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Research Article

Isolation of Monochloroacetic Acid Biodegrading Bacteria from Tigris River

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Abstract: Organic compounds containing halogens are widely dispersed throughout the world, resulting in different types of pollution. One of the most common xenobiotics used in agricultural activities is monochloroacetic (MCA) which was Isolated from Iraqi mud in the Tigris River. This bacterial strain was known as SW2. Both standard universal primers Fd1 and rP1 were used with the colony PCR method for bacterial identification before being sent out for sequencing. Basic Local Alignment Search Tool nucleotide sequences and information were analyzed (BLASTn). The phylogenetic tree was constructed using the 16S rRNA sequence to determine their evolutionary distance. The Neighbor-Joining method was used to infer the evolutionary history. The Neighbor-Joining method was used to infer the evolutionary history. There is a 96 percent match between the SW2 bacterium and another type of aerobic Gram-Negative Bacteria. Strain SW2 (*Pseudomonas* sp.) was inoculated for two days and yielded colonies that were small, non-spore-forming, and rod-shaped. Growth slowed slightly after 48 hours. The release of chloride ions as a result of the degradation of MCA was seen using a halide ion test. Biochemical tests backed up the choice of the genus's name as well. As a result, bacteria found in the river are capable of utilizing and degrading the MCA compound. In conclusion, this study confirmed the potential ability of the isolated bacterial strain of utilizing MCA, especially from contaminated environments with the pragmatic application of the bacterial strains to degrade residual herbicide.

Keywords: *Pseudomonas* sp SW2, Dehalogenase, Monochloroacetic (MCA), Degradation xenobiotic.

1. INTRODUCTION

The use of haloaliphatic compounds has increased over the last century as a result of the widespread human industrial in addition to all agricultural activity [1]. Environmental contamination and toxic chemicals accumulation in our ecosystem especially Pesticides and fungicides, insecticides, solvents and herbicides result from the widespread use of these hazards [2]. Natural sources are also a source of chlorinated compounds entering the environment. Natural organic halogen compounds numbering in the thousands, according to research [3].

When it comes to halogens, the most common one is chlorine, which is responsible for the toxicity of these compounds. There are many examples, such as the production of Monochloroacetic acid (MCA) for agricultural use especially herbicides, which will lead to an increase in pollution and health issues [4].

Biodegradation is a natural process by which Microorganisms have the ability to eliminate xenobiotic chemicals from the environment, such as chloroaliphatic substances [5, 6]. A wide range of microorganisms capable of degrading these chemicals have been discovered, and studies have helped to clarify the workings of various microbial biodegradation mechanisms [7]. One of the major issues is the inability of these compounds to be

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degraded by microorganisms because they are barely water-soluble [8, 9].

For as long as the pollutant serves as both a carbon source and an electron donor, microorganisms will naturally degrade the pollutant. Halo-respiration, on the other hand, uses chlorinated compounds as electron acceptors [10].

It was possible to isolate bacteria that utilize and degrade monochloroacetic acid, identify the isolated bacteria using molecular analysis utilizing the 16S rRNA gene amplification and characterize the bacterium through biochemical testing, all of which were accomplished through this study. One of the most commonly used techniques for the identification and characterization of microorganisms is the Polymerase Chain Reaction (PCR) amplification of the bacterial small subunit ribosomal RNA gene (16S rRNA) [11].

Many bacteria use hydrolytic mechanisms to break down halogenated compounds. Cleavage of the halogen-carbon bond and substitution of hydroxyl for the halogen group are the methods used in this procedure. The primary objective of the study was to determine how to degrade environmental toxins to lessen the environmental pollution. Our ecosystem will continue to function normally and even improve as long as there exist microorganisms that can digest these toxins.

2. MATERIALS AND METHODS

2.1 Preparation of Samples and Minimal Media

Mud samples were collected from five separate locations. In 15 ml of sterile, distilled water, 0.5 g of soil sample was suspended. After adding the mixture, the soil fragments were let to settle. In a 250 ml flask containing soil, 5 mM monochloroacetic acid (MCA) was added as a carbon and energy source to 100 ml of the minimum media (10 g). An incubator at 37 ^{o}C and 200 rpm rotary shakers were used to culture the culture for two days. The experiment began after a few drops of soil solution were applied to solid media. To obtain pure colonies, the sub-culture process had to be repeated several times. To prepare the bare minimum of media for use, we needed two stock solutions. The amount of trace metal salt and basal salt produced increased tenfold. The base salt and metal salt compositions in the minimal medium are shown in the following [12].

2.2 Growth Profile

The growth profile or "growth curve" was plotted to examine the growth of the microorganisms in the described medium. This experiment allowed researchers to distinguish between different stages of bacterial growth. Nylon filters of 0.22 microns were used to sterilize the MCA, which was then aseptically added to the mixture of basal and metal salts. It was incubated at 30 ^{o}C and 200 rpm for 24 hours with a pure colony in the liquid medium. In the following six-hour intervals, a new 1mL cuvette was filled with the sample, and the procedure was repeated. At A600 nm, the BUCK SCIENTIFIC VIS 100 spectrophotometer was used to take the measurements [13].

2.3 Halide Ion Assay

An assay known as the halide ion assay measures the amount of halide ions that can be released from halogen compounds. Fluoride, Chloride, Bromide, and Iodine are all examples of halide ions, and each has a unique set of properties. Monitoring the release of chloride ions was done using the spectrophotometer at A460 nm, as described by Bergmann and Sanik [14]. Ferric ammonium sulfate and mercury thiocyanate were used as reagents in this experiment.

2.4 Preparation of Samples for PCR Analysis

PCR was amplified using the 16S rRNA gene and then sequenced to detect the bacterium isolated from Mud samples. In this experiment, universal primers have been utilized to amplify the 16S rRNA gene. DNA extraction is a standard procedure for isolating DNA. Using Promega Wizard® Genomic DNA Purification Kit, the procedure was carried out [15]. Before the extraction process, some materials and chemicals were prepared, and the standard procedure was strictly adhered to. Evaluation of Quantity and Purity of Extracted DNA, the extracted DNA samples were quantified using a NanoDrop spectrophotometer. The 260/280 nm absorbance ratio and DNA yield (μg) = DNA concentration (μg/μl) × total sample volume (ml) were used to

measure DNA purity and concentration as described by Sambroole and Russell [16]. Moreover, the quality of the isolated DNA was evaluated by 1.5 % Agarose gel electrophoresis. The approximate size of the isolated DNA was calculated using a molecular weight marker, a 100 bp plus DNA ladder (Bioneer, Korea).

2.5 Polymerase Chain Reaction (PCR)

In genetic studies, PCR was used to determine a specific gene sequence in the DNA using a polymerase chain reaction. Universal primers fD1 and rP1 were used for the general amplification of procaryotic 16S rRNA. A conserved region of the 16S rRNA gene was used by scientists in this case for molecular identification of the unknown bacteria and for comparison and species determination among the several kinds of prokaryotic microorganisms that are listed in the database. 16S rRNA gene region has the lowest mutation rate of any gene in bacterial DNA, so it can be used to identify bacterial species. Using universal PCR primers, the 16S rRNA gene was amplified. Phylogenetic information about the isolated bacteria can be obtained through amplification. AGA GTT TGA TCC TGG CTC AG-3' and 5'-ACG GCT ACC TTG TTA CGA CTT-3' are the forward and reverse primer sequences, respectively. PCR requires the following components: a Promega master mix, universal forward and reverse primers for 16S rRNA, and deionized water. PCR master mix (25 mL) was added to a 100 mL microtube, followed by 1 mL of each of the forward and reverse primers. Afterward, 5 L of the rehydrated DNA sample and 50 L of deionized water were added to the mixture. Once the mixture was ready, it was fed into the PCR machine for further analysis.

2.6 Sequencing and Analysis of PCR Products and Phylogenetic Analysis of 16S rRNA gene

The 1st Base® Company received the PCR products used in the "DNA sequencing" process. Eppendorf tubes containing 25 L of 16S rRNA gene amplification products were used to store the PCR products. They were not purified. Separate sterile tubes held 5 L of each primer's forward and reverse primer. For sequencing, the tubes were sealed and labeled. Blastn analysis was used to perform an alignment and comparison of our 16S rRNA sequence with sequences from the Gene Bank at NCBI. The command CULSTAL-W was utilized to align the sequence with the first 5 sequences. MEGA version 5.2.2 used a neighbor-joining method [17] to create phylogenetic tree [18].

3. RESULTS AND DISCUSSION

3.1 Isolation of Bacteria from Water-Capable Degrade MCA

Five different locations in the Tigris River, Baghdad / Iraq were used to collect mud from water samples. At 37 °C, 10 mM monochloroacetic acid was added to the broth minimal media supported by the Microbiology lab to find the bacteria capable of degrading this substrate. Incubation at 37 °C for 2 days resulted in the formation of tiny creamy circular colonies on the plate of the agar master after a few drops were transferred and separated.

To isolate the two colonies, we used the streaking plate method and then incubated them at 37oC for two days. The morphology of one of them had been grown, and it was found to be similar to the other 3 times, the bacteria were re-suspended in the same way. Eventually, the SW2 colonies were found and renamed. There was no sign of growth on the control plate, which was incubated in similar conditions. Figure 1 depicts the outcome.

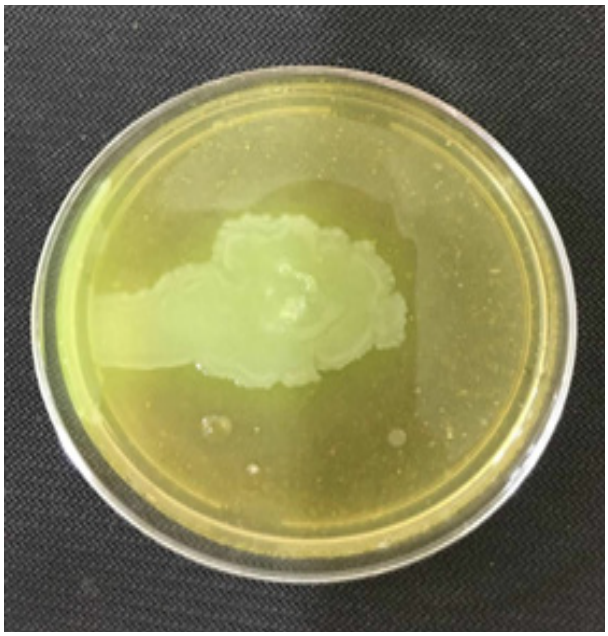

Fig. 1. SW2 strain grown in solid minimal media with 10 mM MCA.

3.2 Halide Ione Assay and Growth Profile

A growth profile had been performed on the isolated bacterium to ascertain its capacity for growth. MCA concentrations of 10 mM, 20 mM, 30 mM, and 40 mM were used to grow SW2 bacteria in liquid minimum at 30 °C in the shaker incubator at 200 °C. Every six hours, following a 24-hour adaptation period, the rate of growth was monitored with a spectrophotometer equipped with an A600 nm wavelength. Figure 2 depicts the result of the experiment.

Different stages of growth were discovered during this experiment by using a growth profile, also known as a "growth curve," to track the growth of the bacteria in the specified medium. There are three different concentrations of MCA that the bacteria can grow in. 10 mM, 20 mM, and 30 mM, whereas the growth was not detected in the medium containing 40 mM of MCA. Bacterial growth may have been stifled by the high concentrations of MCA that had been used. As a result, the halide ion assay for MCA concentrations of 10 mM, 20 mM, and 30 mM will be described in further detail in order to examine the relationship between the growth profile and the released chloride ions. The concentration of released chloride ions was determined by converting the absorbance readings to mM concentration using a standard curve built using sodium chloride as a reference measurement of soluble chloride.

By comparing the absorbance of the sample with a standard curve constructed using sodium chloride, where strain sw2 showed a value of 0.192 mol Cl/ml, the maximum chloride ion liberation is further determined. This finding is in line with that of Wong and Huyop [13], who found that bacteria using halogenated compounds released chloride ions. In addition, it has been demonstrated that the SW2 bacterial strain is capable of using MCA as its sole carbon and energy supply. By producing dehalogenase, microorganisms are capable of breaking down the substance.

In general, the bacteria grow well in MCA minimum media. The growth rate is influenced by the MCA concentration in the media, as shown in Figure 3. The medium with 10 mM MCA had the best growth rate, while media containing 20 mM and 30 mM had lower growth rates. After 24 hours, the bacteria entered the stationary phase in the solution containing 10 mM of carbon and energy. However, after 42 hours bacteria had reached the death phase, indicating an accumulation of the toxin.

3.3 16S rRNA Polymerase Chain Reaction

Figure 4 of the Promega® 1Kb DNA ladder shows the varied sizes of the ladders that were used. Extraction of bacterial DNA and universal primers were used to perform PCR. We used "Forward Primers" and "Reverse Primers" from 1st Base Company in this process. Figure 4 shows the results of gel electrophoresis, which was used to track the progress of the PCR reaction. According to this Figure, the DNA ladder in lane 1 had a band of

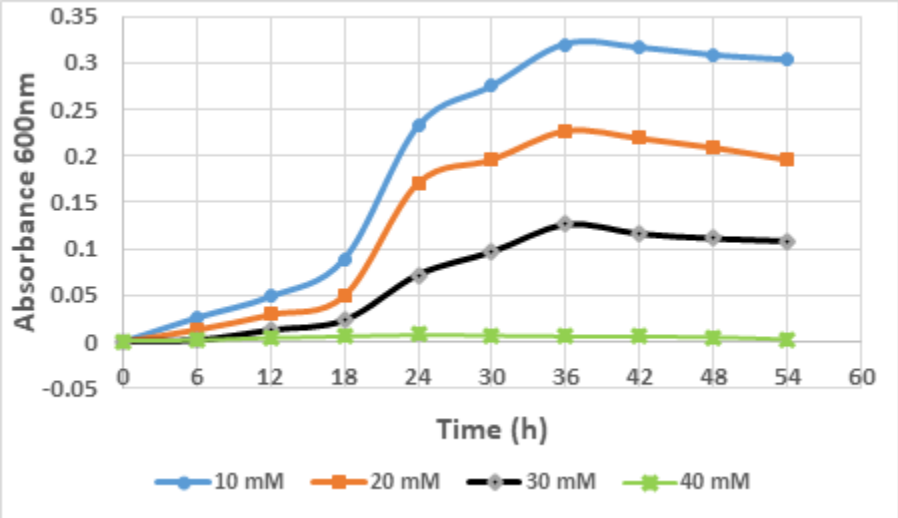

Fig. 2. SW2 strain growth profile at various concentrations.

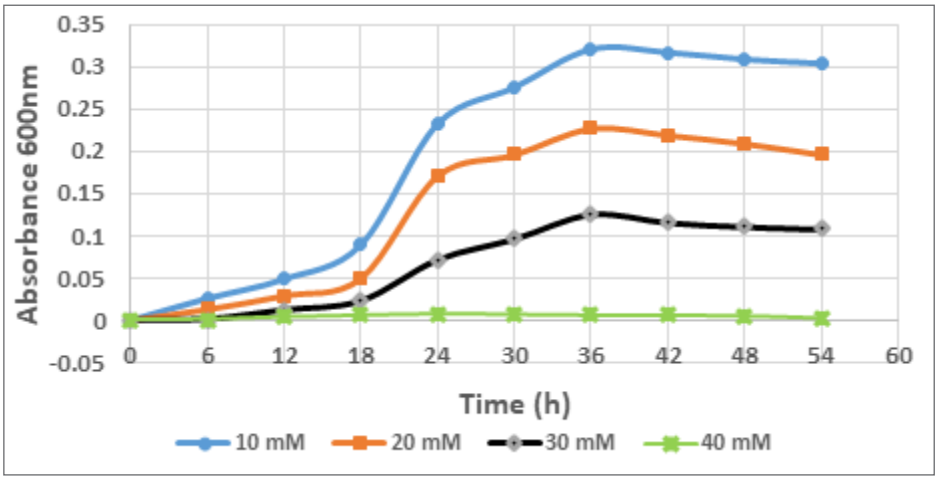

Fig. 3. Correlation between SW2 growth and chloride ion in 10 mM MCA.

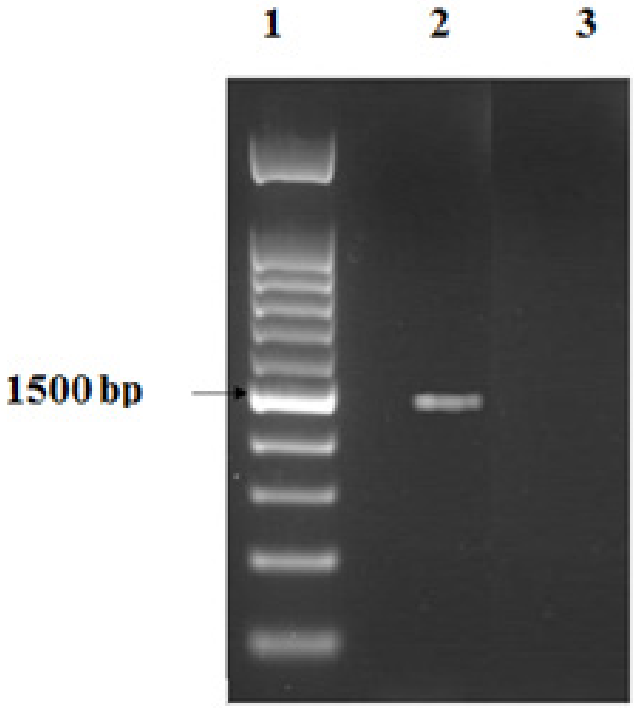

Fig. 4. Electrophoresis on 1 % agarose gel of DNA amplified with 16S rDNA universal primers. Lane 1: M, 1kb ladder, Lane 2: sample of SW2, and Lane 3: Negative control.

around 1500bp while the controls (PCR reactions without the use of forward or reverse primers) were found in lane 5 and 6.

3.4. 16S rRNA Gene Sequencing and Analysis

To sequence our gene of interest, the PCR product had been submitted to the 1st base firm. It was received by email in ".ab1" format for both the forward and reverse sequences, with the results. The signal quality of the sequence result was determined and analyzed using a chromatogram viewer. The low-quality bases were eliminated from both forward and reverse sequences.

It was then used to align forward and reverse sequences, removing the overlapping region between them, in order to retrieve the complete length of the 16S rRNA gene. It was at this point that the total length of our sequence was 1433 base

pairs, which we then used in NCBI's BLAST to compare it with other sequences and determine the similarity percentage. The percentage of similarity between our sequence and the BLAST result is shown in Table 1.

The 16S rRNA gene sequence is conserved in all studied bacterial species. In addition, this served as a target for identifying the bacteria. Before the PCR reaction, the DNA of the direct wild-type bacteria is extracted. Based on the BLASTn results, it was determined that *Pseudomonas* sp. and bacterium SW2 share 96 % sequence identity. As shown in Table 1, the NCBI database's top five species are compared to the BLASTn search results with significant alignments.

3. 5 Phylogenetic Analysis

ClustalW (version 5.2.2) is used to align the 16S rRNA gene sequences of strain SW2 with the nucleotide sequences from the Genbank database (NCBI) [17]. When using BLASTn, all of the identical bacteria are used to build the Neighbor-joining phylogenetic tree. *Pseudomonas* sp. SW2 has been identified as the bacterium SW2.

It was acknowledged by Towner and Cockayne [19] that the molecular approach has long been used to discover new species. Because it is found in all bacteria, the 16S rRNA gene sequence is an excellent tool for identifying bacteria. Aside from that, it is an extremely accurate procedure that is both reliable and reproducible [20]. Nucleotide sequence variations can be used to identify the most specific type of microorganism.

For phylogenetic analysis, bacterial species from the same isolate family were chosen. The 16S rRNA sequence for this species has been gotten from the database found in NCBI and aligned using the CLUSTAL W tool in MEGA version 5.2.2 software. A phylogeny tree of neighbor-junction with bootstrap value has been observed (Figure 5), and it suggests that the bacterial individual SW2 is closely designated as *Pseudomonas.*

Table 1. Sequences similarity with SW2 strain from the NCBI database.

Microorganisms Description	Accession Number	Maximum Score	Maximum Identity (%)
Pseudomonas aeruginosa strain MS14403	CP049161.1	1088	96 %
Pseudomonas aeruginosa strain WG-36	MN793065.1	1088	90 %
Pseudomonas aeruginosa strain SP4527	CP034409.1	1088	96 %
Pseudomonas aeruginosa strain FZD1	MK493327.1	1088	92 %
Pseudomonas aeruginosa strain TO4	MH458773.1	1088	92 %

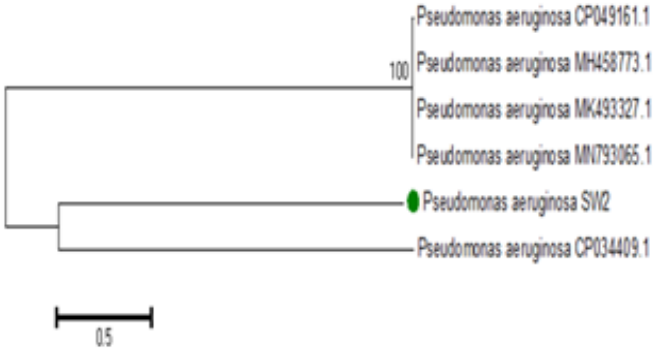

Fig. 5. Phylogenetic tree of the SW2 strain created using the neighbor-joining method and bootstrap values using MEGA software.

4. CONCLUSION

According to the study's findings, *Pseudomonas* sp. SW2, a soil-isolated bacterium that thrived in the minimum medium of 10, 20, and 30 mM, had a high capacity for degrading MCA. As an environmentally benign bacterial strain that produces the dehalogenase enzyme, it can be helpful as a bioremediation agent for detoxifying xenobiotic chemicals by MCA degradation.

5. CONFLICT OF INTEREST

The authors declared no conflict of interest.

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