

Biological Treatment of Used Engine Oil by Single and Mixed Bacterial Cultures Isolated from Soil of Mechanic Workshops

التحلل البيولوجي لزيت المحرك المستهلك بواسطة المزارع البكتيرية المفردة والمختلطة المعزولة من تربة الورش الميكانيكية

Amal A. Hussein

Saad H. Khudhair

Applied Science Department/Branch of Biotechnology/ University of Technology

Directorate of water and environment/ Ministry of science and technology

سعد حسين خضير

امل علي حسين

قسم العلوم التطبيقية / فرع التقنيات الاحيائية/ الجامعة التكنولوجية

دائرة البيئة والمياه/ وزارة العلوم والتكنولوجيا

E-mail: amelali71@yahoo.com

Abstract

The accumulation of hydrocarbon waste, such as used engine oils in environments, has many impacts on humans and other organisms, therefore many researches were achieved to degrade or remove or consume these pollutants. The aim of the current study is to get a local bacterial isolates has high ability to degrade the spent engine oil as a single or mixed culture. Five soil samples contaminated with spent engine oil were collected from mechanic workshops in Baghdad city to isolate degrading bacteria using Bushnell Hans medium (BHM), pH 7 with 5% of used engine oil. While the growth patterns and gravimetric analysis was used to reveal the ability of these isolates to degrade spent engine oil in liquid BHM medium. The best three isolates A4, B6 and D5 were identified and the optimal temperature and pH for biodegradation of spending engine oil were studied. Also, the consortium culture of three isolates was tested their ability to utilize spent engine oil under the same conditions for single isolate. Twenty five bacterial isolates were obtained from contaminated soil samples and three isolates appeared a maximum degradation rate 74.6, 70.2 and 78.5% respectively. The results from identification tests were showing these isolates belong to *Bacillus* sp., *Acinetobacter* sp. and *Pseudomonas* sp., respectively. The studied three isolates gave the best degradation when incubated at 30°C in BHM medium pH 7. While other results were indicated that consortium cultures are more effective 90.2% than all experiments that used single isolate.

Key words: Biodegradation, Bacterial isolates, spent engine oil.

المخلص

تراكم المخلفات الهيدروكربونية، مثل زيوت المحركات المستخدمة في البيئة، لها تأثيرات كثيرة على الانسان و الكائنات الاخرى، ولذلك تم اجراء العديد من الابحاث لتقليل او ازالة او استهلاك هذه الملوثات. الهدف من الدراسة الحالية هو الحصول على عزلات بكتيرية محلية لديها قدرة عالية على تحلل زيوت المحركات المستهلكة بشكل مزارع مفردة او مختلطة. جمعت خمس عينات من التربة الملوثة بزيوت المحركات المستهلكة من ورش الميكانيك في مدينة بغداد لعزل البكتيريا المحللة باستخدام وسط Bushnell Hans (BHM) برقم هيدروجيني 7 وبنسبة 5% من زيت المحرك المستخدم. في حين تم استخدام انماط النمو والتحليل الوزني للكشف عن قدرة هذه العزلات على تحلل زيت المحركات المستهلك في وسط BHM السائل. شخّصت افضل ثلاث عزلات A4، B6 و D5 ودرست درجة الحرارة المثلى ودرجة الحموضة للتحلل الحيوي لزيت المحركات المستهلكة. كما تم اختبار قدرة العزلات الثلاثة بصورة مجتمعة على استهلاك زيت المحرك المستهلك بنفس الظروف للعزلة المفردة. تم الحصول على خمس وعشرين عزلة بكتيرية من عينات التربة الملوثة وقد اظهرت الثلاث عزلات اعلى معدل للتحلل 74.6، 70.2 و 78.5% على التوالي. اظهرت نتائج التشخيص ان هذه العزلات تعود للاجناس *Bacillus* sp.، *Acinetobacter* sp. و *Pseudomonas* sp. على التوالي. العزلات الثلاثة المدروسة اعطت احسن تحلل عندما حضنت بدرجة حرارة 30 °م في وسط BHM السائل برقم هيدروجيني 7. بينما اشارت النتائج ان المزارع المختلطة هي اكثر كفاءة بنسبة 90.2% لكل التجارب التي استخدمت فيها العزلة المفردة.

الكلمات الدالة: التحلل البيولوجي، عزلات بكتيرية، زيت المحركات المستهلك

Introduction

Pollution with hydrocarbon materials and its products such as gasoline, fuel, engine oil, residues of fuel, and spent engine oil, has more attention according to their effects on environment pollution [1]. The most important sources of soil pollution in Baghdad/ Iraq are due to the discharging of spending engine oils from cars and many types of machines used for generating the electricity and other purposes. The main pollutants in spending oils are the polycyclic aromatic hydrocarbons (PAHs) and

metals, these pollutants can be considered the main cause to occur the mutations and therefore the spread of cancer [3, 4]. A huge amounts of spending oils can be generated at each day from the processes to change the oils of cars and other engines in the mechanical workshops, therefore must be recycled. However, most of them is treated using the processes of incineration or dumping. So, the world need to other methods to recycle these pollutants to prevent their harmful effects on environment [5]. The remediation processes using bacteria and other organisms are depending on the ability of these organisms to degrade or remove or consume the hydrocarbon contaminants as a percentage or completely [6]. Harder (2004)[7], showed that the bioremediation process represents about for 5 to 10 percent from the treatment methods used in for all methods used to treat spent oils or clean up the environment from them. The use of mixed cultures in the processes of degradation to degrade the hydrocarbon wastes was achieved in many researches than those used the pure cultures [8]. Also in ecosystems, there are many microbial communities able to work together to degrade the oil pollutants, according to their ability to consume these pollutants and nature of environmental conditions in sites [9].

The aims of this study were focused on the isolation and screening of bacteria that degrade the spent engine oil and study the effect of temperature and pH on degradation of used oil, also the effect of single and mixed cultures on degradation was detected.

Materials and Methods

Collection of contaminated soils

Soils contaminated with mainly spent engine oil were collected from five different areas of mechanic workshops in Baghdad city during 1-28 April, 2016. These selected sites were polluted with spent engine oil for a long time (more than 10 years). The 25 g of soil samples was taken by sterilizing spatula from a depth of 0-5 cm and transferred into a sterilized container [10].

Isolation of Bacteria

The samples of used engine oil-contaminated soils were used to isolate the bacteria by inoculating 1 g from them in flasks 50 ml of liquid Bushnell Haas medium (BH medium) supplemented with 5% spent engine oil as carbon source and then the flasks were incubated in shaker incubator 150 rpm at 30°C for 7 days. After incubation period 0.1 ml from each flask was transferred into the plates of solid BH medium with 5% spent engine oil and incubated at 30°C for 3-5 days, then the single colonies grown on solid medium were sub-cultured into plates of nutrient agar [11].

Monitoring of bacterial growth

The bacterial growth in the liquid BH medium was determined according to the optical density of bacterial cultures at 600 nm using spectrophotometer [11].

Extraction and determination of residual oil

The residual of used engine oil in liquid BH medium was measured by gravimetric method, the BH medium of bacterial cultures 50 ml after incubation period was transferred into separating funnels and treated with dichloromethane 50 ml, and then the organic solvent layer was collected and filtered through 15g of sodium sulfate. The percentage of spending engine oil biodegradation was calculated according to following equation [10].

$$\% \text{biodegradation of used oil} = \frac{\text{weight of oil in control} - \text{weight of oil in test}}{\text{weight of oil in control}} \times 100$$

Screening of bacterial isolates

250 ml flasks of the sterilized liquid BH medium of pH 7 supplemented with 5% spent engine oil were used to screen the bacterial isolates for their efficiency to degrade used engine oil. The flasks were inoculated with isolates (one flask was left uninoculated as a positive control), then all flasks were incubated in shaker incubator 150 rpm for 10 days at 30°C. After incubation period growth of bacteria at 600 nm and residual of used engine oil in liquid medium were determined [12].

Identification of selected isolates

The most active bacterial isolates have been selected depending on their ability to consume the used engine oil in liquid medium, then these isolates were characterized to genus level based on colony morphology, Gram staining, and biochemical tests (such as lactose fermentation, citrate, catalase, indole, methyl red, oxidase and etc.) according to the Bergey's Manual[13].

Effect of temperature and pH on degradation

The sterilized liquid BH medium supplemented with 5% spent engine oil was used to determine the effect of temperatures and pH values on degradation of used engine oil.

The flasks of BH medium of pH 7 were inoculated by selecting isolates and incubated at different temperatures (20, 25, 30, 35 and 40°C) for 10 days, while the effect of pH was studied by preparing BH medium with different pH values (5, 6, 7, 8 and 9) and then were inoculated by selecting isolates and incubated in 30°C for 10 days. After the incubation period the bacterial growth and residual oil were determined [14].

Biodegradation by single and consortium culture

The consortium culture of *Bacillus sp.*, *Acinetobacter sp.* and *Pseudomonas sp.* was prepared by mixing equal volumes of pure bacterial cultures together, then the liquid BH medium, pH 7 with 5% spent engine oil was inoculated by single and consortium culture of selected isolates. The flasks were incubated in shaker incubator 150 rpm at 30°C for 10 days, and then the bacterial growth and biodegradation of used engine oil were determined [15].

Results and discussions

Bacteria isolated from samples

Twenty five bacterial isolates were obtained from the five soil samples collected from mechanic workshops using the solid BH medium, pH 7 supplemented with 5% spent engine oil as carbon source. The results of isolation processes as in Table (1) show a clear difference in the number of isolates obtained from each soil sample, this may be due to the nature of soil samples according to the quantity of contaminants in it and the length of pollution period.

Table (1): Isolation the bacteria from samples of spent engine oil–contaminated soils collected from different regions of Baghdad city, using BH medium with 5% used engine oil as substrate.

Soil samples	Symbol	Collection period	Number of isolates
Al-Jadriya	A	1 th April, 2016	5
Al-Karrada	K	7 th April, 2016	2
Al-Bayaa	B	14 th April, 2016	9
Al-Ghazaliyah	G	21 th April, 2016	2
Al-Dora	D	28 th April, 2016	7
Total isolates =			25

The current work corroborated the exist data on the indigenous bacteria that can utilize or metabolize hydrocarbons in any polluted site. Oil contaminated soil is the best source for the isolation of oil degrading bacteria and may be used for the isolation of other microorganisms. The isolated bacteria can be used for the degradation of different hydrocarbon containing oil and it can also be used for the remediation of contaminated sites.

Screening of bacteria

The twenty five bacterial isolates were investigated for their ability to utilize the used engine oil in liquid BH medium. The bacterial growth and biodegradation of used engine oil for inoculating isolates were measured as optical density at 600 nm and by gravimetric method.

The results in Table (2) exhibited that the isolates have different capability to consume the substrate and the isolates A4, B6 and D5 appeared the highest growth rate 0.67, 0.61 and 0.73 respectively, compared with other isolates in this study.

Table (2): Primary screening of bacterial isolates in liquid BH medium, pH 7 with 5% spent engine oil, flasks incubated for 10 days at 30°C.

Isolates	Bacterial growth at 600 nm	Isolates	Bacterial growth at 600 nm	Isolates	Bacterial growth at 600 nm
A1	0.29	B3	0.33	D1	0.44
A2	0.44	B4	0.26	D2	0.39
A3	0.11	B5	0.50	D3	0.41
A4	0.67	B6	0.61	D4	0.26
A5	0.09	B7	0.46	D5	0.73
K1	0.09	B8	0.17	D6	0.52
K2	0.16	B9	0.31	D7	0.22
B1	0.24	G1	0.28		
B2	0.42	G2	0.08		

However, the experiment of degradation for spent engine oil showed also a high variation between the isolates about their ability to degrade the spent oil as in Table (3), the results also proved that the isolates A4, B6 and D5 are more efficient isolates to degrade the spent oil 74.6%, 70.2% and 78.5% respectively, compared with other isolates.

Table (3): Secondary screening of bacterial isolates in liquid BH medium, pH 7 with 5% spent engine oil, flasks incubated for 10 days at 30°C.

Isolates	Biodegradation (%)	Isolates	Biodegradation (%)	Isolates	Biodegradation (%)
A1	45.3	B3	55.9	D1	63.1
A2	62.6	B4	53.3	D2	58.5
A3	39.4	B5	67.3	D3	59.4
A4	74.6	B6	70.2	D4	49.9
A5	32.9	B7	63.8	D5	78.5
K1	29.4	B8	47.8	D6	69.2
K2	41.2	B9	54.6	D7	51.2
B1	50.1	G1	52.3		
B2	60.7	G2	26.5		

These results indicate that this bacterial isolate utilized engine oil as a sole source of carbon and energy. Enriched culture of five soil samples was spread into the BH agar plates which are the selective media for oil degrading bacteria.

Identification of bacteria

The most active bacterial isolates A4, B6 and D5 for degradation of used engine oil were identified according to morphological and biochemical tests. The results in Table (4) indicate that the isolates A4, B6 and D5 belong to *Bacillus sp.*, *Acinetobacter sp.*, and *Pseudomonas sp.*, respectively.

Table (4): Morphological and biochemical tests to identify the isolates.

Tests	A4	B6	D5
Form	Circular	Round	Circular
Surface	Smooth	Smooth	Smooth
Color	Cream	Cream	Whitish
Margin	Undulate	Entire	Entire
Elevation	Raised	Slightly raised	Convex
Opacity	Opaque	Opaque	Translucent
Gram stain	Positive	negative	negative
Catalase	+	+	+
Oxidase	+	-	+
Citrate utilization	+	+	+
Lactose ferment.	-	-	-
H₂S production	+	-	-
Methyl red	-	-	-
Nitrate reduction	+	-	+
Indol	-	-	-
Urease	-	-	-
Motility	+	-	+

Such results are similar to those obtained by [16] who revealed that the *Pseudomonas*, *Bacillus*, *Proteus*, *Salmonella* and *Streptococcus* were isolated from oil contaminated soil samples, two of these bacteria (*Pseudomonas* and *Bacillus*) have been reported to be among the most frequently isolated bacteria from hydrocarbon-polluted sites [17,18]. Thenmozhi *et. al.*, (2011) [11] have isolated three bacterial isolates which are able to consume spent oil as substrate. The isolate *Pseudomonas aeruginosa* was given the best result for oil degradation 81% after 30 days of incubation, while the *Serratia marcescens* and *Bacillus licheniformis* gave 72% and 60% respectively under the same conditions.

The results of this study have proven *Pseudomonas* species are the most common bacterial hydrocarbon degraders which is agreed with that reported in the literature [19-20]. *Acinetobacter spp.* is widespread in nature and can remove or degrade a wide range of organic compounds [21] and inorganic compounds [22].

Effect of temperature and pH on degradation

The optimal temperature to degrade used engine oil by *Bacillus sp.*, *Acinetobacter sp.* and *Pseudomonas sp.* were studied in liquid BH medium supplemented with 5% substrate as carbon source. The results of the growth rate and biodegradation are demonstrated in Figures (1,2) respectively, which show that the best growth and degradation rate were obtained at 30°C compared with other studied temperatures. Also the results revealed that the *Pseudomonas sp.* has given the highest ability to degrade spent engine oil 78.9%, while other isolates *Bacillus sp.* and *Acinetobacter sp.* have given 74.7% and 70.4% respectively.

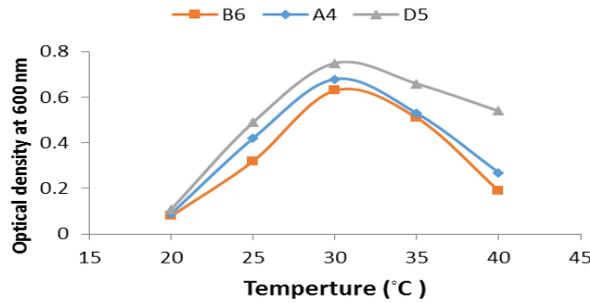


Fig. (1): Effect of temperature on bacterial growth of three isolates in liquid BH medium with 5% used engine oil, pH 7 after 10 days of incubation.

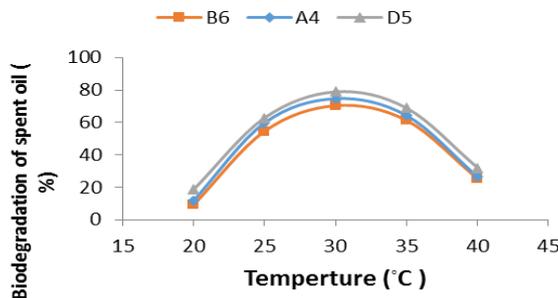


Fig. (2): Effect of temperature on degradation rate of used engine oil by three isolates in liquid BH medium, pH 7 after 10 days of incubation.

The effect of pH on using engine oil degradation by *Bacillus sp.*, *Acinetobacter sp.* and *Pseudomonas sp.* were investigated using liquid BH medium with 5% spent oil as substrate. Figures (3,4) show different ability to grow and degrade the spent oil by three isolates with different pH values. The maximum growth and degradation has been obtained at pH 7 by three isolates, while the *Pseudomonas sp.* has appeared maximum degradation (79.1%) for spending engine oil compared with other isolates *Bacillus sp.* and *Acinetobacter sp.* which has given 74.8% and 70.5% respectively.

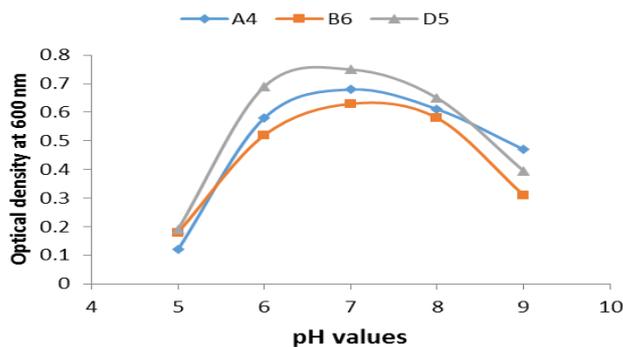


Fig. (3): Effect of pH values on bacterial growth of three isolates in liquid BH medium, pH 7 with 5% used engine oil after 10 days of incubation.

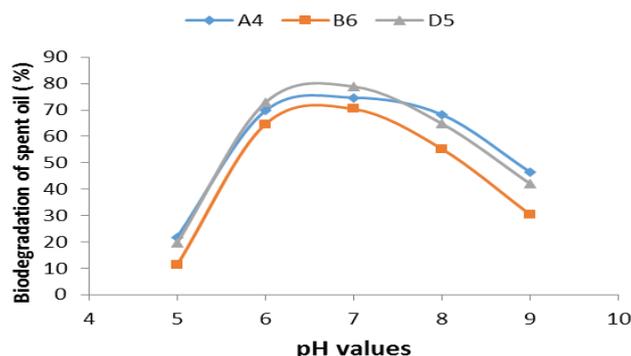


Fig. (4): Effect of pH values on degradation rate of used engine oil by three isolates in liquid BH medium after 10 days of incubation.

These results, at a temperature of 30°C, is in good agreement with that reported by [23] who found that the temperature between 30-37°C showed the maximum degradation for spent engine oil by a microbial consortium at neutral or slightly alkaline pH of the medium.

Biodegradation by single mixed culture

The ability of single and a consortium culture of *Bacillus sp.*, *Acinetobacter sp.* and *Pseudomonas sp.* were tested in order to determine their ability to grow and consume the spent engine oil using BH medium with 5% substrate, after 10 days of incubation the bacterial growth and remaining spent oil in flasks will be calculated.

According to the results from a Table (5) the consortium culture has appeared the highest ability 0.94 and 90.2% as bacterial growth and oil degradation respectively, compared with single isolate of *Bacillus sp.*, *Acinetobacter sp.* and *Pseudomonas sp.*

Table (5): Effect of single and consortium culture on bacterial growth and biodegradation of used engine oil in liquid BH medium after 10 days.

Type of inoculum	Bacterial growth at 600 nm	Percentage of biodegradation %
<i>Bacillus sp.</i>	0.68	74.8
<i>Acinetobacter sp.</i>	0.63	70.5
<i>Pseudomonas sp.</i>	0.75	79.1
Consortium culture	0.94	90.2

This is in accordance with other reports [24,25] who have revealed that the biodegradation of the used engine oil by mixed cultures was more effective, because some species in mixed culture can be consume the intermediates compounds released from degradation processes of oil, their products and hydrocarbon wastes, while other species in the mixed culture can be lead to utilize all these pollutants and their byproducts [31; 32]. The most important reduction is related to the nature of species in the consortium culture that can consume 90% of aliphatic compounds during 30 days under same environmental conditions.

The best reduction of hydrocarbons was seen when used the *Pseudomonas aeruginosa* [11]. The pure culture of *Pseudomonas aeruginosa* was appeared the maximum ability to degrade 53.44% of engine oil within 30 days, while *Bacillus sp.* gave 34.92% value for engine oil degradation under the same conditions [25].

The single organisms often prefer to use when the process done on one hydrocarbon substrate [26]. However, a mixed culture of different bacterial species is usually used to produce all enzymes necessary for complete degradation of complex mixtures of hydrocarbons [27].

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