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Exploring the frequency of isolated bacteria from oil and assessing their efficiency of polycyclic aromatic hydrocarbons PAHs compounds Biodegradation

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Abstract

One of the main methods for removing petrol and other hydrocarbon chemical pollutants from the environment is through the use of microorganisms that are already present in contaminated soils. Exploring the frequency of isolated bacteria from oil and measure the ability of modified bacteria to hydrocarbon degradation. Twelve bacterial isolates from different oil factories. The samples were routine culture methods and test utilized to detect and as well they further confirmed using Vitek 2. Furthermore, hydrocarbon degradation test done by depended on the color change in the nutrient medium as an indicator of. Three isolates were obtained from the screening these isolates, namely *Pseudomonas aeruginosa*, *Escherichia coli*, and *Sphingomonas paucimobilis*. The *E. coli* and *Ps.aeruginosa* were high frequencies isolated from oil felid (60%, 30%, respectively). Furthermore, based on the Biodegradation Productivity, results after twenty-eight days the top-performing bacterial isolate for phenanthrene degradation were (*Sphingomonas paucimobilis)* wild type (43.6%). In terms of naphthalene breakdown, the highest degradation effectiveness was observed in (*E. coli)* type with a ratio of (97.7%). As for acenaphthene, (*E. coli)* wild type exhibited excellent degradation efficiency (88.6%).

Keywords: *Pseudomonas aeruginosa*, *Escherichia coli*, bioremediation, PAHs

Introduction

Poly cyclic aromatic hydrocarbons (PAHs) are notorious for their resistance to degradation due to their low reactivity [1]. A multitude of microorganism gradually break down these mixtures. The study focused on the examination of hydrocarbon-degrading bacteria's role in the biodegradation improvement of hydrocarbons in polluted location. The degradation of mixtures of hydrocarbons typically requires the cooperation of multiple microorganisms [2]. This is particularly important when dealing with contaminants that consist of various compounds, like crude oil or petroleum. The ultimate goal in these cases is to achieve complete mineralization into CO2 and H2O [3].

The spread of oil pollution, stemming from various sources such as refineries, accidental spills, leaks of oil and its byproducts, and the disposal of oil-based waste by humans, has led to a significant rise in sites contaminated with crude oil [4]. Among the most dangerous components of these pollutants are polycyclic aromatic hydrocarbons (PAHs), which are toxic, semi-volatile organic compounds with long-term environmental impact [5]. PAHs persist in soil and can enter the environment through the food chain, raising serious health and ecological concerns.

They can infiltrate the environment, impacting the food chain and causing genetic toxicity in humans, as well as possessing mutagenic and carcinogenic properties. Additionally, their harmful effects extend to plants and animals, leading to reduced soil quality [6].

Fertility, leading to reduced agricultural productivity. The chemical composition of crude oil includes aliphatic and aromatic hydrocarbons, asphaltenes, resins, and organic compounds containing carbon, hydrogen, nitrogen, sulfur, and oxygen, as well as numerous heavy metal-containing compounds [7]. Various methods exist for treating oil pollution, including physical and chemical treatments [8]. However, emphasized that physical technologies are often expensive, have numerous drawbacks, require significant energy, and can be environmentally damaging due to their inability to effectively decompose oil pollutants, leading to their persistence in the soil and continued environmental contamination. Additionally, pointed out that the use of physical technologies can emit toxic pollutants into the air and generate secondary pollution [9-10].

The objectives of this study involve isolating, screening, and identifying bacterial isolates from a polluted site containing hydrocarbons. Additionally, the study aims to these bacterial isolates in order to obtain strains that are highly efficient in degrading PAHs. The biodegradation efficiency (BE %) will be measured to assess the effectiveness of these bacterial strains.

Material and Method

Model Collection

Soil models polluted with oil were gathered from the oil production sites of Doura oil factory (Baghdad), Al-wassat Refinerie Comp. (Al-kout) and Karbalaa oil factory during the period of 5-11-2019 to 17−12−2019. The gathered specimens were carefully stored in clean zip-lock polyethylene pouches and conveyed to research laboratory for extra examination [11].

Isolation of bacteria

The Bushnell−Hass broth medium (BH) was utilized to isolate microorganisms with the ability to degrade hydrocarbons from soil samples. This medium consisted of (0.22gram of Magnesium sulfate, 0.02 gram of Calcium chloride, 1 gram of Monopotassium phosphate, 1 gram of Dipotassium phosphate, 1 gram of Ammonium nitrate, 0.05 gram of ferric chloride) dissolved in distill water (1000 ml). Additionally, the medium was supplemented with sterilized crude oil at a concentration of 1% (V/V) as only carbon source [12]. 0.1ml from each serial dilutions ($10^{\hat{}1}$ to $10^{\hat{}8}$) were applied onto Bushnell−Hass agar containing decontaminated raw material (1% V/V of oil) as only energy element. The petri dishes were then placed in an incubator at 37^{o}C for a duration of 7 days. After the incubation period, distinct colonies were selected and subjected to sub-culturing and purification by cultivating them on a plate of nutrient agar [13-15].

Selection of oil consuming isolates

Under the conditions of BH broth enriched with naphthalen (1% wt/v), as only carbon source, the purified isolates were cultivated and incubated with condition of (37°C, 150 rpm for a period of 7 days). In order to validate the proliferation of degrading bacterial isolates, a 0.5 ml volume (specifically from growing isolates) was evenly distributed on BH agar plates. Subsequently, a drop of 0.5 ml crude oil was carefully placed upon the media [16-18]. To ascertain the ability of bacteria to decompose aromatic hydrocarbons, a improved Simmon-citrate medium was employed, incorporating the bromothymol-blue pointer. Each test tube contains (4ml) of Simon-citrate liquid broth media enhanced with 0.04gram (1.0% w/v) of naphthalen and bromuthymol pointer, were inoculated with 100 µL of bacterial isolate cultivated on crude oil. Following inoculation, the test tubes were incubated at 37 °C, and daily observations were carried out to track the disappearance of the green color and the emergence of the yellow color [19-20].

Bacterial isolates identification

To identify and characterize pure colonies exhibiting a positive outcome (yellow color), following the characterization scheme provided in Bergey's Manual of Determinative Bacteriology [21-22]. To validate the diagnosis, the isolates were further analyzed using the (VITEK 2) technique.

Biodegradation of PAH

To degrade PAHs material, three highly effective bacterial strains (SD 1, SD 2 and SK 1) employed in BH broth media as the single energy and carbon source. 20 ml of BH media containing 0.2gram (1% wt/v) of naphthalen, acenaphthen, and phenanthren was prepared for the experiment. Subsequently, the cells were separately inoculated into liquid BH media, 20 m apart, with the respective strains (SD1, SD2, and SK1), and incubated for a period of 28 days at 37°C and 150 rpm [23].

Results

The isolation and screening processes using a single carbon source (PAHs) carefully selected to match the environmental conditions of the bacterial nutrient medium resulted in several isolates.

In order to select the most effective bacteria for degrading hydrocarbons, an alternative carbon source, specifically PAHs (naphthalene), was used instead of citrate in the modified Simmon citrate medium. To confirm the presence of efficient H.C degrading bacteria, bromothymol blue indicator was added to the BH media. This indicator changes the color of the media from green to yellow, indicating the presence of acidic compound metabolites resulting from naphthalene oxidation.

Validation of bacterial isolates was carried out using the VITEK 2 system, which effectively confirmed the standard diagnosis. The acquired results from VITEK 2 analysis revealed that the best three isolates, namely S.D 1, S.D $_2$, and S.K 1, were recognized respectively as *Pseudomonas aeruginosa* (with a probability of 99%), *E.coli* (with a probability of 89%), and *Sphingomonas paucimobilis* (with a probability of 93%).

In the figure1 presents the distribution of bacteria isolated from different solid oil field. *E. coli* was high frequencies isolated from soil contaminated by oil; it reaches to (60%) as well. Following those the *Ps aeruginosa* was reached to (30%). Finally, *Sph. Paucimobilis* was isolated from oil with percent (10%).

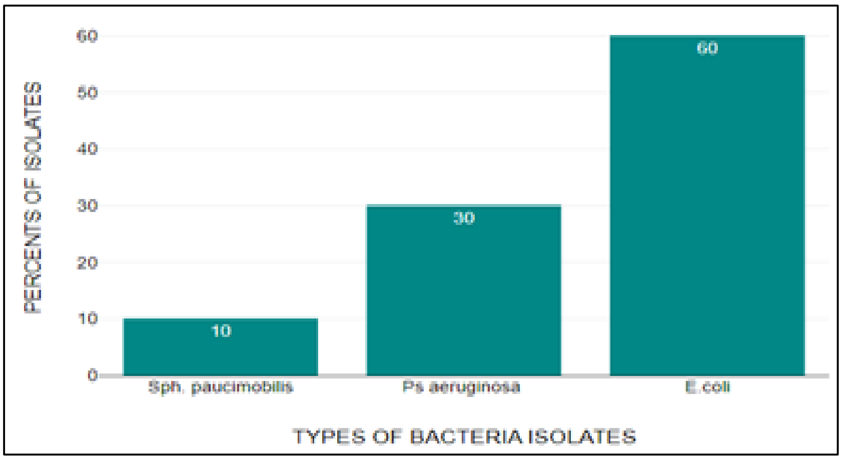

Fig 1: Frequencies of bacteria isolated from oil field

The control samples for polycyclic aromatic hydrocarbon compounds were measured by GC Mass, and the purity of each substance was determined in its natural state. The measurement was repeated after 4 weeks of cultivation in modified Bushnell Haas medium, with the addition of three bacterial isolates, *E. coli*, *Pseudomonas*, and *Sphingomonas* one strain for each compound the results were obtained using the GC Mass device shown below for both cases before and after incubation.

Table 1: The purity of PAHs used in processes by GC Mass spectrometry

Numb.	Sample	Rt min.	Corr. area	Total areas	Purity
1.	Naphthalene control	10.217	*7179468783*	*7179468783*	100 %
2.	Phananthrine control	16.118	318093272	324904536	97.90 %
3.	Acenaphthrine control	13.794	400854196	411448724	97.42 %

Below are the results of Jesse Maas's examinations for the control which don't contain any bacterial strain but only the pure PAHs, and tests samples containing one hydrocarbon component and one bacterial isolate after 4 weeks of incubation in the shaking incubator at a temperature of approximately 37 degrees Celsius and with a solar lamp illuminated to simulate outdoor conditions.

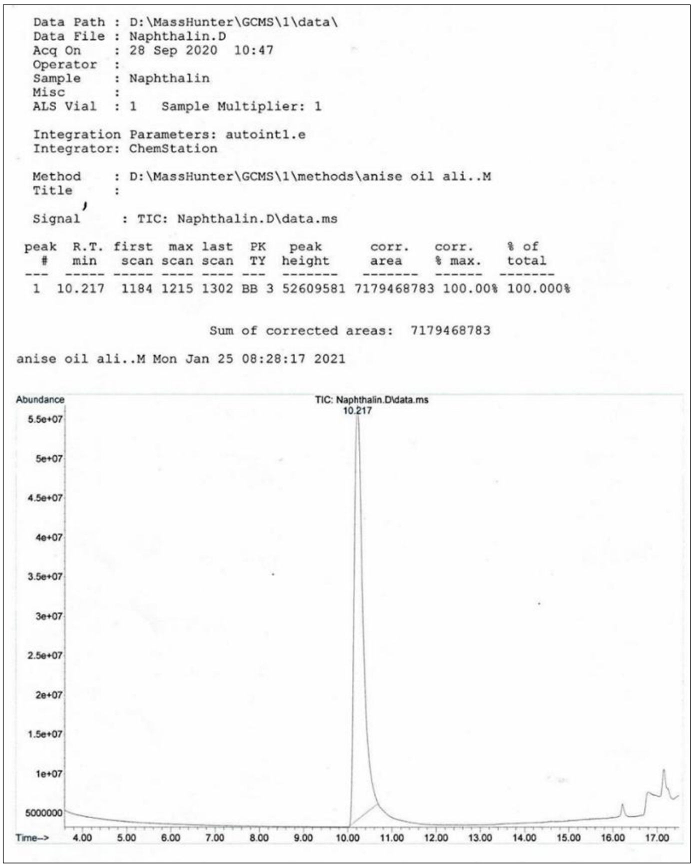

Fig 2: Naphthalene control sample GC Mass results

```
Data Path : D:\MassHunter\GCMS\1\data\
Data File : phenathrine.D
Acq On    : 28 Sep 2020  11:16
Operator  :
Sample    : phenathrine
Misc      :
ALS Vial  : 2   Sample Multiplier: 1

Integration Parameters: rteint.p
Integrator: RTE
Smoothing : OFF                            Filtering: 5
Sampling  : 1                               Min Area: 1 % of largest Peak
Start Thrs: 0.2                            Max Peaks: 100
Stop Thrs : 0                          Peak Location: TOP

If leading or trailing edge < 100 prefer < Baseline drop else tangent >
Peak separation: 5

Method    : D:\MassHunter\GCMS\1\methods\anise oil ali..M
Title     :

Signal     : TIC: phenathrine.D\data.ms
```

peak #	R.T. min	first scan	max scan	last scan	PK TY	peak height	corr. area	corr. % max.	% of total
1	15.976	2261	2271	2280	rBV2	1456686	6811264	2.14%	2.096%
2	16.118	2282	2297	2304	rBV8	72736318	318093272	100.00%	97.904%

```
                    Sum of corrected areas:    324904536

anise oil ali..M Mon Jan 25 08:47:54 2021
```

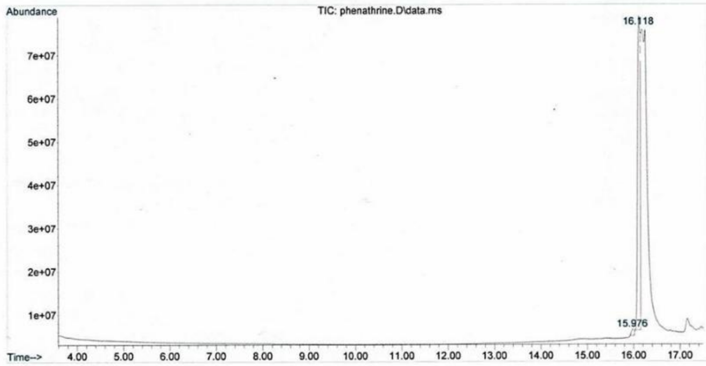

Fig 3: Phenanthrene control sample GC Mass results.

```
Data Path : D:\MassHunter\GCMS\1\data\
Data File : Acenaphrine.D
Acq On    : 28 Sep 2020  11:45
Operator  :
Sample    : Acenaphrine
Misc      :
ALS Vial  : 3   Sample Multiplier: 1

Integration Parameters: rteint.p
Integrator: RTE
Smoothing : OFF                            Filtering: 5
Sampling  : 1                               Min Area: 1 % of largest Peak
Start Thrs: 0.2                            Max Peaks: 100
Stop Thrs : 0                          Peak Location: TOP

If leading or trailing edge < 100 prefer < Baseline drop else tangent >
Peak separation: 5

Method    : D:\MassHunter\GCMS\1\methods\anise oil ali..M
Title     :

Signal     : TIC: Acenaphrine.D\data.ms
```

peak #	R.T. min	first scan	max scan	last scan	PK TY	peak height	corr. area	corr. % max.	% of total
1	13.794	1851	1871	1881	rBV5	69914935	400854196	100.00%	97.425%
2	17.159	2477	2488	2499	rBV2	2246851	10594528	2.64%	2.575%

```
                    Sum of corrected areas:    411448724

anise oil ali..M Mon Jan 25 08:33:51 2021
```

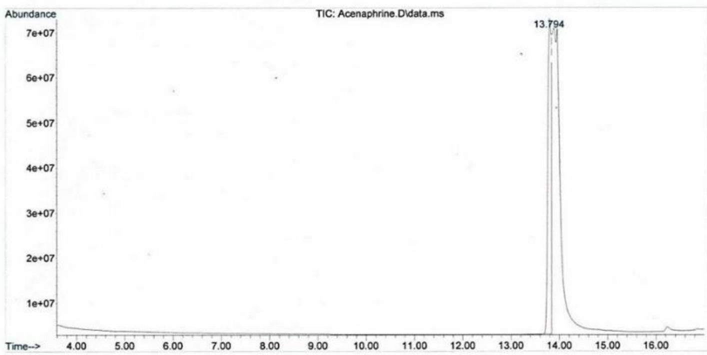

Fig 4: Acenaphthene control sample GC Mass results

```
Data Path : D:\MassHunter\GCMS\1\data\
Data File : Naphthaline E.coli After 28day .D
Acq On    : 08 Nov 2020  09:50
Operator  :
Sample    : Naphthaline E.coli After 28day
Misc      :
ALS Vial  : 1   Sample Multiplier: 1

Integration Parameters: autoint1.e
Integrator: ChemStation

Method    : D:\MassHunter\GCMS\1\methods\Drug screen.M
Title     :

Signal    : TIC: Naphthaline E.coli After 28day .D\data.ms
```

peak #	R.T. min	first scan	max scan	last scan	PK TY	peak height	corr. area	corr. % max.	% of total
1	17.312	2510	2608	2704	BB 6	173795	23359030	34.91%	6.948%
2	20.013	2710	3103	3144	BV 6	261481	32148808	48.04%	9.563%
3	20.345	3144	3164	3219	VB 7	97961	11888428	17.77%	3.536%
4	21.378	3236	3353	3413	BV 9	265007	50363178	75.27%	14.980%
5	22.610	3413	3579	3631	VV 9	64510	43850797	65.53%	13.043%
6	23.147	3631	3677	3716	VV 10	105228	19013220	28.41%	5.655%
7	23.474	3716	3737	3795	VB 9	65798	10072444	15.05%	2.996%
8	26.286	3840	4253	4287	BV 9	72624	-14583341	-21.79%	-4.338%
9	26.623	4287	4314	4343	VB 6	31795	4148854	6.20%	1.234%
10	28.574	4375	4672	4687	BV 6	24201	-8819804	-13.18%	-2.623%
11	31.170	4687	5148	5169	PV 6	149340	54767624	81.85%	16.290%
12	31.575	5169	5222	5244	VV 8	227850	43072613	64.37%	12.812%
13	32.203	5244	5337	5423	VB 8	139381	66914237	100.00%	19.903%

Sum of corrected areas: 336196087

Drug screen.M Thu Jan 21 09:37:59 2021

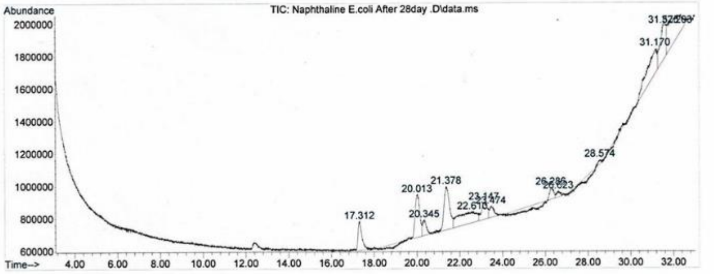

Fig 5: E. coli + naphthalene sample degradation results by GC Mass

```
Data Path : D:\MassHunter\GCMS\1\data\
Data File : Sphingo W phenathrin.D
Acq On    : 12 Nov 2020  09:25
Operator  :
Sample    : Sphingo W phenathrin
Misc      :
ALS Vial  : 2   Sample Multiplier: 1

Integration Parameters: autoint1.e
Integrator: ChemStation

Method    : D:\MassHunter\GCMS\1\methods\Drug screen.M
Title     :

Signal    : TIC: Sphingo W phenathrin.D\data.ms
```

peak #	R.T. min	first scan	max scan	last scan	PK TY	peak height	corr. area	corr. % max.	% of total
1	17.304	2552	2606	2697	BB 5	116379	16338414	21.51%	8.919%
2	20.013	2849	3103	3142	BV 4	203979	30951793	40.74%	16.896%
3	20.322	3142	3159	3218	VV 5	78791	10021750	13.19%	5.471%
4	23.179	3218	3683	3710	PV 6	75316	19208861	25.28%	10.486%
5	23.480	3710	3738	3793	VB 2	59001	9033831	11.89%	4.931%
6	26.259	3798	4248	4285	BV 4	51061	7381704	9.72%	4.029%
7	27.693	4285	4510	4535	PV 3	28999	787645	1.04%	0.430%
8	29.121	4535	4772	4784	PV 8	27286	-6435167	-8.47%	-3.513%
9	31.746	4784	5254	5296	PV 8	259005	75970209	100.00%	41.470%
10	32.273	5296	5350	5392	VBA3	109454	19934369	26.24%	10.882%

Sum of corrected areas: 183193411

Drug screen.M Thu Jan 21 09:34:53 2021

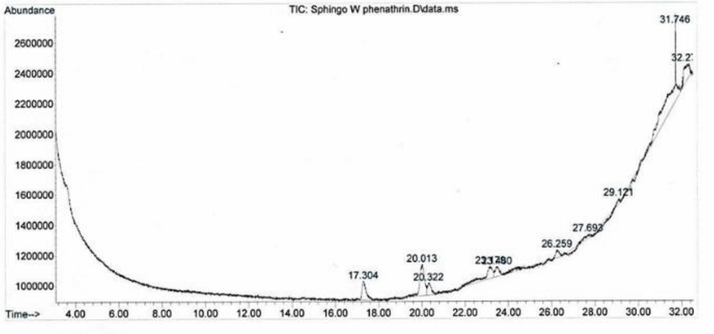

Fig 6: Sphingomonas + phenanthrene sample degradation results by GC Mass

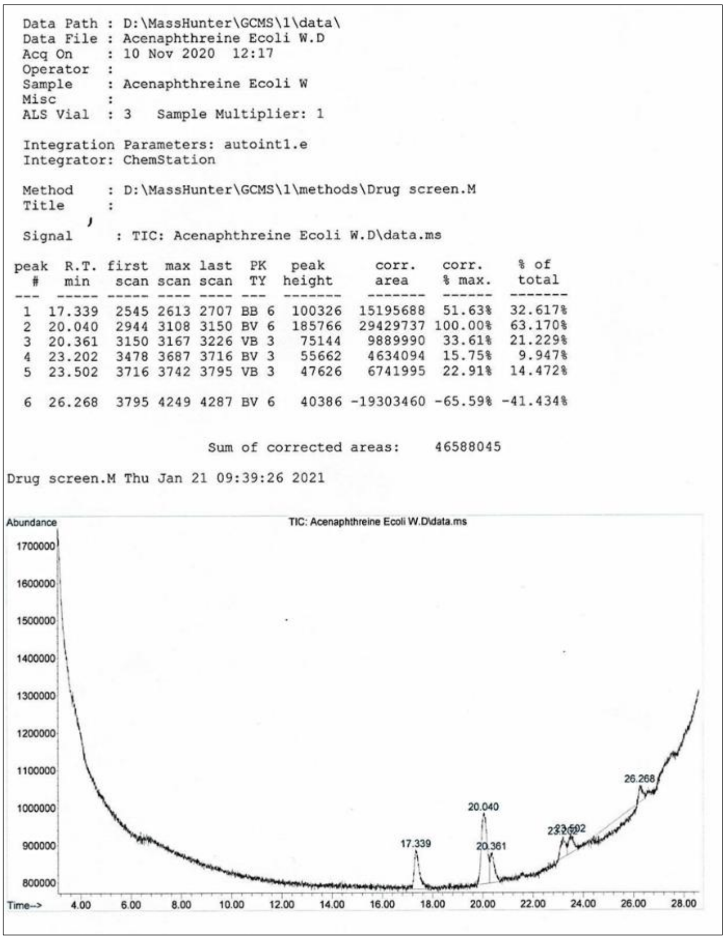

Fig 7: E.coli + acenaphthrene sample degradation results by GC Mass

Table 2: bacterial strains biodegradation efficiency for PAHs

Compounds.	Bacteria.	Control total area.	Sample total area.	Biodegradation efficiency (BE %).
Naphthalene	E. coli	7179468783	163557919	97.7 %
Phenanthrene	Sphingomonas	324904536	183193411	43.6 %
Acenaphthene	E. coli	411488724	46588045	88.6 %

Discussion

In assessment of the previous results, the efficiency of bacterial isolates was proven through the use of a culture medium with a specialized carbon source, which in this experiment was modified Bushnell Haas medium. The carbon source was replaced in the screening process with crude oil first and then with the hydrocarbon elements mentioned above. This proved the ability of the isolates to consume carbon. The process of transformation and consumption was observed by adding bromothymol blue reagent. The results obtained from the GC Mass device in Table 1 show the purity of the polycyclic aromatic hydrocarbon materials used. As in fig (2) show the Naphthalene GC-Mass result for control sample showed high purity by 100% for the compound. The test used to confirm the type of sample, purity and chemical configuration of polycyclic compound. Figure (2) clarify the retention time of naphthalene (10.217) with only one peak number as evidence for purity and corrected areas (7179468783), the previous number used as control number to explain the biodegradation efficiency of samples contain naphthalene. In Figure 3, the results of the GC-Mass test for phenanthrene in the control sample showed a high purity of 97.904%. This test was used to confirm the type of sample used in the experiment, as well as the purity and chemical structure of the polycyclic compound. The figure illustrates the retention time of phenanthrene (16.118) and its corrected area (324,904,536), which serves as a reference for evaluating the biodegradation efficiency of samples containing phenanthrene. Similarly, in Figure 4, the GC-Mass test for acenaphthene in the control sample recorded a purity of 100%. This test also confirmed the type of sample, its purity, and the chemical configuration of the polycyclic compound. Figure 4 shows the retention time of acenaphthene (13.794) and its corrected area (411,448,724), which is used as a reference to assess the biodegradation efficiency of samples containing acenaphthene. Also, after comparing them with the results after 4 weeks of incubation, it is noted in Figures 2 to 4 that some secondary materials resulting from the breakdown of the original materials that were used as a carbon source in the middle appear, and this is due to The ability of these isolates adapted to that environment and soil contaminated with oil and its derivatives to consume aromatic compounds at a high rate.

Figure (5) above describe the metabolites produced in media from naphthalene degradation by *E. coli* wild type mechanism, each peak in the figure refer to a specific organic compound as a metabolites product. Most products are naphthalene derivatives. Figure (6) above explain the metabolites results from phenanthrene degradation by *Sphingomonas paucimobilis* wild type activity, each peak in the result represent specific organic compound as a metabolites product. Figure (7) above describe the metabolites produced in media from acenaphthene degradation by *E. coli* wild type activity, each peak in the figure refer to a specific organic compound as a metabolites product. Most products for tow last figures are acenaphthene and phenanthrene derivatives.

As shown in Table (2). The naphthalene compound was broken down by E. coli bacteria to a rate of approximately 98 % because it consists of two rings and a short carbon chain numbering 10 carbon atoms. To a lesser extent, amounting to 88 %, the bacterial isolate E. coli analyzed the acenaphthene compound because it is a tricyclic compound with ethylene bridges and a 12-atom carbon chain. As for Sphingomonas bacteria, they consumed phenanthrene by 43 %. This can be attributed to the fact that the compound is poorly mixed with water, in addition to containing three hexagonal rings that are difficult to break and 14 carbon atoms. Despite the presence of rings and carbon chains, E. coli and Sphingomonas bacteria have shown a high ability to consume naphthalene, phenathrene, and acenaphthene in moderate or excellent proportions. Although the presence of Pseudomonas was twice as high as the presence of Sphingomonas in the soil, its ability to consume compounds was not as required despite the availability of suitable conditions for growth. This may be due to it not obtaining a sufficient period of adaption in the medium or its lack of adaption and nutrition. These suggests agreed with the study was indicated that that P. mirabilis and R. qinshengi degraded nearly 94% of naphthalene in the incubated media. Rhodococcus qinshengi demonstrated a significant ability to degrade pyrene and naphthalene under different pH and temperature conditions, with mineralization exceeding 50% across the tested ranges. This suggests that the isolated strains are well-suited for the biodegradation of sediment contaminated with naphthalene and pyrene [24-25]. Other study demonstrated that isolate and characterize a newly discovered bacterium, designated as strain W10, which is capable of degrading polycyclic aromatic hydrocarbons (PAHs) and producing biosurfactants [26]. Strain W10 belongs to the Pseudomonas genus and is closely related to Pseudomonas aeruginosa, with a 99.1% similarity in the 16S rRNA gene sequence. According to GC-MS analyses, this strain degraded approximately 80% of phenanthrene, using it as the sole carbon and energy source, at an initial concentration of 200 mg/l after 30 days of incubation at 37°C and 180 rpm [27]. The surface tension was reduced from 56.1 to 42 mN/m after 4 days of incubation. Additionally, strain W10 degraded about 10%, 20%, 90%, and 99% of hexadecane (C16), pyrene, fluoranthene, and crude oil, respectively, after 30 days of incubation under the same conditions. Metabolites identified during the degradation of phenanthrene and fluoranthene indicated the biodegradation pathways of these PAHs [28]. Notably, strain W10 also exhibited a strong potential to produce surface-active agents, reducing the surface tension to 32 mN/m and achieving a production of around 2 g/l after 48 hours of incubation with olive oil (1%, v/v) as a substrate at 37°C and 180 rpm. The biosurfactant produced by strain W10, named BSW10, demonstrated significant emulsification activity and maintained high stability across a broad range of salinity (0-150 g/l), temperature (0–100°C), and pH (2–12). These characteristics suggest that strain W10 and its biosurfactant BSW10 hold promising potential for bioremediation applications, particularly in the removal of used motor oil and crude oil from contaminated soils [29-30].

Conclusion

The method of modifying Bushnell-Haas medium has proven effective in screening and isolating isolates consuming polycyclic aromatic hydrocarbons (PAHs) by replacing the carbon element with one of the hydrocarbon compounds. The use of bromothymol blue reagent is important for observing the gradual color change resulting from the consumption of compounds as evidence that the bacteria have adapted to the medium and begun to grow in it. The consumption of naphthalene by isolates is largely due to the fact that it mixes quickly with water and is more consumable, unlike the remaining compounds, acenaphthene and phenathrene, which have multiple benzene rings and are difficult to break down. *Ps. aeruginosa* didn't show high degradation efficiency while *Escherichia coli* and *Sphingomonas paucimobilis* were high frequencies isolate from oil field and have highly efficacy to biodegradation for PAHs.

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