Original Article

Isolation and Identification of Phenanthrene Degrading Bacteria from Polluted Soil in Iraq

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Abstract

Phenanthrene is a one of polycyclic aromatic hydrocarbon, it's considered slow decomposition pollutants in nature, and therefore they are treated in several ways and removed from the environment. The most important of these methods is biological treatment by microorganisms that are already present in the areas of presence of these pollutants. Soil samples were collected from oil-contaminated areas of Al-Dora and Sheikh Omar refinery area with a depth of (5-10) cm and a series of dilutions from (10-1) to (10-8) were made for them, then 0.1 ml was cultured on solid culture medium (nutrient agar). Twenty bacterial colonies were isolated and then cultured inside wells in Bushnell Haas Media agar that contain Phenanthrene as the sole source of carbon and energy. They were incubated at a temperature of 37°C for 7 days, then the growth zone was observed on two dishes. The two isolates grown on liquid Bushnell Haas Media were incubated in the shaker incubator at a temperature of 37°C and 150 rpm for 7 days. Then it was examined by HPLC High-performance liquid chromatography to know the percentage of bacteria degradation of Phenanthrene. After this, the most efficient bacterial isolates were diagnosed with the VITEK 2 device. Serratia ficaria and Bacillus subtilis showed the high degradation rates for Phenanthrene. Serratia ficaria degraded Phenanthrene by 91.36%, and Bacillus subtilis degradation was 91.72%.

Keywords: Phenanthrene, Degrading, Bacteria, Pollution.

Introduction

Phenanthrene is a one of polycyclic aromatic hydrocarbons (PAHs), it's pollutants which are ubiquitous[1]. Phenanthrene with formula C14H10, Anthropogenic and pyrolytic activities are the main causes of a polycyclic aromatic hydrocarbon with three condensed rings fused in an angular form being widely disseminated in the environment. It is the simplest non-linear polycyclic aromatic hydrocarbon and is formed from the Latin words "phenos" and "anthrax," which stand for "benzene" and "coal," respectively. Two words, "phenyl" and "anthracene," are additional derivations of these Latin words [2]. Phenanthrene appears as colourless crystals [3]. The following signs and symptoms will appear after exposure to phenanthrene: skin irritation, respiratory irritation, dermatitis, cough, bronchitis, respiratory neoplasm, dyspnea, and kidney neoplasm [4].

Demonstrates no evidence of a connection between the risk of cancer in humans and animals [5]. Different organic substances, including insecticides, fungicides, detergents, dyes, and mothballs, have all been synthesized using phenanthrene [6]. They are extremely significant in the realm of medicine. Analgesic, antitussive, antimalarial, cytotoxic, and anti-constipation are only a few of the numerous therapeutic advantages of phenanthrene derivates.

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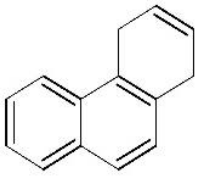
Phenanthrene serves as the fundamental nucleus in a number of opioid medicines and their derivatives, which are a potent class of analgesics from a pharmacological standpoint [7]. By examining trials on its derivatives, this study was conducted to highlight the many pharmacological applications of phenanthrene derivatives [8]. These compounds are prioritized pollutants in natural resources according to the United States Environmental Protection Agency (US EPA). But a large variety of bacteria slowly destroy these substances [9,10,11]. Volatilization, photooxidation, chemical oxidation, sorption, leaching, and biodegradation are the six basic methods of dissipation, or disappearance, in the environment. The main method used to dissipate PAHs is thought to be microbial degradation [12]. Thus, the biodegradation of PAHs is becoming a more popular area of research. Some microbes have the ability to use PAHs as a source of carbon and energy, which enables PAHs to be converted into other non-toxic or low-toxic compounds or degraded into carbon dioxide and water [13]. The elimination of PAHs via biodegradation is anticipated to be more cost-effective and environmentally benign than other physical and chemical techniques such combustion, photolysis, landfill, and ultrasonic decomposition [14]. Our study aimed to isolate most efficient bacterial strains from two sides that degrade Phenanthrene and diagnoses them.



Phenanthrene

Materials and Methods

Sample collection

Twenty soil samples were taken from Baghdad's Al-Doura refinery. Samples were collected with a depth of 5-10cm by sterilized zip-lock polythene containers and moved to laboratory and kept at 4°C.

Isolation of Bacteria

A sterilized L rod was used to spread 0.1 ml of a 10-5 dilution of the soil suspension over the nutrient agar plates after serial dilution. after a 24-hour incubation period. Mucous colonies developed on the plates at 37°C and 20 colonies taken to study on.

Screening of Bacterial Isolates

Primary Screening (By Solid Media)

Bushnell Haas Medium was prepared and glucose was added by 1% for adaptation of bacteria to consume Phenanthrene and agar-agar was added to the medium to solidify then sterilized by autoclave at 121°C for 15 minutes. Then, it is left to cool and Phenanthrene was sterilized by 0.45 Millipore filter and added to the medium as sole source of carbon the media was poured into plates and left to solidify. Make wells inside the dish and fill the wells with bacterial broth to see the size of bacterial growth zone around each well which indicates the ability of bacteria to degrade Phenanthrene.

Secondary Screening

Preparation of Secondary Screening Samples

Bushnell Haas Media broth was prepared in 100ml and sterilized by autoclave at 121°C for 15 minutes, after cool the Phenanthrene was added by 0.45 millipore filter for sterilization then separated into 50ml in two flasks each one inoculated with one bacterial strain based on primary screening results, these two flasks were incubated for 7 days at 37°C and 150rpm.

Secondary Screening by High Performance Liquid Chromatography (HPLC)

The concentrations of Phenanthrene, was calculated before and after treatment with bacterial isolate using (HPLC) according to (15), under the following conditions as shown in Table (1).

Table (1): Conditions of HPLC analysis.

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× 5 µm
Detector UV	220 nm
Flow rate	0.8 ml/min
Sample volume	100 µl
Temperature	30°C

The equation below was used to calculate the concentration of the compound in 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 PAHs was calculated using the equation below:

$$\mathbf{C}_{(\mathbf{Sample})} = \frac{\mathrm{C}_{(\mathrm{Standard})} * \mathrm{A}_{(\mathrm{Sample})}}{\mathrm{A}_{(\mathrm{Standard})}}$$

$$\textbf{Sample concentration percentage} = \frac{\mathrm{C}_{(\mathrm{Sample})}}{\mathrm{C}_{(\mathrm{Control})}} * 100\%$$

Degradation percentage=100-Sample concentration percentage

Conventional Diagnosis of Bacteria

The growing colonies of bacteria isolates were initially diagnosed depending on:

Microscopic Examination

The reaction pattern of bacterial cells with gram-stain had been studied. This include the examination of shape, Gram stain reaction, and arrangement of cells(16).

VITEK 2 Compact Identification System

The pure isolates to be identified were culture on Brain heart agar (BHA); the plates were incubated in a incubator at 37°C for 18-24 hrs. Test Card Setup procedure included(17):

1. Inoculums were prepared from a pure culture.

2. Aseptically transferred 3.0 ml of sterile saline aqueous (0.45% to 0.50% NaCl, pH 4.5 to 7.0) into a clear plastic test tube (12 mm × 75 mm) polystyrene test tube. The turbidity is adjusted and measured using turbidity meter called the DensiChek.

3. A sterile swab or applicator stick is used to transfer a sufficient number of morphologically similar colonies to the saline tube prepared in step 2. A homogenous organism is prepared by the suspension with a density equivalent to the appropriate McFarland standard using the VITEK 2 Densi CHEK plus.

4. In a second tube containing 3.0 ml of saline, transferred 280 µl of the suspension prepared in step 3 for AST-GP cards. Then place this tube in the cassette with a susceptibility card. The tube with the initial bacterial suspension could also be used for the inoculation of an identification card. Microorganisms were then identified by VITEK later, the tube with the initial bacterial suspension could also be used for the inoculation of an identification with the automated phenotypic method.

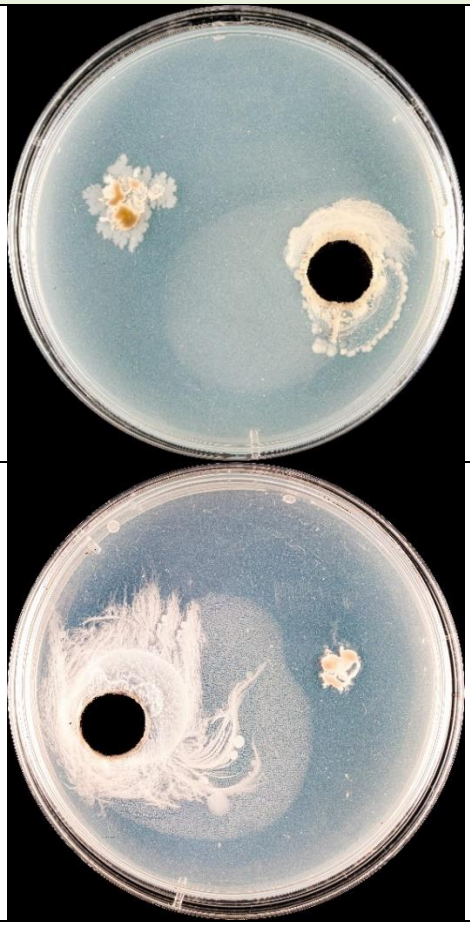
Results and Discussion

Primary Screening by Solid Media

The incubation of bacterial isolates is done in wells in solid Bushnell Haas Media with Phenanthrene as sole source of carbon at 37°C for 7 days according to the research(18) to measure the size of growth zone around each well. So, the size of growth zone is an appropriate method to measure the efficiency of bacterial degradation of Phenanthrene as shown in the Table (2).

The results showed that only two of the 20 plates showed the bacterial growth zone.

Table (2): First Screening.

Bacterial isolate code	PAH type	Growth zone size	Plate picture
Z1	Phenanthrene	21mm	
Z3	Phenanthrene	35mm	

Z1 isolate shows 21 mm growth zone and Z3 also shows 35 mm growth zone which means these bacterial steranes are capable of degrading Phenanthrene.

Secondary Screening Measurement of PAHs Degradation Using High Performance Liquid Chromatography (HPLC)

the HPLC result for control sample showed high purity by 100% for the compound. The test is used to confirm the type of sample, purity and chemical configuration of polycyclic compound. Figure (1) clarifies the retention time of Phenanthrene (8.077) with only one peak number as evidence for purity and standard solution concentration was (10 ppm) and corrected areas (658.888). The previous number is used as control number to explain the biodegradation efficiency of samples contain Phenanthrene.

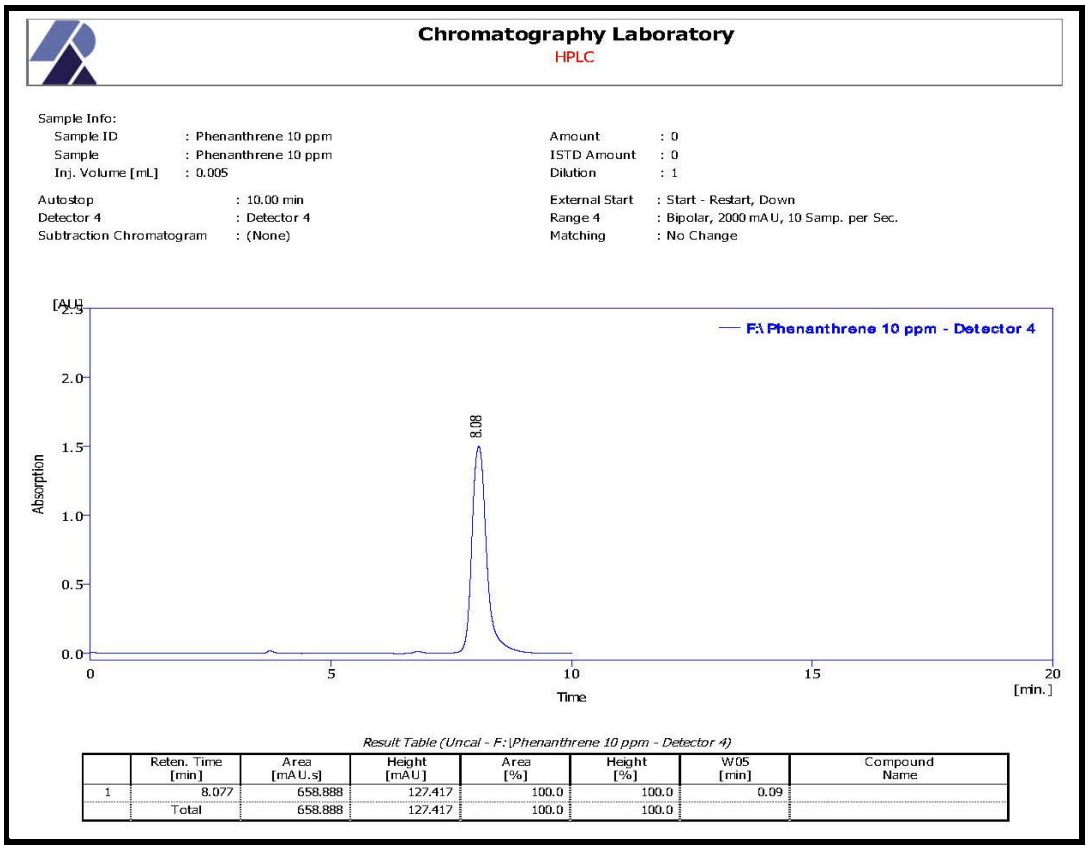
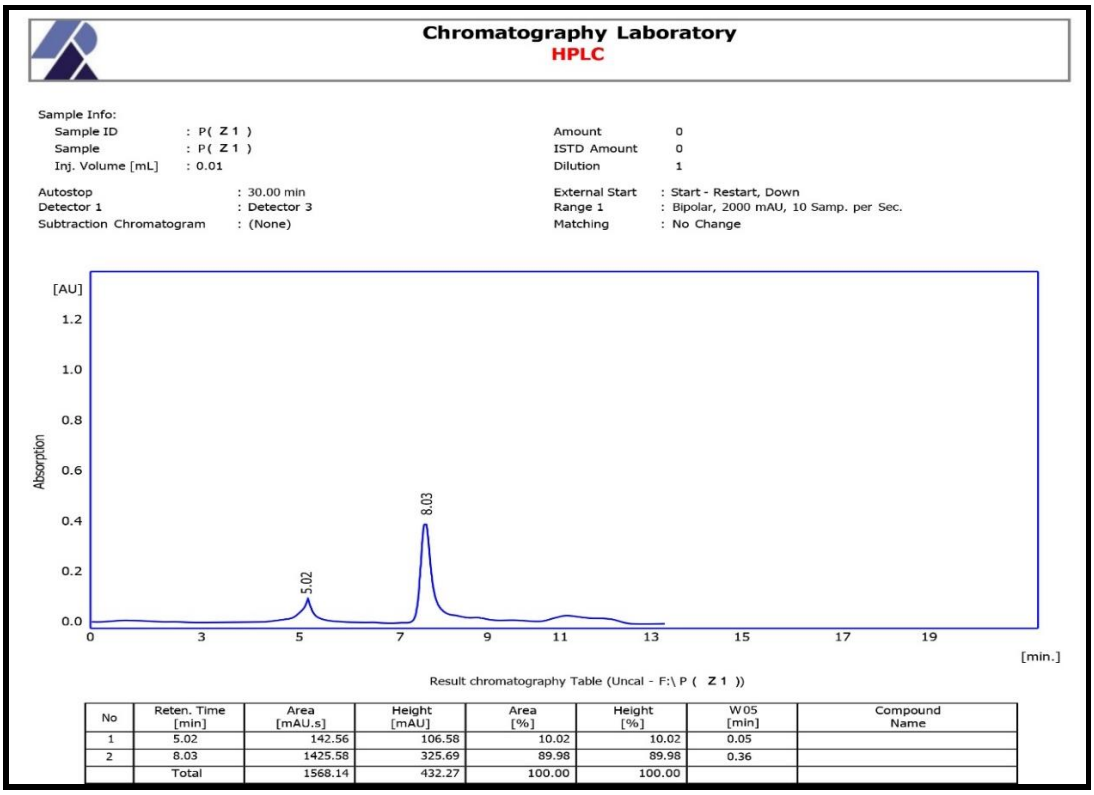
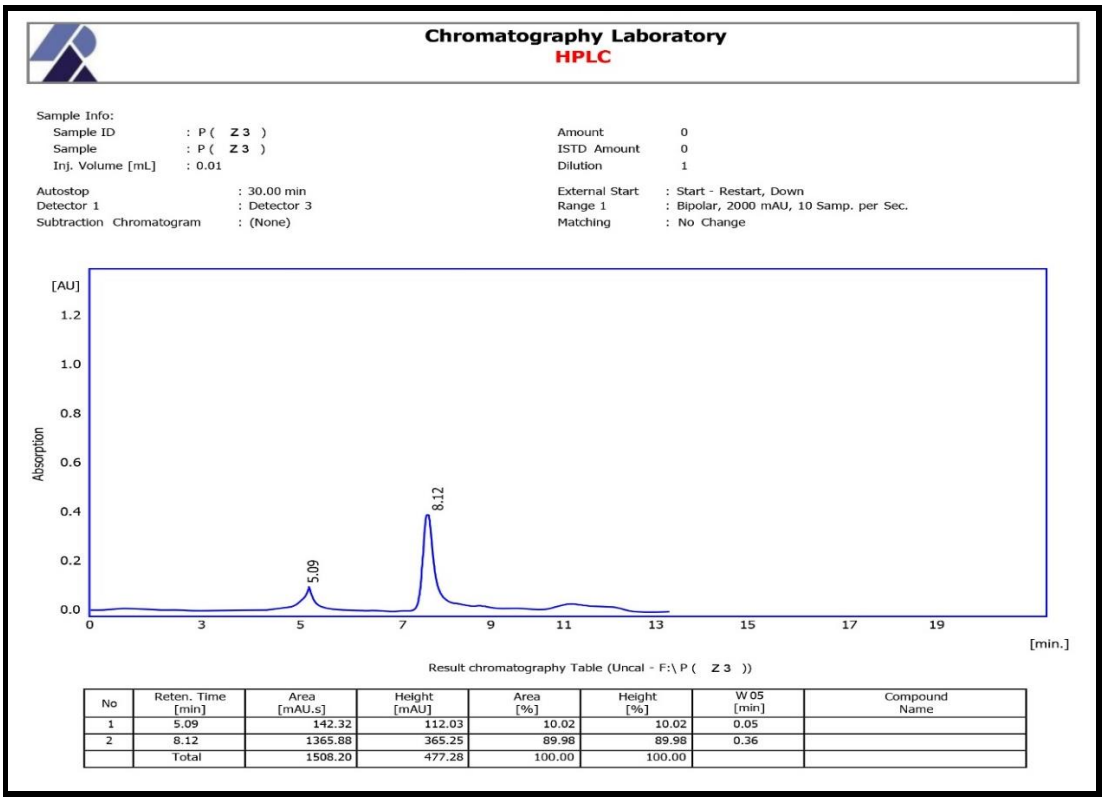


Figure (1): Phenanthrene control sample HPLC results.



No	Reten. Time [min]	Area [mAU.s]	Height [mAU]	Area [%]	Height [%]	W 05 [min]	Compound Name
1	5.02	142.56	106.58	10.02	10.02	0.05	
2	8.03	1425.58	325.69	89.98	89.98	0.36	
	Total	1568.14	432.27	100.00	100.00		

Figure (2): Phenanthrene Z1 sample HPLC results.



No	Reten. Time [min]	Area [mAU.s]	Height [mAU]	Area [%]	Height [%]	W 05 [min]	Compound Name
1	5.09	142.32	112.03	10.02	10.02	0.05	
2	8.12	1365.88	365.25	89.98	89.98	0.36	
	Total	1508.20	477.28	100.00	100.00		

Figure (3): Phenanthrene Z3 sample HPLC results.

The HPLC result indicated that the isolate (Z1) degrade (91.36%) of Phenanthrene.

The HPLC result indicated that the isolate (Z3) degrade (91.72%) of Phenanthrene.

Secondary screening was done by HPLC device, the bacterial isolates were cultured in Bushnell Haas Media containing PAHs as sole source of carbon to force bacteria to degrade them.

In addition creating bacteria-free solutions to be tested by HPLC as control solutions and PAHs test after adding the degrading bacteria to know the percentage of degradation, bacteria were added to the solution and incubated in the shaking incubator at a temperature of 37°C and 150 rpm for 7 days according to the research (18).

Table (3): Percentage of PAHs Degradation by bacteria.

PAH type	Bacteria Code	Degradation percentage
Phenanthrene	Z1	91.36%
	Z3	91.72%

Isolate Z1 showed degradation of Phenanthrene was 91.36% and Isolate Z3 showed Phenanthrene degradation by 91.72%.

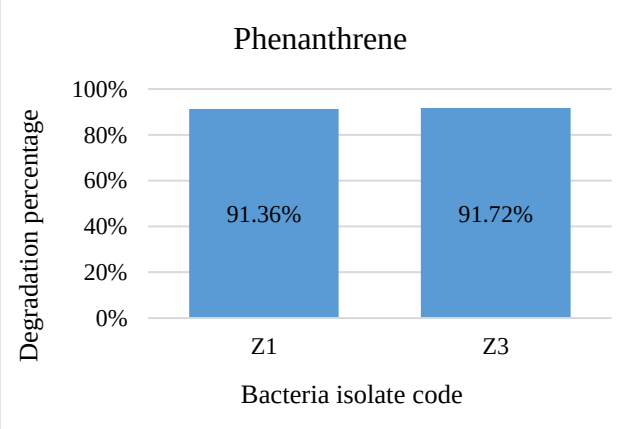


Figure (4): Rates of Phenanthrene degradation by isolates over 7 days of shaking incubation at 150 rpm and 37°C.

Identification of Bacterial Isolates

MacConkey Agar Test

The MacConkey agar test shows a growth of Z1 isolate (Serratia ficaria) colonies which means it is a gram negative bacteria. No growth of Z3 (Bacillus subtilis) colonies means it is a gram positive bacteria.

Microscopic Examination

The microscopic examination revealed the identity of the two efficient bacterial isolates that analyze PAHs, which are gram negative (Serratia ficaria (Z1)) and gram positive bacteria (Bacillus subtilis (Z3)) as shown in Figures (5) and (6) and Table (4).

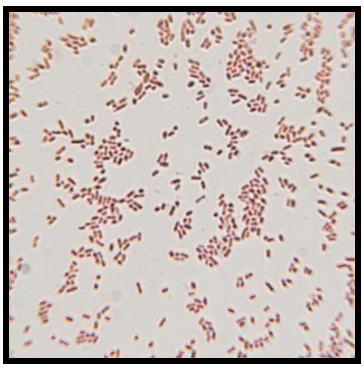


Figure (5): Serratia ficaria (Z1).

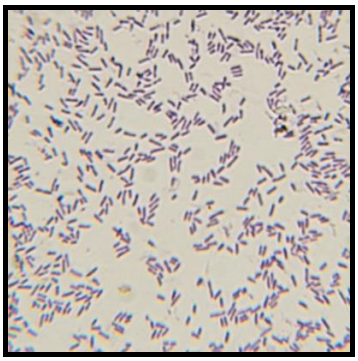


Figure (6): Bacillus subtilis (Z3).

Table (4): Gram stain test results.

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

VITEK 2 Identification Test

Bacterial Isolates have been identified for the validation by VITEK 2 which has been used to confirm the conventional diagnosis. According to the results from VITEK2 technique the two isolates were identified as Serratia ficaria (probability 94%) and Bacillus subtilis. (probability 85%).

This study found that S. ficaria was able to degrade Phenanthrene by 91.36% and we did not find any research that use this strain of bacteria to degrade Phenanthrene and Bacillus subtilis degrade Phenanthrene by 91.72% where in [19] study Bacillus subtilis degraded 24.8% phenanthrene by 7 days. Another study proved vibrio vulnificus showed degradation percentage of 84.23% of phenanthrene [20]. While in [21] research the phenanthrene degraded by 88.8% by P. aeruginosa with 28 days. Where in [22] research Rhodococcus sp. degraded phenanthrene by 31%. And in [23] research the phenanthrene was degraded by 75%

Conclusions

Through the study we concluded that S. ficaria degrade naphthalene by 86.60% and Bacillus subtilis degradation was 93.36% and it took short period of time (7 days), and the

cultivation of bacteria on a solid medium that contains phenanthrene as soule source of energy and calculating the growth zone size give a good idea of bacterial degradation of this compound.

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