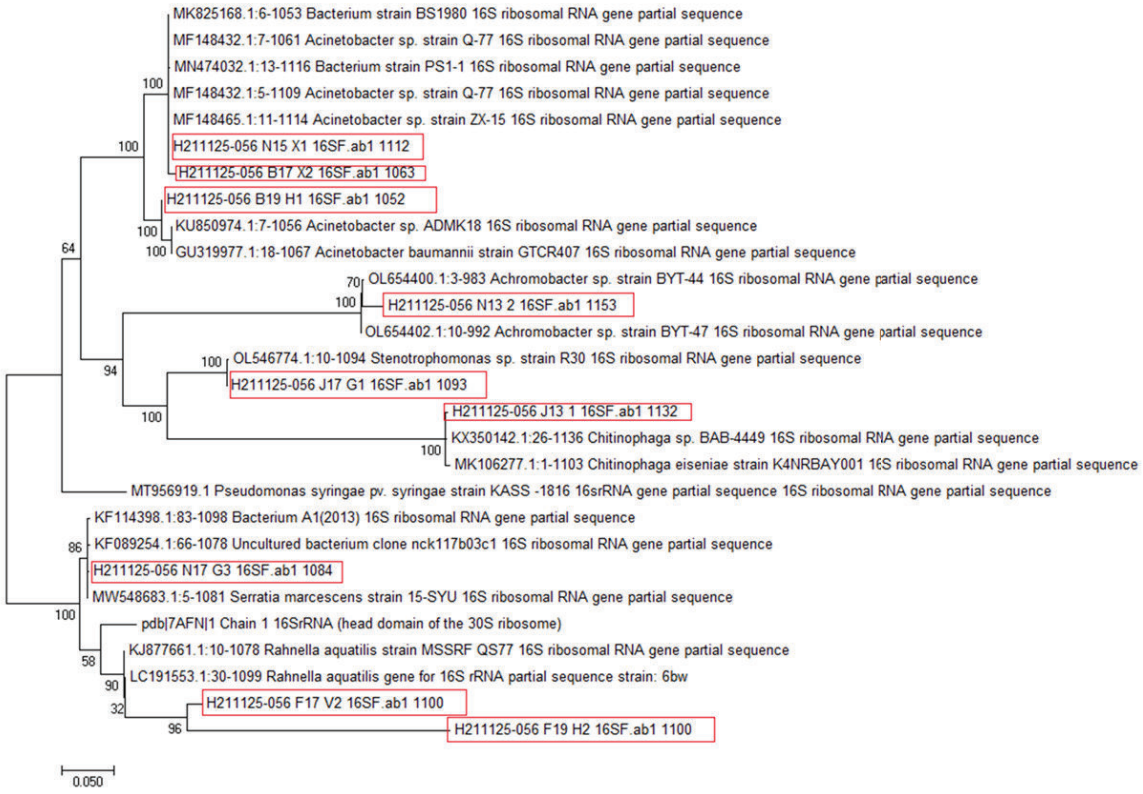

Fig. 3. *The phylogenetic analysis of soil isolates*

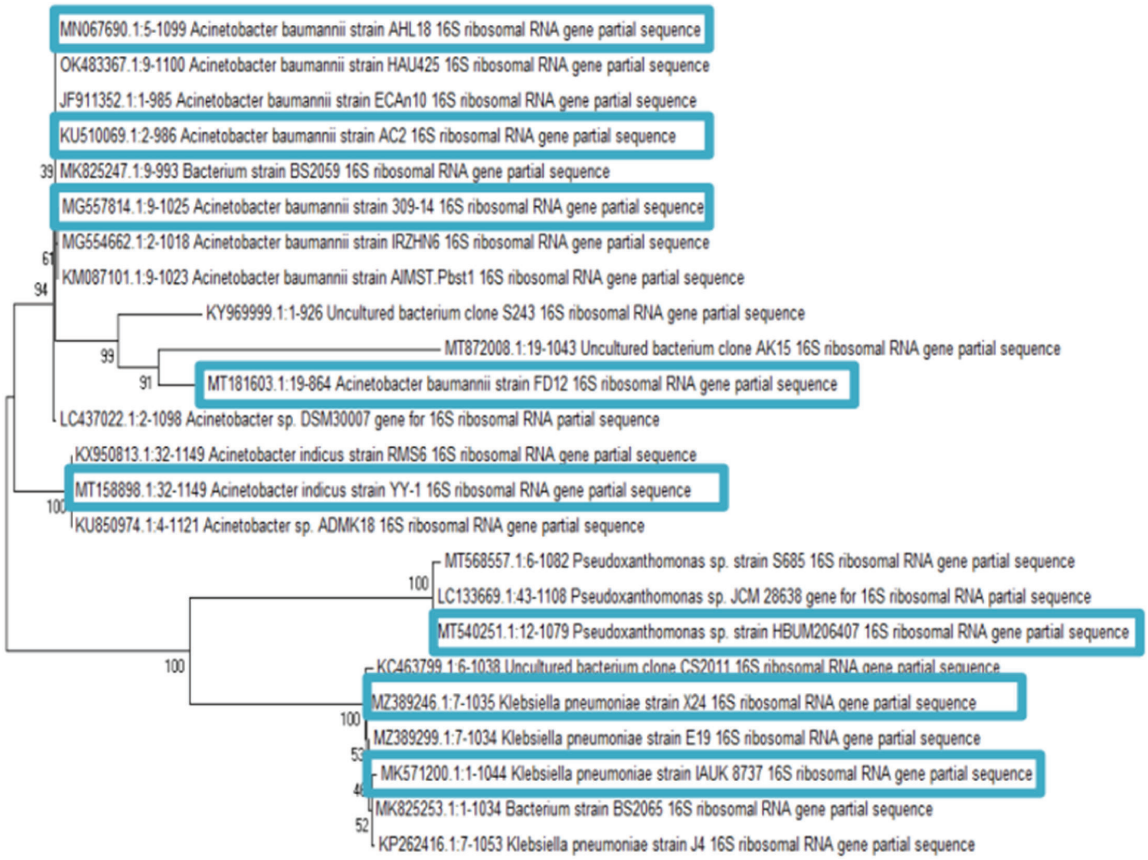

Fig. 4. *The phylogenetic analysis of water isolates*

Discussion

Although they are a necessary part of agriculture, pesticides are actually dangerous substances that are disguised by good output and financial growth[16]. (Ralf Bernhard and Caquet, 2007). Yet, the use of bioremediation is now the only way to stop the curse while keeping the beneficial part. By utilizing them as an energy source, bacteria can break down dangerous pesticides like carbofuran, preventing their bioaccumulation and the negative consequences they have on ecosystems[4,18].

The research identified certain microbes whose contribution to pesticide breakdown had not previously received considerable attention. The ability to breakdown carbofuran up to quantities of 0.15 percent was demonstrated by *Chitinophaga*, *Stenotrophomonas*, *Rahnella*, *Acinetobacter*, *Serratia,* and others. This is good since it correlates with the sprayed pesticide concentration on fields, which is 1-5000 g/kg, or 0.0005%. Consequently, using these microorganisms to break down the herbicide has the potential to provide unheard-of results[2,8,22,23].

This conclusion was reached after profiling the bacterial isolates on all microscopic, colony morphology, biochemical, and, finally, molecular levels by sequencing. Because DNA extraction and PCR are molecular procedures that demand great precision and sensitivity, necessary precautions were taken to avoid any contamination. The results also showed that the processes were carried out correctly. All forms of profiling produced data that were consistent and devoid of anomalies. The 99% identity rate of the BLAST results, which verified the isolates' identities, attested to the accuracy and dependability of the findings. The phylogenetic analysis showed the evolutionary relationships between the bacteria as well as their resemblances to other, more widely distributed microorganisms.

In addition to being one of the destinations for hospital waste and drainage lines, the water bodies from which samples were taken were also the final resting place for drained pesticides and fertilizers[11,19]. Although the bacterial isolates' identities were established, their capacity to break down carbofurans was demonstrated, and their capacity to break down abamectin was also demonstrated, all of these tests were conducted at lab scales and in narrow ranges. To qualify them as potential candidates for industrial production for use in bioremediation, field-level experiments, strain-improvement techniques, optimization of growing conditions, and the impact of environmental factors like pH, temperature, salinity needs to be examined and studied[5,7].

Therefore, it can be conferred that the work has paved the path to establish more than one potential bioremediation candidates and scopes for extensive research and study.

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