Bioremediation of Organic Contamination Using Molecularly Identified Bacillus Subtilis Bacteria

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Abstract: Soil bioremediation primarily depends on the enzymatic activity of living microorganisms, to facilitate the degradation of contaminants or their conversion into less dangerous forms. Ten soil samples were collected from different locations in the city of Sharqat and the Rumana area of Qayyarah district. The Physical (concentration and Electrical Conductivity) and chemical tests (pH) conducted on soil samples. The *B. subtilis* were cultured and identified by Gram stain and VITEK2 compact system, also DNA extraction and Genome sequencing of *16S RNA* ribosome gene of bacteria was carried out to complete the diagnosis of *B. subtilis. Bacillus subtilis* is a characteristic bacterium that is rod-shaped. When cultivated on standard nutritional agar, the shape of this bacterium's circular colony is rough, opaque, fuzzy white or slightly yellow, exhibiting jagged edges. Sequencing revealed that most of the isolated strains were *B. subtilis*. The result of PCR showed that U1 gene has found in 100% of the *B. subtilis* isolates. BLAST results show 98% nucleotide sequence similarity and similarity for the *16RNA* ribosomal gene of *B. subtilis* with no gaps. The sequence for *16RNA* ribosomal gene was sub-mitted to NCBI, Gene Bank with accession No. KY962959.1. The alignment was done through Clustal Omega that recognized partial variations on some bases. The concentration of spontaneous wastes was decreased with bioremediation (3.864) for controlled temp. and (3.21) with un controlled temp. the Electrical Conductivity also decreased (3647) for controlled temp. and (2265) with uncontrolled temp. The pH increase in controlled temp. (8.699) and uncontrolled temp. (8.23). *B. subtilis* has a large efficiency in bioremediation process which had a benefit in ecosystem. It has a high effect in uncontrolled environmental condition(temperature) which lead to decrease the concentration of Spontaneous wastes and decease the electrical conductivity and increase the pH to more alkaline pH.

1 INTRODUCTION

Organic pollution of the environment is a critical concern that impacts the planet's soil, water, and air, presenting a substantial risk to ecosystems and living organisms, therefore there is an increased focus on formulating solutions to address environmental pollutants via physical, chemical, and biological methods. Conventional soil remediation techniques, including excavation, transportation to specialist waste facilities, incineration, stabilization, coagulation, or ion exchange, are complex, disruptive, and frequently lead to abrupt alterations in the soil's chemical, physical, and biological characteristics. Bioremediation provides an alternate approach by employing biological processes to detoxify, degrade, or convert contaminants into a harmless form [1], [2]. Bioremediation is typically used microorganisms, to remediate polluted environments[3]. This method offers several advantages over physical and chemical treatments, chiefly due to its lower cost and greater environmental sustainability. Furthermore, organic pollutants can be entirely mineralized or biodegraded into simple inorganic compounds, e.g. CO_2, H_2O, and Cl using bioremediation, while physical and chemical processes, such as vaporization, adsorption, and extraction, compromise soil structure and disrupt normal environmental processes, frequently merely relocating contaminants from one medium to another, for instance, from soil to the atmosphere [4]. Microorganisms are employed in bioremediation, an attractive, novel, cost-effective, and environmentally friendly method, to remove petroleum hydrocarbon pollutants from water or soil.

The predominant microorganisms employed in bioremediation include bacteria, fungi, and yeasts. Bacteria are reported to be the most effective microbial community in decomposing petroleum hydrocarbons[5]. Using the soil's native microbial populations' inherent capacity to biodegrade and remove pollutants, natural attenuation is a type of organic bioremediation.

The success of bioremediation was highly dependent on the type of soil, the pollution level, and environmental factors that includes pH, moisture level, temperatures, conductivity of electricity, and nutrient availability. Soil contamination samples must be defined and analyzed before biodegradation experiments may begin[6]. *Bacillus subtilis (B. subtilis)**:*** One species that is often used in bioengineering and biotechnology is *Bacillus subtilis (B. subtilis)***,** which is also available for purchase. The fact that *B. subtilis* can produce biofilms and has growth-promoting characteristics in plants suggests that it could replace fertilisers and pesticides made from petroleum [7]. It has been demonstrated that the matrix of biofilm of *Bacillus subtilis* mainly comprises three separate components, and that the formation of biofilm is controlled by complex regulatory processes.

The first part is an exopolysaccharide, which is both a signalling molecule and a moisture retainer in the biofilm. The non-wetting qualities of the biofilm are caused by the second component, the protein BslA. The biofilm's structural integrity, particularly its attachment to plant roots, is ensured by the third element of the matrix, the protein TasA, in conjunction with the accessory protein TapA [8]. *B. subtilis* have developed strategies such as active efflux or sequestration utilizing proteins or isolates genes, insoluble substances, via which they can resist, detoxify, or metabolize these contamination from soil and water samples [9].

2 MANUSCRIPT PREPARATION

2.1 Sample Collection

Ten soil samples were collected from different locations in the city of Sharqat and the Rumana area of Qayyarah district (see Fig. 1). This study is considered the first of its kind in this area because it is rich in organic pollutants, especially (hydrocarbons). The samples were collected from (November 2023 - June 2024), where the samples were taken from different depths ranging between (5-8 cm). The samples were collected in sterile plastic bags with information such as (sample number - date - size) written on them. They were transferred to the laboratory for the purpose of dilution and isolation of microorganisms present in this contaminated soil.

Figure 1: The locations for collecting soil samples.

2.2 Physical and Chemical Tests Conducted on Soil Samples

2.2.1 Measurement of Physical Characteristic

2.2.1.1 Temperature

A digital cooking thermometer was used to directly check the temperature while taking samples from the places and the temperatures measured by this device range between (-50-300°C).

2.2.1.2 Electrical Conductivity

The soil's electrical conductivity was assessed utilizing the suspension method (1:5). Subsequent to the preparation of the suspension and filtration, the electrical conductivity of the filter was measured using a conductivity meter, taking three measurements, and the average was calculated to determine the error rate following the calibration of the device with standard solutions**.** As for water samples, the ability of water to conduct electricity

was tested by immersing the electrode in water after placing it in special glass bottles for a minute until the reading was fixed, then it was recorded in microsemens/cm (APHA, 2003). The final value of conductivity was then extracted

2.2.2 Measurement Chemical Characteristic

The pH was measured using a Professional Benchtop pH meter type BP3001 and the device was pre-calibrated before measurement using solutions with a pH of (9-7-4) and tested immediately within the sampling time of the same day in the laboratory.

2.3 Preparation of Soil Samples for Microbiological Culture

One gram of soil contaminated with organic matter (hydrocarbons) was taken and sieve it using a sieve with a diameter of 2 mm. place the samples in 10 ml tubes. Place 9 ml of D.W in tube. In addition to the soil sample. The samples were mixed well using a Vortex mixed device for one minute to obtain the colony forming unit CFU/ml. Then, a series of successive decimal dilutions were made until we reached dilution number 10^6.

Then 0.1 ml of the 10^4, 10^5 and 10^6 dilutions were taken using a Micropipette and then spread on the following culture media: nutrient agar - MacConkey agar - potato dextrose agar - starch casein agar. They were spread on the surface of the culture media using a clean and sterile glass spreader. The plates were incubated for 24 hours at 37°C for bacterial growth.

2.4 Isolation and Identification of Bacteria From Soil

After preparing the series of dilutions in the previous paragraph in order to isolate and purify bacteria from soil , 1 ml of each dilution, which is 10^4-10^6-10^5, which we prepared from soil was transferred to the surface of the culture media, which are nutrient agar and MacConkey agar and blood agar Starch casein agar-, distributed by spreading method and the plates were incubated at a temperature of 37°C for 24-48 hours for bacteria [10].

2.5 Diagnosis of Isolated Bacteria

2.5.1 Microscopic Examination

A gram stained bacterial slides were examined using an oil lens with a magnification power of (100X) in light microscope, the shapes of the bacteria were identified and whether they were positive or negative.

Also the biochemical test was performed on the isolates and the diagnosis were confirmed by VITEK 2 Compact system.

2.5.2 DNA Extraction

The analysis kit provided by (mepep biosoence) was relied upon to extract DNA from samples of bacteria and filamentous bacteria.

2.5.3 Estimation of the Concentration and Purity of the Extracted DNA

The concentration and purity of the extracted DNA from the bacterial isolates belonging to the unknown bacteria were measured using the Nano-drop spectrophotometer device equipped by the BioDrop Company.

2.5.4 Electrophoresis of DNA in Agarose Gel

To transport and detect DNA, agarose gel is prepared at a concentration of 1%. To obtain this level of concentration, 0.35 g of agarose powder is mixed in 35 ml of Sodium Borate (S.B), and 3 microliters of red safe dye are included. This is accomplished by employing a heat source with constant agitation until boiling, then allowing it to cool to a temperature of 50-60 degrees. The gel solution is subsequently poured into the transport device's tray following the installation of a specialized comb to create wells at the gel's edges, ensuring that the pouring is executed gently to prevent bubble formation. If they develop, they are extracted using a pipette. The gel is allowed to solidify, after which the tray is positioned in the electrophoresis tank holding a suitable volume of X1 TBE solution. The comb is thereafter elevated with care. The transport samples are created by

combining 7 microliters of the DNA sample with 3 microliters of loading buffer. Subsequently, the relay device is activated by transmitting an electric current with a voltage differential of 5 volts/cm, and the procedure lasts between 1.5 to 2 hours. Subsequently, the gel is imaged under ultraviolet light utilizing a UV transilluminator to visualize the DNA bands and the outcome of the PCR reaction.

2.5.5 PCR Reaction Kits for Determining the 16srRNA Gene to Diagnose Unknown Bacteria

2.5.5.1 Primer

To determine the 16srRNA gene, a universal primer of bacteria was used in this study, prepared by the manufacturer in lyophilized form. To prepare the working solution, the primer was dissolved in nuclease-free water to obtain a concentration of 10 picomoles/microliter. The primer used in this study is shown in the following Table 1. The multiplication reaction conditions for PCR additives are summarized in Table 2.

Table 1: Sequence of the primer used in the study.

Primer	Sequence	Size bp
U1 Forward (21)	CCAGACTCCTACGGGAGGCAG	180
U2 Reverse (62)	CGTATTACCGCGGCTGCTG	

Table 2: Multiplication reaction of PCR additives.

No	Cycle number	Time	Temperature	Stage
1	1 cycle	5min	94	Initial denaturation
2	30 cycle	35min	94	Denaturation
3	30 cycle	1min	55	Annealing
4	30 cycle	1min	72	Extension
5	1 cycle	7min	72	Final extension

2.5.5.2 DNA Extraction from Agarose Gel

The bands obtained from the PCR reaction were excised from the gel for purification and subsequent nucleotide sequencing, based on various analyses conducted by the company. (Geneaid).

2.5.5.3 Nucleotide Sequence Determination of the Amplified Fragments Using DNA Sequencing

The nitrogenous base sequence of the samples was ascertained by dispatching the PCR reaction products, along with the primer from the resultant package, to North Korea, where the gene sequence was analyzed using the Hitachi 3130 Genetic Analyzer. The gene sequences were compared with those recorded in the National Center for Biotechnology Information (NCBI), and the results were assessed utilizing the BLAST program.

3 RESULTS AND DISCUSSION

3.1 Isolation and Identification of Bacillus Subtilis

The result of cultural growth with biochemical test and Vitek result revealed the species of *B. subtilis*. *Bacillus subtilis* is a characteristic bacterium that is rod-shaped. When cultivated on standard nutritional agar, the shape of this bacterium's circular colony is rough, opaque, fuzzy white or slightly yellow, exhibiting jagged edges, Figure 2. The results was presented in Figure 3, showed that that this bacterium belong to the Gram-positive class by morphological observations and was identified as Gram-positive *bacillus*, which can form spores.

Figure 2: Bacillus subtilis on nutrient media.

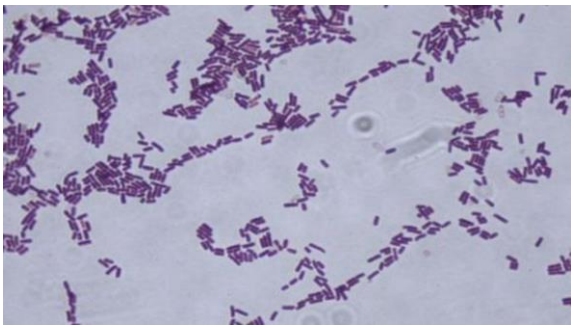

Figure 3: Gram positive B. subtilis under microscope.

3.2 Molecular Identification of B. Subtilis

The result of PCR showed that *U1* gene has found in 100% of the *B. subtilis* isolates as showed in Figure 4.

The number and location of genetic mutations of the *U1* gene (180 bp) in isolate of *B. subtilis* was showed in Figure 5. The gene was registered at the American International Bank of Genes NCBI under the serial number (KY962959.1). Sequencing revealed that most of the isolated strains were *B. subtilis* (Fig. 4). BLAST results show 98% nucleotide sequence similarity and similarity for the 16RNA ribosomal gene of *B. subtilis* with no gaps (Fig. 6). The sequence for 16RNA ribosomal gene was sub-mitted to NCBI, Gene Bank with accession No. KY962959.1. The alignment was done through Clustal Omega that recognized partial variations on some bases.

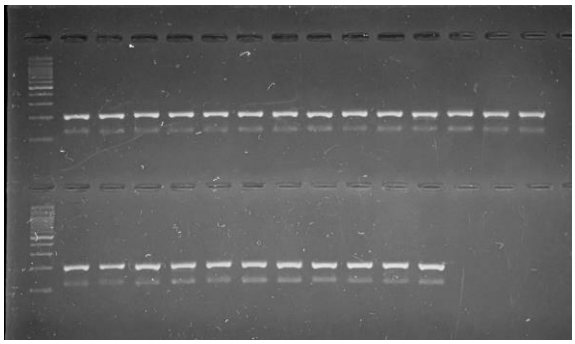

Figure 4: PCR products for the amplified product of U1 gene in B. subtilis after electrophoresis isolates on agarose gel (1%) at 5 V/cm for 60 min. M: DNA ladder marker; Lane (1-25): B. subtilis isolates.

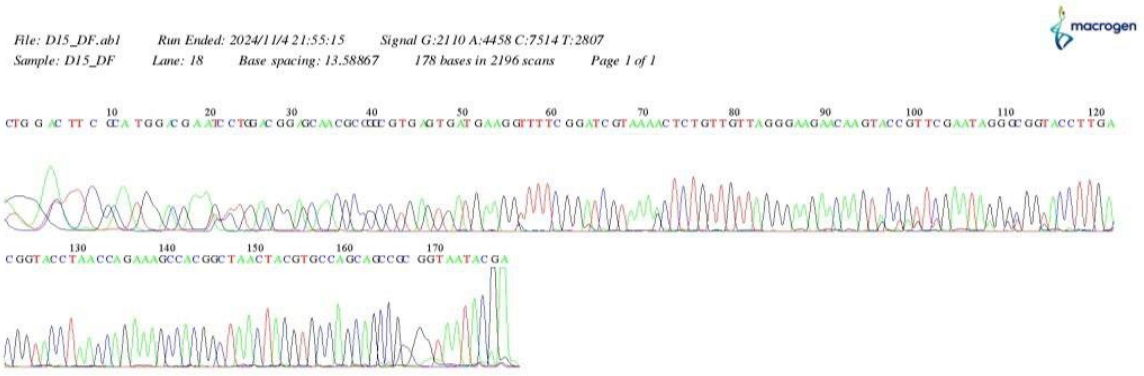

Figure 5: DNA sequencing of B. subtilis gene.

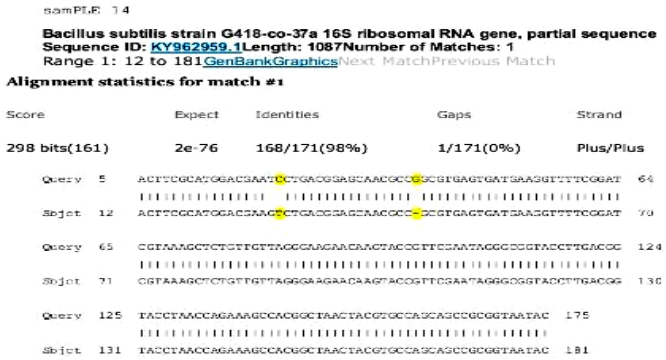

Figure 6: Confirmation of *B. subtilis* strain 16SrRNA gene sequence through BLAST.

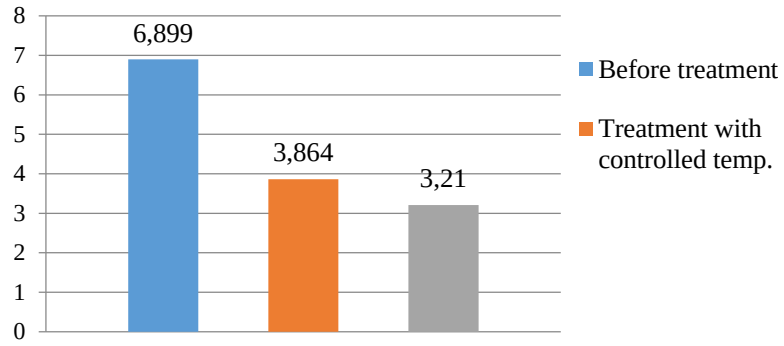

Figure 7: Organic contamination concentration during bioremediation with B. Subtilis.

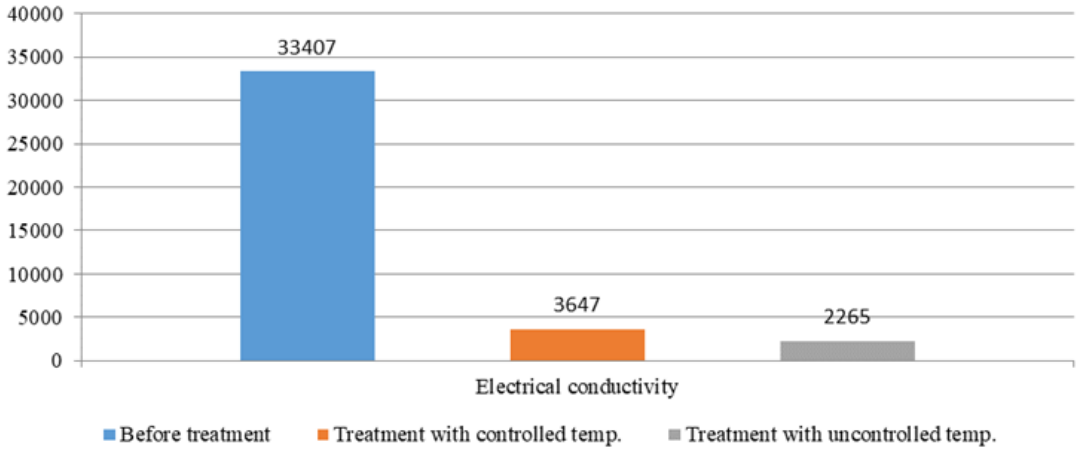

Figure 8: Electrical conductivity during bioremediation with B. Subtilis.

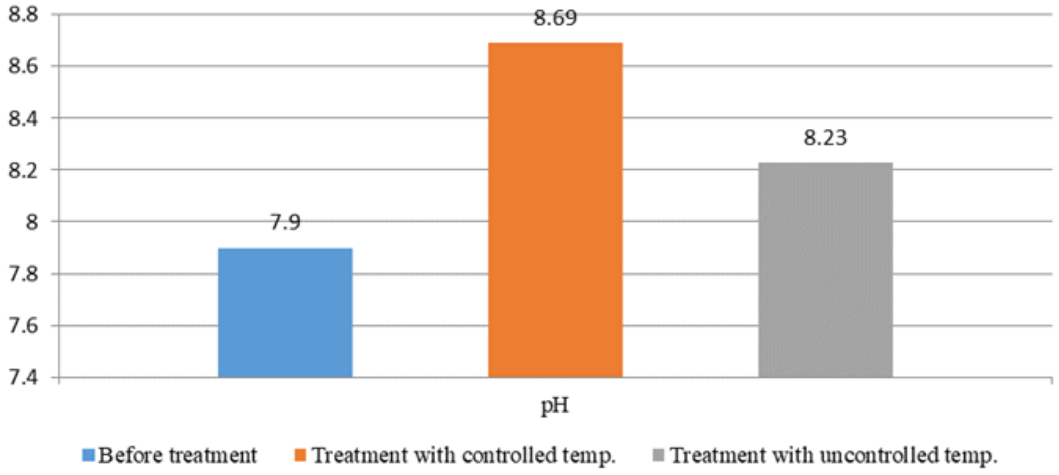

Figure 9: pH during bioremediation with B. Subtilis.

3.3 Bioremediation with B. Subtilis

The bacterium were used for spontaneous wastes bioremediation, the process were carried out in two condition, controlled temperature and uncontrolled temperature. The concentration of spontaneous wastes were decreased with bioremediation 3.864 for controlled temp. and 3.21 with un controlled Temp. Electrical Conductivity also decreased 3647 for controlled temp. and 2265 with uncontrolled temp. The pH increase in controlled temp.8.699 and uncontrolled Temp.8.23, which mean that bacteria has an effective role in bioremediation, Figures 7, 8, and 9.

Bioremediation has garnered significant scholarly attention over the past decade due to the superior environmental sustainability and cost-effectiveness of microorganisms compared to physical and chemical approaches for the removal of pollutants from soil and water samples [11]. Many studies have proven that most types of bacteria, such as Pseudomonas aeruginosa, Bacillus subtilis, and Bacillus melanogaster, are effective in decomposing many dangerous pollutants.

Bacillus subtilis emerged as the most extensively researched species within the Bacillus genus owing to its inherent competence for the uptake of extracellular DNA, which enables straightforward genetic manipulation and the occurrence of sporulation, one of the earliest investigated processes of bacterial cell development [12]. The current investigation identifies *B. subtilis* as gram-positive rods, characterized by a rough, opaque, fuzzy white or slightly yellow appearance with jagged edges on culture media [13].

The species of Bacillus spp. most well characterized for their bioremediation capability are *B. subtilis*, *B. cereus*, and *B. thuringiensis*. This bacterium genus employs many bioremediation techniques, such as biosorption, EPS-mediated biosorption, bioaccumulation, and bioprecipitation. Consequently, Bacillus spp. strains can diminish the concentrations of metals in the environment. Furthermore, Bacillus spp can promote growth of plants and enhance the bioaccumulation of pollutants in soil, making them one of the more effective sustainable alternatives for pollutant remediation in diverse habitats, especially soil. This makes them a promising candidate for phytoremediation [14].

Molecular detection of Bacillus species has only been documented in a few studies that have reported amplification, sequencing, and sequence analysis of the 16S rRNA gene [15], [16]. In their groundbreaking investigation, Geetha et al. [17] successfully used a sequencing analysis of the 16S rRNA gene to identify and categorize seven native strains of *B. subtilis* based on their taxonomy. We were able to identify the Bacillus species and two more subspecies using this molecular technology. Some problems have emerged in the Bacillus generation, despite the *16S rRNA* gene sequence's great success in bacterial species identification [18].

By comparing twenty-six strains having *16S rRNA* sequences with forty-eight incomplete Bacillus subtilis sequences from GenBank, Sabie et al. were able to build a phylogenetic tree. While the GenBank sequences for these 26 isolates ranged from 1153 to 1559 nucleotides in length, the in silico study showed that their lengths varied from 995 to 1233 nucleotides. The percentage of GC content ranged from 55% to 56% across all 68 sequences of *16S rRNA* genes [19].

Akinsemolu *et al*. [20] emphasised the potential importance of *B. subtilis* in environmental cleanup, in addition to its significant contributions to biotechnology, agriculture, and medicine. The more we learn about *B. subtilis*, the more important its role becomes in finding long-term answers to global problems.

Elenga-Wilson *et al*. [21] proven that Bacillus subtilis have remarkable bioremediation capabilities, particularly in the metabolism and destruction of various organic pollutants. Hydrocarbons, PAHs (polycyclic aromatic hydrocarbons), pesticides, and industrial chemicals are all part of this category. Decomposition and conversion of these pollutants into less hazardous compounds are made possible by *B.subtilis*'s extensive metabolic capabilities. Restoring and reviving ecosystems that have been polluted need it, thus.

JALILZADEH et al. [22] proved that *B. subtilis*' biodegradative potential stems from its capacity to create several biosurfactants, enzymes, and biodegradative chemicals. By utilising their complex metabolic and enzymatic interactions, *B. subtilis* is able to break down complex organic pollutants into their smaller, less dangerous components. The waste treatment and management processes are greatly improved by *B. subtilis*. A bacterium's enzymes may break down even the most complicated chemical compounds into simpler, less harmful ones. When these two processes work together, they reduce garbage volume and eliminate unpleasant odours. Using *B. subtilis* improves the efficiency and longevity of waste management processes by encouraging the creation of

ecologically friendly methods of trash disposal and recycling [23].

Soil type of soil, pH, and temperatures, the presence of oxygen or other electron acceptors, nutrients, and the presence of microbial populations that are good at degrading pollutants are some of the environmental variables that have a significant impact on the control and enhancement of biological treatment processes. Another factor is the accessibility of pollutants to these communities [24].

B.subtilis is more effective in bioremediation when exposed to unregulated temperatures, according to the present study. Sakthipriya et al. [25], showed that temperatures, salinity, and pH, affected the stability of the biosurfactant made by *B. subtilis* for improved oil recovery, therefore our results are in line with theirs.

4 CONCLUSIONS

The present study demonstrated the high efficiency of Bacillus subtilis in bioremediation of organic contaminants from soil environments with hydrocarbon pollution. The bacterium was successfully isolated and molecularly identified through morphological characteristics, biochemical tests, VITEK2 system, and 16S rRNA gene sequencing, with confirmed identification (NCBI accession: KY962959.1). B.subtilis has a large efficiency in bioremediation process which had a benefit in ecosystem. It has a high effect in uncontrolled environmental condition(temperature) which lead to decrease the concentration of organic contamination and decease the electrical conductivity and increase the pH to more alkaline pH. The study highlights that *B. subtilis* performs well even in fluctuating environmental conditions, making it a reliable candidate for large-scale bioremediation projects, particularly in hydrocarbon-contaminated zones. Its ability to produce biofilms and biosurfactants further enhances pollutant degradation efficiency while contributing positively to the ecosystem.

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