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Enhancement of oil biodegradation by using the biosurfactant produced from local *Bacillus subtilis* isolate

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Abstract

Background: Oil contamination poses a significant threat to the global environment and has attracted widespread attention in recent years. Given its significance, further exploration of biodegradation options for this contamination has never been more crucial than it is today. Therefore, the objective of the study was to improve the biodegradation of oil by utilizing local bacterial isolates alongside a biosurfactant produced by a *Bacillus subtilis* isolate. Twenty-two bacterial isolates were collected from four samples of hydrocarbon-contaminated soil. These isolates underwent screening to assess their effectiveness in degrading crude oil using two distinct methods.

Methods: Soil samples were collected from two contaminated sites with oil pollutants in Baghdad city. Biodegradation ability was tested on liquid Bushnell Haas medium (BH), pH 7 supplemented with 1% of crude oil and then screened primarily using the 2,6-dichlorophenol indophenol method to determine the ability of isolates to degrade the crude oil. All isolates were identified morphologically and biochemically. biosurfactant was extracted from Bacillus subtilis previously isolated.

Results: Isolate SCS1 showed the highest ability reaching 52.6% compared with other isolates, also, the results of secondary screening confirmed that isolate SCS1 gave the best biodegradation reaching 53.8%. The isolate SCS1 was identified as *Pseudomonas aeruginosa*, this isolate was used to study the effect of biosurfactant on crude oil biodegradation. Results exhibited a high biodegradation efficiency reaching 85.1% in culture broths supplemented with 100 mg/100. It was noted that the use of 150 and 200 mg of biosurfactant led to a decrease in the biodegradation of crude oil.

Conclusion: The use of biosurfactant led to an increase in the degradation rate by up to 61.7% of crude oil by *Pseudomonas aeruginosa* when using 25-100 mg of crude biosurfactant that produced by local isolate *Bacillus subtilis*.

Introduction

Hydrocarbon pollutants are one of the most important toxic pollutants of biological components in the environment, and the use of physical and chemical methods to get rid of them is often complex, economically costly, takes a long time, and is not environmentally friendly because these methods produce other pollutants after treatment, which may have a greater impact on the environment [1]. Therefore, the bioremediation method was considered the ideal way to remove hydrocarbon pollutants from the environment without any other effects from the treatment processes [2,3]. The main cause of environmental pollution with hydrocarbon pollutants is the accidental release of hydrocarbons or as a result of human activities. This type of pollution is the most widespread in the world and significantly impacts human health and various environmental systems. Therefore, interest has increased in the past few periods to develop the efficiency of treating these pollutants by using special microorganisms or using materials that accelerate the biodegradation processes [4]. Many microorganisms capable of degrading hydrocarbon pollutants were obtained from natural environments, where many studies were conducted to develop their ability to degrade these pollutants by using them as an energy source or converting them into harmless substances [5]. Among the methods used to improve the bioremediation of environmental sites contaminated with hydrocarbons is the use of biosurfactants [6]. The rates of biodegradation of environmental pollutants are among the critical factors in the success or failure of treatment processes, and according to the fact that the processes of biological treatment of hydrocarbon pollutants are generally limited because these materials are hydrophobic, which leads to a decrease in its solubility in water. Therefore, the studies tended to increase its solubility with water by adding surfactants during microbial treatment [7,8]. Increasing the solubility of organic pollutants in liquid media is one of the basic methods to enhance the biodegradation of these pollutants due to the increased availability of the organic pollutants for microorganisms or their enzymes [9]. Previous studies have consistently shown that incorporating a biosurfactant into hydrocarbon pollutant treatment processes enhances the rate of biodegradation. This is attributed to the biosurfactant's ability to increase the accessibility of hydrocarbon pollutants, thereby promoting their breakdown [10-12]. Recently, interest in biosurfactants has increased due to some of their unique properties, including the low toxicity of these materials and their high efficacy in reducing the interfacial tension between the oil and water phases. Numerous studies have been conducted to evaluate the ability of biosurfactants to enhance the solubility of organic pollutants and then increase the bioavailability of these pollutants towards microbial cells [13-15]. Most studies have focused on the effect of adding biosurfactants on the rate of biodegradation of pure hydrocarbon pollutants, and not on mixtures of hydrocarbon pollutants, but current studies focus on studying the effect of adding biosurfactants on the biodegradation of hydrocarbon mixtures, which makes these studies closer to the reality of environmental pollutants [16].

Methods

Samples of oil-polluted soil

Four samples of hydrocarbon-contaminated soils were collected from two sites contaminated by oil pollutants in Baghdad city, where sterile containers were used to collect the soil samples, and then the samples were kept in the refrigerator at a temperature of 4°C until use.

Isolation of oil-degrading bacteria

One gram from each soil sample was added to 9 ml of sterile distilled water, shaken well for 15 minutes, and then left for 10 minutes at room temperature. After that 5 ml of the supernatant from each soil sample was added to flasks containing 100 ml of liquid Bushnell Haas medium [or BH which is commonly used in biodegradation experiments], pH 7 supplemented with 1% of crude oil. The shaker incubator was used to incubate the flasks at 35°C at 150 rpm for 7 days, then 0.1 ml from each culture medium were transferred and spread to agar plates of solid BH medium with 1% crude oil. Plates were incubated at 35°C for 3-4 days, the bacterial colonies were transferred to plates of nutrient agar for purification and stored for further steps [17,18].

Screening of bacterial isolates

The bacterial isolates were screened primarily using the 2,6-dichlorophenol indophenol method to determine the ability of isolates to degrade the crude oil. 7.5 ml of liquid BH medium was mixed with 50 µl of crude oil and then 40 µl of 2,6-dichlorophenol indophenol as an indicator was added. Thereafter, the test tubes were inoculated by loopful from each isolate, and the control tube was prepared without inoculum. All tubes were incubated at 35°C for five days. The isolates that caused a rapid discoloration of the medium from blue to colorless compared to the control were selected. After 5 days of incubation, the bacterial biomass in the tubes was separated by filtrating the mixture and after that, centrifuged the filtrate at 8000 rpm for 15 min, then the UV–VIS spectrophotometer was used to read the absorbance of the supernatant at 609 nm [19].

Subsequently, the equation below was used to estimate the percentage of biodegradation [19].

$$\%\ of\ biodegradation = 1 - \frac{Absorbance\ of\ treated\ sample}{Absorbance\ of\ control} \times 100$$

The secondary screening of isolates was achieved using a liquid BH medium, pH 7. The 25 ml of BH medium with 1% crude oil was inoculated by loopful from each isolate and incubated for 24 h. in a shaker incubator with 150 rpm at 35°C, thereafter, 1 ml from each overnight culture was used to inoculate new 100 ml of BH medium with 1% crude oil, then the shaker incubator was used to incubate the flasks at 150 rpm for 7 days at 35°C, also three flasks were prepared as control [medium without inoculum]. After a period of incubation, 100 ml of chloroform was used to extract the oil residues in each flask using a separator funnel and then the rotary evaporator at 50 °C was used to remove the chloroform. Subsequently, the equation below was used to estimate the rate of degradation [20].

$$Rate\ of\ biodegradation = \frac{Oil\ content\ of\ control - Oil\ content\ of\ treated\ sample}{Oil\ content\ of\ control} \times 100$$

Identification of selected isolate

The more efficient local bacterial isolate for oil biodegradation was identified according to the results of selected tests (morphological, cultural, and biochemical tests) that were described in Bergey's manual[21]. All biochemical tests plus its ability to grow on cetrimide agar were typical for *P. aeruginosa.* Further confirmation was done as described earlier [22] using universal primers.

Biosurfactant production by *Bacillus subtilis*

The local bacterial isolate *Bacillus subtilis* was selected as the best isolate in a previous study to produce the crude biosurfactant [23]. The 1 ml from the old culture (24 h) of *Bacillus subtilis* was used to inoculate each flask containing 100 ml of BH medium supplemented with 1% of crude oil and then the shaker incubator was used to incubate the flasks at 35°C for 7 days at 150 rpm. Thereafter, the crude biosurfactant was extracted from the whole culture [24].

Extraction of crude biosurfactant

After 7 days of incubation, the culture broth of *Bacillus subtilis* was centrifuged at 9000 rpm for 30 min to remove the bacterial cells. Then, the pH of cell-free broth was adjusted to pH 2 using the solution of sulfuric acid (1 M), thereafter the crude biosurfactant was extracted using an equal volume of the broth and, solvents mixture which was prepared from a ratio (2:1) of the chloroform and methanol. The cell-free broth was transferred to a separator funnel and then added an equal volume of solvents mixture was to extract the crude biosurfactant, thereafter, the organic layer was separated, and the solvents were evaporated by rotary evaporator and then dried at 60°C to obtain the constant weight [25] . For each batch of extracted crude biosurfactants, an emulsification index (E24) was determined before using it to prove the effectiveness of the biosurfactant [26].

Influence of crude biosurfactant on oil biodegradation

The sterilized liquid BH medium, pH 7 supplemented with 1% crude oil was used to study the influence of biosurfactant on crude oil biodegradation. The experiment was designed to know the effect of adding a biosurfactant to the BH medium inoculated with the most active isolate on crude oil biodegradation. Different weights (25, 50, 75, 100, 150, and 200 mg) of crude biosurfactant were added to each flask containing 100 ml of the culture medium of the most active isolate. Three flasks containing culture medium without crude biosurfactant were used as a positive control, also negative control was prepared from BH medium with 1% crude oil without bacterial inoculum. All flasks were incubated in a shaker incubator at 150 rpm at 35 °C for 7 days, then the oil residues were extracted from each flask. The method described above was used to calculate the percentage of biodegradation [27,28].

Results

Isolation of oil-degrading bacteria

The results indicate that only 22 bacterial isolates were isolated from the four samples that can consume crude oil as a carbon source, also Table (1) shows a discrepancy in the number of bacterial isolates that were obtained from each sample of soil contaminated with oil waste.

No.	Samples symbol	Number of isolates
1	ZSF	4
2	ZSS	7
3	SCF	5
4	SCS	6

Table 1: The number of bacterial isolates from contaminated two sites with oil pollutants in Baghdad city.

The current research used four contaminated soil samples to obtain the local bacterial isolates that can consume crude oil as an energy source.

Selection and identification of the most active isolate

The primary screening results as in Table (2) indicate a clear discrepancy in the ability of the isolates to degrade the crude oil, it was also observed that isolate SCS1 gave the highest ability to degrade the oil, reaching 52.6%, while eight isolates showed a very low ability that ranged between 3.4 – 8.4%, whilst the rest of the bacterial isolates gave the good ability to degrade the crude oil ranged from 18.4 – 46.1%.

NO.	Bacterial isolates	Biodegradation [%]	NO.	Bacterial isolates	Biodegradation [%]
1	ZSF1	41.3	12	SCF1	34.6
2	ZSF2	29.5	13	SCF2	7.1
3	ZSF3	5.8	14	SCF3	38.7
4	ZSF4	18.4	15	SCF4	26.4
5	ZSS1	44.9	16	SCF5	3.4
6	ZSS2	4.2	17	SCF6	41.9
7	ZSS3	45.7	18	SCS1	52.6
8	ZSS4	3.9	19	SCS2	8.4
9	ZSS5	22.8	20	SCS3	44.3
10	ZSS6	46.1	21	SCS4	6.7
11	ZSS7	6.4	22	SCS5	41.7

Table 2: The primary test of bacterial isolates for crude oil biodegradation using the 2,6-DCPIP indicator.

The results in Table (3) confirm the results of the primary screening, which indicate that isolate SCS1 showed the highest ability to degrade the crude oil, which reached 53.8%, while eight isolates also gave the lowest effect on oil degradation, whilst the rest of the isolates appeared a good ability to degrade the oil with different degradation rates. Therefore, the local isolate SCS1 was selected as the best isolate for crude oil degradation and to study the effect of different concentrations of biosurfactant on crude oil biodegradation. So, this isolate was identified as *Pseudomonas aeruginosa* according to its selected morphological characteristics and some biochemical tests.

No.	Bacterial isolates	Biodegradation [%]	No.	Bacterial isolates	Biodegradation [%]
1	ZSF1	41.8	12	SCF1	37.2
2	ZSF2	30.2	13	SCF2	7.6
3	ZSF3	5.6	14	SCF3	40.6
4	ZSF4	16.9	15	SCF4	24.9
5	ZSS1	45.7	16	SCF5	3.6
6	ZSS2	4.9	17	SCF6	40.1
7	ZSS3	44.5	18	SCS1	53.8
8	ZSS4	4.1	19	SCS2	8.2
9	ZSS5	29.4	20	SCS3	43.1
10	ZSS6	44.9	21	SCS4	6.9
11	ZSS7	6.4	22	SCS5	44.6

Table 3: The secondary test for estimating the efficiency of local bacterial isolates on crude oil biodegradation.

Crude oil biodegradation with and without biosurfactant

Figure (1) shows a positive effect on the efficiency of the crude oil degradation process by local isolate Pseudomonas aeruginosa when using biosurfactant at a concentration ranging between 25-100 mg/100 ml of the culture broth compared to the oil degradation process in the culture broth of Pseudomonas aeruginosa without biosurfactant. The results also indicate a negative effect when using the biosurfactant with concentrations of 150 and 200 mg on the degradation rate of crude oil, This may be due to the fact that the use of a high concentration of biosurfactant led to consuming biosurfactant first by local isolate and then consuming the crude oil.

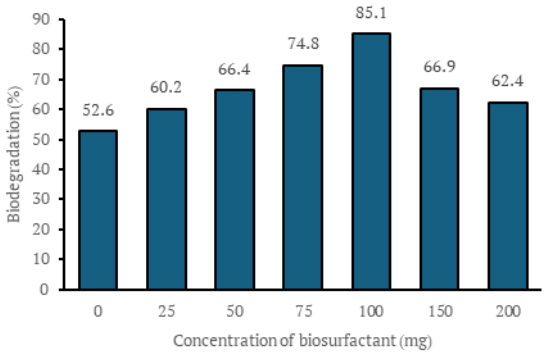

Figure 1: The efficiency of *Pseudomonas aeruginosa* to degrade the crude oil without and with different amounts of biosurfactant produced from *Bacillus subtilis* isolate.

Discussion

Our study aims to uncover a locally sourced bacterial strain proficient in breaking down crude oil. Additionally, we seek to explore how these bacteria perform in degrading crude oil, both with and without the assistance of a biosurfactant, shedding light on their potential applications in environmental cleanup efforts. One of the most important environmental problems diagnosed on a large scale is oil pollution, whether by crude oil or an oil product, as it represents at the present time a major problem that threatens all elements of the environment, especially soil and water, therefore many previous studies were conducted to get rid of these pollutants by using chemical, physical, or biological methods with the aim of collecting or breaking them down chemically or biologically into simpler materials that are not harmful to the environment [29,30]. Biological research is one of the best methods used to treat these pollutants because they are environmentally friendly and inexpensive, therefore, recent studies have tended to use vital emulsions with microorganisms used in treatment processes in order to increase oil degradation processes, since these emulsions increase the process of mixing oil waste with water and thus increasing the microbial ability to decompose and consume it as an energy source or convert it into simpler, non-toxic materials for the environment [31,32]. Many studies have been done to isolate the indigenous bacteria from the sites contaminated with spilled oil. The presence of indigenous bacteria in environments contaminated with oil was described in much previous literature as an essential factor for biodegradation, , also various studies showed that the bacterial isolates consuming oil and present in contaminated sites have an excellent role in degrading the oil pollutants when the oil spill occurred [33,34]. The isolates of *Bacillus* spp. were isolated in many studies from different sites contaminated with oil pollutants, most of these isolates showed a good ability to degrade several types of oil

pollutants because these bacterial isolates can consume the hydrocarbons pollutants for their growth, Therefore, bacterial species that are identified in the genus *Bacillus* are widely used in bio-treatment processes and other biological applications [35,36]. It was found that many isolates of the genus *Bacillus* which belongs to the species *B. subtilis, B. cereus*, have shown the ability to consume many types of hydrocarbon compounds during the biological treatment of crude oil [36]. It was also observed that isolates of *Bacillus* spp. showed a good ability to exist and grow in environments containing high concentrations of complex hydrocarbons because they have endospores, so the isolates they identified as *Bacillus* sp. appeared good efficacy in treating and removing various hydrocarbon pollutants, including polycyclic aromatic hydrocarbons [37]. Several reports indicated that bacterial isolates known as hydrocarbon-degrading bacteria can increase their ability to consume or degrade hydrophobic hydrocarbons by producing biosurfactants [38]. Bacterial isolates belonging to the genus *Bacillus* can produce biosurfactants with high efficiency, which are lipopeptide compounds. Recently, it was found through several studies that the use of biological surfactants led to an increased degradation capacity of the degradation of hydrocarbon pollutants [39,40]. It was found that stimulating the production of bio-surfactants by bacterial isolates during the biological treatment of hydrocarbon pollutants led to an increase in the capacity of bacterial isolates to degrade these pollutants due to the increased readiness of these pollutants for the bacterial isolates used in the biological treatment [40,41].

Through the results of the current study, it was found that supplementing the growth medium of the bacterial isolate used in experiments, *Bacillus subtilis*, with biosurfactants, led to an increase in the rate of biodegradation in all studied concentrations from 25-100 mg/ 100 ml medium compared to the growth medium [42] without the biosurfactant, while it was noted that the use of higher concentrations than that led to a decrease of biodegradation rates in the growth medium of the *Bacillus subtilis* isolate.

Author Contributions

Melad Khalaf. Mohammed: devised the idea for the study and sample collection,
Saad Hussein Khudhair: study design and microbial identification.
Ahmed Darweesh Jabbar: Biodegradation assay
All authors contributed to preparing the initial manuscript draft.

Conflict of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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