STM JOURNALS

International Journal of
Pathogens

Volume 1, Issue 2, 2024
July—December
DOI (Journal): 10.37591/IJPG

https://journals.stmjournals.com/ijpg/

Research IJPG

Bioremediation Potential: Discovering Naphthalene-Degrading Microbes in Iraqi Contaminated Soil

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Abstract

A polycyclic aromatic hydrocarbon, napthalene is an environmental contaminant with a slow rate of disintegration. Biological treatment by microbes present in contaminated places is the most important of the several approaches applied to remove it from the environment. At depths of 5 to 10 cm, soil samples were collected from areas polluted by oil near the Al-Dora and Sheikh Omar refineries. Nutrient agar was cultured with 0.1 ml of each dilution, which ranged from 10-1 to 10-8. Twenty separate bacterial colonies were isolated and cultured on petri dishes using Bushnell Haas Media agar with naphthalene as the sole carbon and energy source. For seven days, the cultures were kept in an incubator set at 37°C. Two dishes were subsequently examined to determine the locations of the growth. Two separate cultures were grown in liquid Bushnell Haas Media and then incubated in a shaker incubator for seven days at 37°C and 150 rpm. Next, High-Performance Liquid Chromatography (HPLC) was employed to measure the amount of naphthalene degraded by these microorganisms. The most effective bacterial isolates were identified using the VITEK 2 technology. The naphthalene degradation rates of Serratia ficaria and Bacillus subtilis were rather high; Serratia ficaria at 85.16 percent and Bacillus subtilis at 93.36 percent, respectively.

Keywords: Naphthalene, Degrading, Bacteria, Pollution, soil

INTRODUCTION

Naphthalene is one of the polycyclic aromatic hydrocarbons (PAHs) and a ubiquitous pollutant [1]. Naphthalene (NAP) is an organic compound with the formula $C_{10}H_8$ [2]. Naphthalene is a white, volatile solid that emits a strong mothball odor when in contact with flames. It is sourced from fossil deposits and generated from incomplete combustion, and it is used to synthesize phthalic anhydride and also as a moth repellent [3].

Investigations into the degradation of naphthalene are important due to its widespread presence as a contaminant and its usefulness as a representative chemical for studying the degradation of polycyclic aromatic hydrocarbons (PAHs) structure shown in Figure 1 [4]. Naphthalene, as the initial member of the PAH group, is a prevalent micropollutant found in drinking water, and its toxicity has been extensively recorded [5]. Naphthalene is a constituent of crude oil and is present in fuels and consumer goods made from petroleum. Mothballs are the primary application of naphthalene in consumer goods. Ingesting naphthalene can cause both immediate and delayed toxicity, primarily affecting the blood and eyes as the main target organs of toxicity [6].

Naphthalene is mostly utilised in the manufacturing process of phthalic anhydride. People can come into contact with naphthalene by using mothballs. Workers may face exposure to

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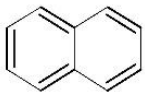
Received Date: June 03, 2024
Accepted Date: July 12, 2024
Published Date: August 01, 2024

Citation: Zahraa M. Abdulhussein, Amal A. Hussein. Bioremediation Potential: Discovering Naphthalene-Degrading Microbes in Iraqi Contaminated Soil. International Journal of Pathogens. 2024; 1(2): 26–35p.

naphthalene during its production and use, especially in processes like coal tar manufacturing, wood preservation, leather tanning, and the production of inks and dyes. Naphthalene is emitted into the atmosphere by the combustion of coal and oil, as well as through the utilisation of mothballs [7].

The United States Environmental Protection Agency (US EPA) has classified these compounds as priority pollutants in natural resources. Nevertheless, a diverse array of microbes have the ability to gradually break down these compounds [8–10]. The environment recognizes volatilization, photooxidation, chemical oxidation, sorption, leaching, and biodegradation as the six main mechanisms of dissipation. The dispersion of PAHs is primarily attributed to microbial degradation [11]. Consequently, an increasing amount of study is focusing on the biodegradation of PAHs. Certain bacteria have the ability to use PAHs as a carbon and energy source. This enables them to break down PAHs into carbon dioxide and water, or convert them into other chemicals that are not harmful or have low toxicity levels [12]. Biodegradation is regarded as a cost-effective and environmentally benign option for eliminating PAHs, in comparison to other processes such combustion, photolysis, landfill, and ultrasonic decomposition [13].

It has been shown that the ability of microorganisms to degrade contaminants is not simply attributable to the addition of certain species but to the ability of individual strains to create suitable biosurfactants, establish tight biodegrading collaboration, and develop in the soil under environmental circumstances with minimal nutrients and water [14]. The objective of our investigation was to identify the most effective bacterial strains from two locations that may breakdown naphthalene and conduct a diagnosis on them.



Naphthalene
Figure 1. Structure of Napthalene.

MATERIALS AND METHODS

Collection of Samples

A total of twenty soil samples were gathered from the Al-Doura refinery located in Baghdad. The specimens were collected from a depth of 5-10 cm using sterilised zip-lock polythene containers, subsequently transferred to the laboratory, and stored at a temperature of 4°C.

Bacterial Isolation

After serial dilution, a sterile L rod was used to apply and evenly distribute 0.1 ml of a 10-5 dilution of soil suspension over the nutrient agar plates. Subsequently, the plates were incubated at 37°C for 24 hours, resulting in the formation of mucous colonies. Twenty of these colonies were selected for further analysis.

SCREENING OF BACTERIAL ISOLATES

Primary Screening (via Solid Media)

Haskell & Bushnell In order to help the bacteria adapt to absorb naphthalene, 1% glucose was added to the medium when it was being prepared. The medium was further thickened with agar-agar before being autoclaved at 121°C for 15 minutes to kill any bacteria. After that, we sterilise the naphthalene with a 0.45 Millipore filter and let the liquid cool. After that, it's added to the mixture as the sole carbon source. When the mixture is finished, it is poured onto plates and allowed to solidify. To observe the level of bacterial growth around each indentation, make indentations in the plate and fill them with bacterial broth. The ability of the microorganisms to degrade naphthalene will be shown by this.

Secondary Screening Analysis

Preparation of samples for secondary screening

Bushnell Haas Medium was prepared with the addition of 1% glucose to facilitate bacterial adaptation to naphthalene consumption. Agar-agar was then incorporated into the medium for solidification, followed by sterilization using an autoclave at 121°C for 15 minutes. Following the chilling process, naphthalene underwent sterilisation using a 0.45 Millipore filter and was subsequently introduced into the medium as the only carbon source. The media that had been produced were poured into plates and left to harden. Subsequently, wells were established within the plates, and each well was filled with bacterial broth to assess the extent of bacterial growth zone surrounding it, which serves as an indicator of the bacteria's capacity to break down naphthalene.

Secondary screening using High Performance Liquid Chromatography (HPLC)

The concentrations of naphthalene were determined before and after treatment with the bacterial isolate using High-Performance Liquid Chromatography (HPLC) according to [15], under the conditions outlined in Table 1.

Table 1. HPLC analysis conditions.

Pump model	**S 2100 Quaternary Gradient Pump**
The mobile phase	acetonitrile–water (70:30 v\v)
Column	C18-ODS
Column dimension	25 cm × 4.6 mm × µm
Detector UV	220 nm
Flow rate	0.8 ml/min
Sample volume	100 µl
Temperature	30°C

The following equation was utilized to determine the concentration of the compound within the plant:

$$C.\ sam = \frac{Cst * Asam}{Ast} * \frac{D\ .\ F}{Wt}$$

C. sam= concentration of sample, **Cst**= concentration of standard, **Asam**= area of sample, **Ast**= area of standard, **D.F**= dilution factor, **Wt**= weight of sample.

The percentage of Polycyclic Aromatic Hydrocarbons (PAHs) was computed using the equation provided below:

$$\mathbf{C_{(Sample)}} = \frac{\mathbf{C_{(Standard)}} * \mathbf{A_{(Sample)}}}{\mathbf{A_{(Standard)}}}$$

$$\textbf{Sample concentration percentage} = \frac{\mathbf{C_{(Sample)}}}{\mathbf{C_{(Control)}}} * 100\%$$

$$\textbf{Degradation percentage} = 100 - \text{Sample concentration percentage}$$

Conventional Diagnosis of Bacteria

The first diagnosis of the proliferating colonies of bacterial isolates was established through:

Microscopic Examination

An analysis was conducted on the reaction pattern of bacterial cells using Gramme staining. This involved examining the form, Gramme stain reaction, and arrangement of cells, as outlined in reference [16].

VITEK 2 Compact Identification System

The isolated specimens designated for identification were cultivated on Brain Heart Agar (BHA) plates and subsequently placed in an incubator set at a temperature of 37°C for a period of 18-24 hours. The technique for setting up the Test Card is outlined in reference [17]:

1. The inoculum was derived from a culture that was completely free of contaminants.
2. Using sterile technique, a volume of 3.0 ml of a sterile saline solution containing 0.45% to 0.50% NaCl and with a pH ranging from 4.5 to 7.0 was transferred into a transparent plastic test tube made of polystyrene, measuring 12 mm in diameter and 75 mm in length. The turbidity was calibrated and quantified using a turbidity metre called the DensiChek.
3. In step 3, a sterile swab or applicator stick was employed to transfer an adequate quantity of colonies that have similar physical characteristics to the saline tube generated in step 2. A uniform mixture of organisms was created by changing its density to the suitable McFarland standard using the VITEK 2 Densi CHEK plus.
4. For the AST-GP cards, 280 µl of the suspension made in step 3 was added to a second tube that held 3.0 ml of saline. After that, a susceptibility card and the tube were placed into the cassette. The tube carrying the initial bacterial suspension can also serve as a means to inoculate an identification card. The VITEK system was used to identify microorganisms by automated phenotypic approaches.

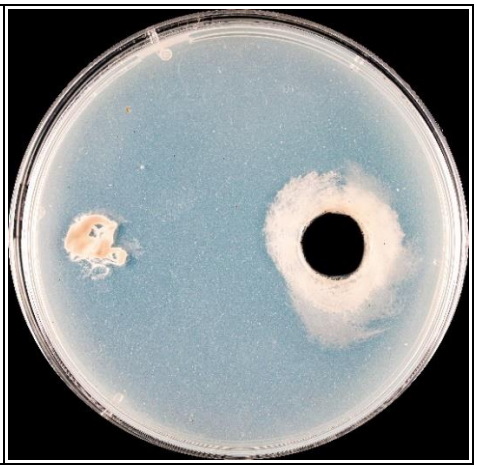
RESULTS AND DISCUSSION

Initial Screening via Solid Media

The bacterial isolates were incubated in wells containing solid Bushnell Haas Media with naphthalene as the sole carbon source at 37°C for 7 days, following the protocol outlined in reference [18]. This allowed for the measurement of the size of the growth zone around each well. The size of the growth zone serves as a suitable method to assess the efficiency of bacterial degradation of naphthalene, as indicated in Table 2.

Table 2. First screening.

Bacterial isolate code	PAH type	Growth zone size	Plate picture
Z1	Naphthalene	19mm	

Z3	Naphthalene	25mm	

The Z1 isolate exhibited a growth zone of 19 mm, while Z3 displayed a growth zone of 25 mm, indicating the capability of these bacterial strains to degrade naphthalene.

Secondary screening for the measurement of PAHs degradation was conducted using High-Performance Liquid Chromatography (HPLC)

Naphthalene

The naphthalene control sample showed a purity level of 100% according to the HPLC results. The purpose of this test is to verify the identity, composition, and purity of the polycyclic compound in the sample. Figure 2 shows that naphthalene has a retention time of 5.36, with only one peak number, indicating that it is pure. With a total of 1955.08 corrected areas, the standard solution concentration was 10 ppm. This number can be used as a benchmark to understand how well naphthalene-containing materials degrade. The HPLC results for the sample of naphthalene labeled as Z1 shown in Figure 3. The HPLC results for the sample of naphthalene labeled as Z3 shown in Figure 4.

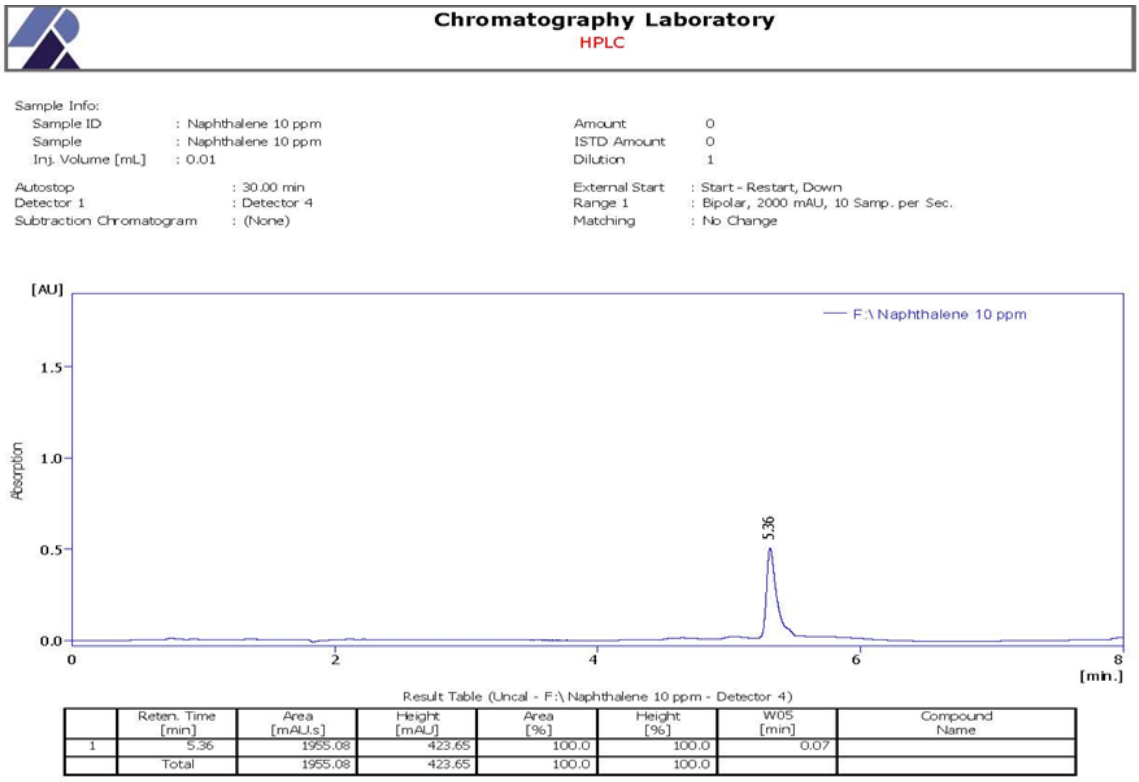
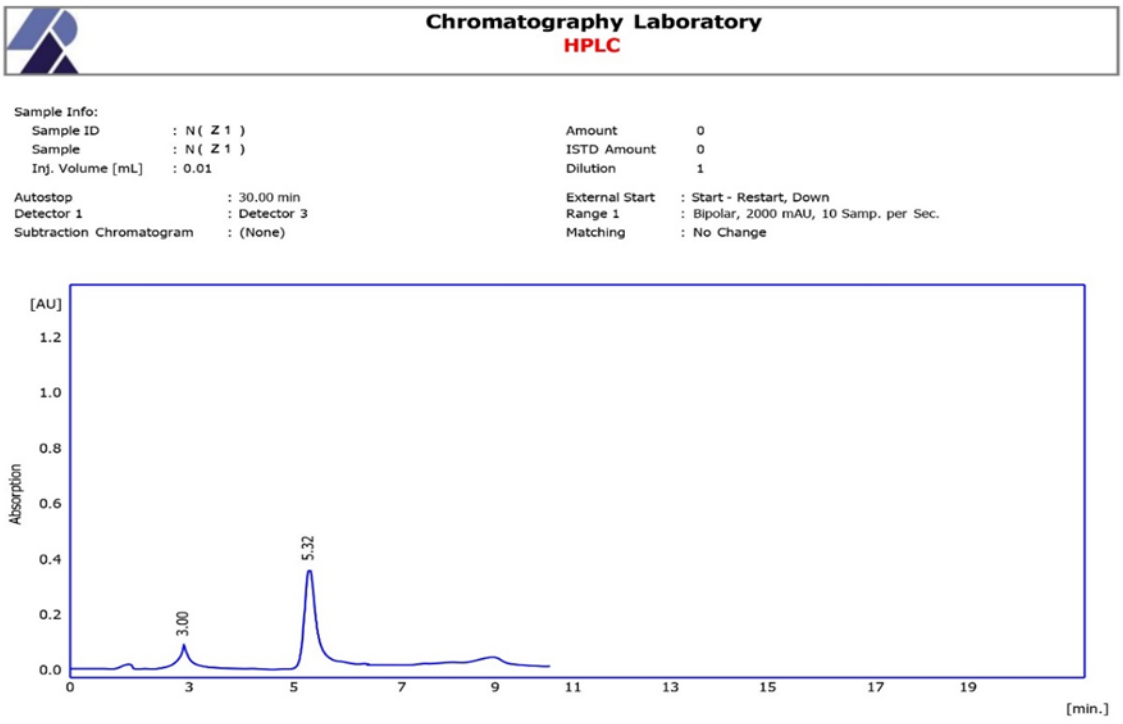


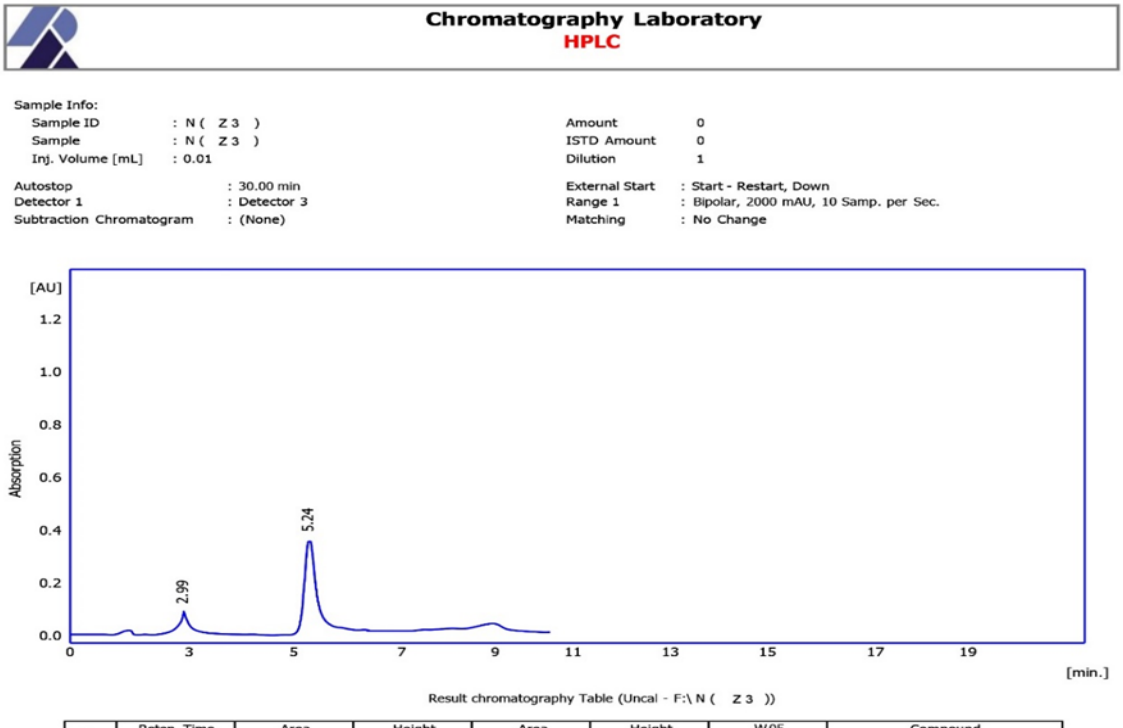
Figure 2. The HPLC results for the control sample of naphthalene.



Result chromatography Table (Uncal - F:\ N (Z 1)

No	Reten. Time [min]	Area [mAU.s]	Height [mAU]	Area [%]	Height [%]	W 05 [min]	Compound Name
1	3.00	99.15	32.58	10.00	10.00	0.06	
2	5.32	7256.25	422.69	90.00	90.00	0.18	
	Total	7355.40	455.27	100.00	100.00		

Figure 3. The HPLC results for the sample of naphthalene labeled as Z1.



Result chromatography Table (Uncal - F:\ N (Z 3))

No	Reten. Time [min]	Area [mAU.s]	Height [mAU]	Area [%]	Height [%]	W 05 [min]	Compound Name
1	2.99	84.59	38.58	10.00	10.00	0.06	
2	5.24	3256.25	325.69	90.00	90.00	0.18	
	Total	3340.84	364.27	100.00	100.00		

Figure 4. The HPLC results for the sample of naphthalene labeled as Z3.

The HPLC results indicated that the isolate Z1 degraded 85.16% of naphthalene, while the isolate Z3 degraded 93.36% of naphthalene.

During the subsequent screening process, bacterial isolates were cultivated in Bushnell Haas Media that contained polycyclic aromatic hydrocarbons (PAHs) as the only carbon source, which stimulated the degradation process. In addition, sterile solutions were prepared to serve as control solutions. The PAHs test was performed subsequent to the introduction of the degrading bacteria in order to ascertain the degree of degradation. The bacterial solutions were thereafter placed in a shaking incubator set at a temperature of 37°C and a speed of 150 rpm for a duration of 7 days, (shown in Figure 5) in accordance with the procedure described in reference[18].

Table 3. The percentage of PAHs degradation by bacteria.

PAH type	Bacteria Code	Degradation percentage
Naphthalene	Z1	85.16%
	Z3	93.36%

Isolate Z1 exhibited a naphthalene degradation rate of 85.16%, while Isolate Z3 demonstrated a naphthalene degradation rate of 93.36%.

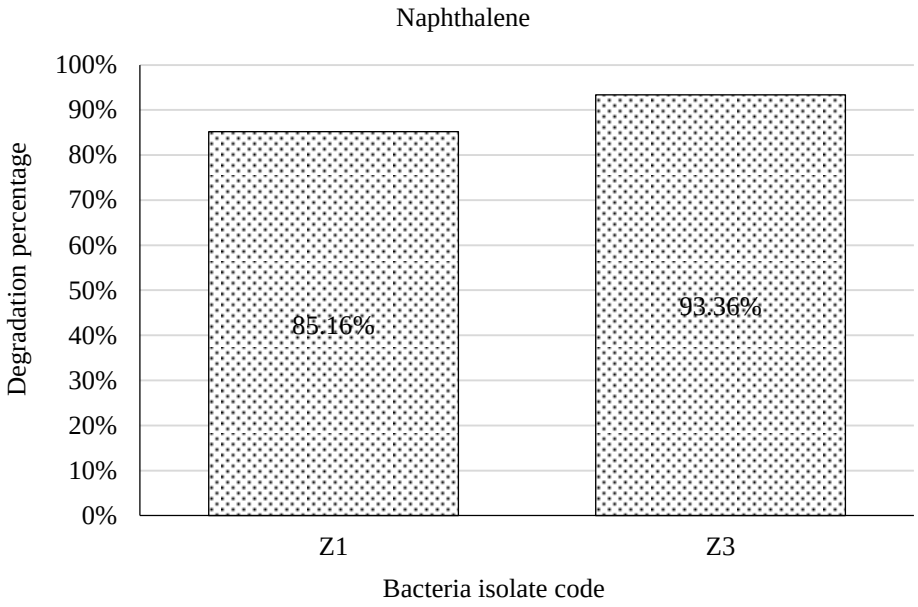


Figure 5. The rates of naphthalene degradation by the isolates over a 7-day period of shaking incubation at 150 rpm and 37°C.

Bacterial isolates identification

MacConkey Agar Testing

The MacConkey agar test demonstrated the proliferation of colonies originating from the Z1 isolate (identified as Serratia ficaria), thereby confirming its classification as a gram-negative bacterium. In contrast, the Z3 isolate (identified as Bacillus subtilis) did not exhibit any colony growth, suggesting that it is a gram-positive bacterium.

Microscopic Examination

Microscopic analysis verified the two effective bacterial isolates used in the PAHs experiment. Figures 6 and 7 show the results, and Table 4 summarises them, but the isolates were found to be gram-negative Serratia ficaria (Z1) and gram-positive Bacillus subtilis (Z3).

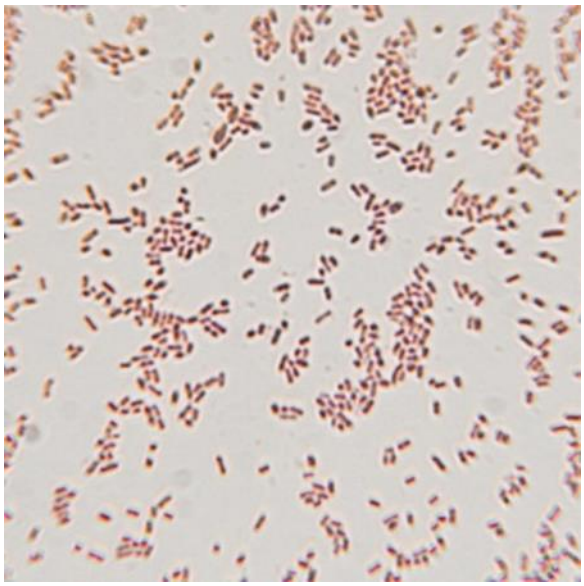


Figure 6. *Serratia ficaria* (Z1).

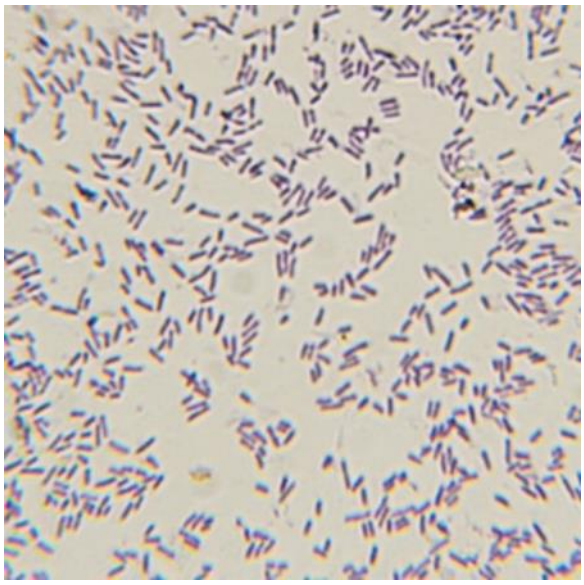


Figure 7. *Bacillus subtilis* (Z3).

Table 4. Results of Gram stain testing.

Name of Bacteria	Gram stain
Serratia ficaria (Z1)	-
Bacillus subtilis (Z3)	+

VITEK 2 Identification Testing

The bacterial isolates underwent identification for validation using the VITEK 2 system, which confirmed the conventional diagnosis. The two isolates were identified as Serratia ficaria with a likelihood of 94% and Bacillus subtilis with an 85% based on the data acquired using the VITEK 2 technology.

This study revealed that *S. ficaria* degraded naphthalene by 85.16%. Interestingly, there is limited prior research on the use of this strain of bacteria for naphthalene degradation. On the other hand, *Bacillus subtilis* demonstrated a naphthalene degradation rate of 93.36%. Previous studies, as

referenced in [19], reported naphthalene degradation rates of 70.5% by *Bacillus subtilis* in 7 days, 70.8% by E. coli in 28 days [20], and 75% by another organism [21].

CONCLUSIONS

Based on our study findings, we have concluded that *Serratia ficaria* degrades naphthalene by 86.60%, while *Bacillus subtilis* exhibited a degradation rate of 93.36%. Remarkably, both strains achieved these degradation rates within a relatively short period of time, specifically (7) days.

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