BIOSYNTHESIS OF IRON NANOPARTICLES BY PETROLEUM DEGRADING BACTERIA AND EVALUATION OF THEIR POTENTIAL FOR REMOVAL OF PETROLEUM CONTAMINANTS

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ABSTRACT

Environmental pollution by petroleum hydrocarbons is one of the significant concerns of the contemporary world. This study aimed to evaluate the enhancement of petroleum oil biodegradation using biosynthetic nanoparticles. Seventeen petroleum-degrading bacteria were isolated from an oil-polluted water samples in Alexandria, Egypt, by the Oil Refinery Company. The magnetic nanoparticles were biosynthesized by different bacterial isolates and the selection of the highest nanoparticles producer was investigated. The biosynthesis of nanoparticles and their role in the biodegradation process was determined. However, one bacterial species showed the highest production of nanoparticles. The synthesized nanoparticles were then analyzed using the dynamic light scattering technique (DLS) to determine the size of nanoparticles which was found to be 71.21nm. This isolate was identified by 16S rRNA gene sequence analysis into Pseudomonas aeruginosa ATCC 10145with a similarity of 99.53 %. Pure and bacterial consortium were utilized to study the bioremediation process

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with/without bio-nanoparticles. The oil remaining after bioremediation was extracted. After 14 days of incubation, the maximum degradation of crude oil was found to be 96.7 % with mixture of bacterial isolates was determined by GC analysis without bio-nanoparticles. The nanoparticles catalyst increases the microbiological reaction rates by stimulating the activity of microbes during the biodegradation process. The percentage biodegradation was increased with bacterial consortium and magnetic bio-nanoparticles to reach 98.9% after 7 days of incubation. With this excellent biodegradation efficiency, we believe that the bacterial consortium with bio-nanoparticles entails high potential treatment for industrial applications for the biodegradation of oil-contaminated sites.
Keywords: Biodegradation, biosynthesized nanoparticles, Bacteria, Crude oil

INTRODUCTION

Nanotechnology as a field of research is progressing day by day, creating an impact on almost every sphere of human life and generating a growing excitement in the biological science field particularly biotechnology and biomedical science (Prashanth *et al*. 2011).

Metal nanoparticles with at least one dimension approximately of 1–100 nm have received great attention in both technological and scientific areas due to their unique and unusual physicochemical properties (Vasileva *et al*. 2011). The importance of metal nanoparticles could be attributed to their catalytic activity, electronic characteristics, optical properties, antimicrobial, anticancer and magnetic activity (Khan *et al*. 2019). Furthermore, bio-nanotechnology is arising and combines principles of biology with physical and chemical procedures to synthesize nano-sized particles with particular functions (Kathiresan *et al*. 2009). Over the past several years, the synthesis

of various nanoparticles such as palladium, selenium, platinum, gold and silver using algae, fungi, bacteria and plant extracts has been reported (Torres *et al*. 2012).Whereas the available chemical methods are often expensive and comparatively complex, the synthesis of nano-materials by biological agents is both economically and environmentally green as they are established on green chemistry basics and are simple, comparatively inexpensive and can be scaled up for larger-scale production(Yasmin *et al*. 2020).

Green synthesis provides various advantages over the physical and chemical methods of nanoparticles synthesis as it is a cost-effective, eco-friendly, and easy modification procedure for high-scale production of nanoparticles as this method of synthesis does not use any hazardous chemicals, high pressure, temperature, and a high amount of energy (Ravindra *et al*. 2012; Prasad 2014).

These particles have unique and modified physical and chemical properties because of their particular electronic structure; large reactive, open surface area; and quantum size effects. Nanoparticles nowadays find their applications in many research areas such as biomedicine, electronics, cosmetics, textiles, etc. (Prasad *et al*. 2014).

Various methods can be employed for the synthesis of NPs, but these methods are broadly divided into two main classes i.e. (1) Bottom-up approach and (2) Top-down approach. These approaches are further divided

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into various subclasses based on the operation, reaction conditions and adopted protocols (Wang and Xia, 2004)

Different techniques can be used to estimate the size of the NPs. These include SEM, TEM, XRD, AFM, and dynamic light scattering (DLS). SEM, TEM, XRD and AFM can give a better idea about the particle size (Kestens *et al.*, 2016), but the zeta potential size analyzer/DLS can be used to find the NPs size at an extremely low level.

Among various types of nanomaterials, iron oxide nanoparticles (Fe3O4 NPs) have excellent catalytic and reductive properties to be used for wastewater treatment for removal of heavy metals, dyes, antibiotics and it has the advantage of ease of separation as compared to other nanomaterials requiring highly expensive centrifugation for separation. (Devi *et al*., 2019). The iron-based nanoparticles were found to be effective against various pathogenic bacterial strains and fungi as they can produce highly reactive oxygen species (ROS) (Muthukumar *et al*., 2019).

The developing countries with weak environmental law practices are facing severe oil spillage problem especially in the marine environment. Environmental pollution is increasing day by day due to wrong channeling and discharging of used engine oil in Open Ocean. These hydrocarbon contaminates are hazardous to the health of plants, as well as, they have carcinogenic, mutagenic and potent immuno-toxicants effects on human and animal health (Hossain *et al*., 2022).

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The higher activity of nanoparticles is usually due to their unique properties and high available active specific surface areas (Singh and Ahmed, 2010). Generally, nanoparticle catalysts increase the microbiological reaction rates by locating on the cells to stimulate the activity of microbes (Shan *et al*., 2005).

Water contamination by petroleum hydrocarbons in the Oil Refinery Companies, Egypt has caused environmental and health defects. So, the present study deals with the cleaning up of this contamination by bioremediation process. The degradation capacity of crude oil samples by pure and mixed bacterial cultures isolated from contaminated area is studied. Also, the aim of this work is to evaluate the enhancement of crude oil biodegradation (bioremediation) using biosynthetic nanoparticles

MATERIALS AND METHODS

1) **Sample collection:** Polluted water samples were obtained from two different contaminated sites in Alexandria, Egypt, at the Oil Refinery Company. The first sample was taken from the primary pond (before the mechanical treatment pond) (site1), and the second sample was taken from the secondary pond (after the mechanical treatment pond) (site 2).
2) **The chemical components:** All of the chemicals used in this investigation were of analytical grade, and were purchased from Sigma-Aldrich: sodium hydroxide, and ferric chloride (Steinheim, Germany). Oxide Ltd was used

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to purchase the Muller-Hinton-Broth and other materials (Hampshire, England).

3) **Physicochemical properties of water samples:** Standard test procedures were used to examine the pH value, electrical conductivity, total dissolved solids (TDS), density, specific gravity, and salinity value (The American Society for Testing and Materials, ASTM (El-Sheshtawy *et al*., 2014).
4) **Isolation & screening of oil degrading bacteria:** Mineral salts medium (MSM) and Bushnell Hass medium (BHM) were used for the isolation of crude oil degrading bacteria. The MSM medium (g/L) contains the following: $MgSO_4 \cdot 7H_2O$, 0.5; KH_2PO_4, 0.5; KCl, 0.1; NH_4NO_3, 4.0; K_2HPO_4, 1.0; NaCl, 2.0; $CaCl_2$, 0.01 and $FeSO_4 \cdot 7H_2O$, 0.01. Additionally, the composition of BHM (g/L) includes the following: KH_2PO_4, 1; K_2HPO_4, 0.2; NH_4NO_3, 1; NaCl, 2; $MgSO_4.7H_2O$, 0.2; $CaCl_2$, 0.02; $FeCl_3$ 2-dropes. The pH of the two media were adjusted at 7.0 ± 0.2, using 1% crude oil as a sole carbon source separately. The 250 mL conical flasks containing 100 mL of MSM and BHM broth were inoculated. Then, the inoculated sample flasks were incubated at an agitation of 150 rpm in a shaking incubator at 30 ± 2 °C, for 7 days. The MSM and BHM without inoculum were taken as control samples at constant culture conditions (Larik *et al*., 2019). The bacterial colonies formed on the plates were selected for further study.

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5) **Enumeration of total bacterial counts from contaminated water samples:** The total number of bacterial isolates was determined from oil contaminated water samples. The bacterial counts were implemented by using the plate count technique, where the polluted seawater samples (1 mL) were serially diluted in sterile saline. Then inoculation in Luria broth (LB) plates medium containing g/L: NaCl 10.0; tryptone 10.0; yeast extract 5.0. The medium was adjusted to pH 7.2±2. The cultures were then incubated at 30 oC for 24 h. Thereafter, a plate count in the range of 30 to 300 colonies was recorded. The experiments were conducted in three independent replicates. The bacterial count per mL was calculated from the following equation:

Bacterial count/mL= colony count per plate x dilution factor

The colonies with different morphological features were selected and purified on LB plates medium (Benson, 2001).

6) **The synthesis of nanoparticles by different bacterial isolates:** Biosynthesis refers to the phenomena which take place by means of biological processes or enzymatic reactions. These eco-friendly processes, referred to as green technology, can be used to obtain better metal nanoparticles from microbial cells (Mandal *et al*. 2006).

7) **Growth media for the production of nanoparticles:** The microbial test strains were inoculated in Muller-Hinton-Broth (MH) (g/L): Beef dehydrated infusion of 300.0, Casein hydrolysate 17.5, starch 1.5, and

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adjusted pH of 7.3 ±0.1. The cultivation of bacterial isolates was carried out by 1mL of fresh microbial culture suspension which corresponds to 10^6 CFU/mL, and then inoculated in autoclaved Muller-Hinton-Broth and incubated at 37 ºC for 18-24 h. After the incubation period, bacterial culture supernatants were collected by separation of cells by centrifugation at 5000 rpm for 30 min at room temperature. Then, the culture supernatants were filtrated by membrane-filtration technology using a vacuum and the cell-free supernatants were collected for further study. The controlled autoclaved Muller-Hinton-Broth without inoculation was used to check the sterility of the media (El-Sheshtawy *et al*., 2021).

8) **Extracellular synthesis of nanoparticles using microbial strains:** The extracellular synthesis of different nanoparticles, Zn-NPs and Fe-NPs, were performed by adding 1mM $ZnSO_4.7H_2O$ and $FeCl_3$ as nanoparticle precursors respectively to the harvested culture supernatants with a volume ratio (5:50).

All mixtures were incubated at ambient temperature and maintained in the dark for 24 h to avoid light effects.

After incubation time, the mixture was centrifuged at 5000 rpm for 30 min to precipitate the harvested metals-NPs, then washed with deionized water and dried in the oven at 80 ºC for 24 h. A control experiment was conducted with uninoculated media, to check for the role of bacteria in the synthesis of nanoparticles (Sunkar and Nachiyar 2012).

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9) Characterization of the biosynthesized nanoparticles

a) <u>Dynamic light scattering (DLS):</u> The particle sizes of the prepared samples were determined by the dynamic light scattering analytical method. The particle sizes were measured using a Zeta Sizer Nano instrument supplied by Malvern Instruments Ltd. For these popups, a 3 mg sample was dissolved in 10 mL of water and subjected to ultra-sonication for 5 min for stable suspension formation. The sample was then loaded into the cell area on the top of the instrument. The cell was then subjected to a laser beam to measure its luminescence. The particle size distribution curve was then taken by the apparatus software. The measurement was repeated 5 times for each sample to ensure reproducibility (El-Sheshtawy *et al*., 2021).

10) Identification and phylogenic affiliation of the potent bacterial isolate: The selected bacterial isolates which produced the nanoparticles were genomically identified using 16s rRNA by Sigma Scientific Services Co., Egypt. For 16S rRNA gene amplification, DNA was extracted using a protocol of the Gene Jet genomic DNA purification Kit (Thermo) (Sigma Scientific Services Co., Egypt). The 16S rRNA genes were amplified using a polymerase chain reaction (PCR) with (5'-AGA GTT TGA TCC TGG CTCAG-3') (5'-GGT TAC CTT ACG ACT T-3') as universal bacterial forward and reverse primers respectively. The PCR Purification Kit GeneJET™ applied for the cleanup of PCR for the production of pure

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PCR. A 45 µL of binding buffer was added to the completed PCR mixture. This mixture was thereafter fully transferred from step 1 to the GeneJET™ cleaning column. After that, the mixture was centrifuged for 30-60 s at >12000 xg, then the flow was discarded. A 100 µL wash buffer was added to the GeneJET™ cleaning column, centrifuged for 30-60 s, and ignored the flow-through and space the purification column back into the aggregation tube. The blend was centrifuged at the empty GeneJET™ purification column for a supplementary 1 min to eliminate any residual wash buffer. The purification column was transferred to a clean 1.5 mL micro-centrifuge tube. A 25 µL of elution buffer was then added to the center of the column membrane, which was then centrifuged for 1 min, discarding the column and storing the purified DNA at -20 °C. After the purification of the PCR products, the DNA sequence of the positive clone was subjected to a similar search, BLAST, on the NCBI website (http://www.ncbi.nlm.nih.gov), and deposited into GenBank. Many appropriate 16S rRNA gene sequences with validly exported names were chosen as references from the Gen- Bank.

11) **Crude oil degradation trial:** A 100 ml of mineral salt medium supplemented with 1% crude oil was prepared in 250 ml flasks. The medium contained (g/l): Na_2HPO_4 2.0, KH_2 PO_4 2.0, $MgSO_4$.$7H_2O$ 0.01, $NaNO_3$ 2.5, NaCl 0.8, $CaCl_2$, 0.2, KCl, 0.8, $FeSO_4$.$7H_2O$, 0.001, yeast extract, 3%, using crude oil as a carbon source. The pure bacterial isolates

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(B1 –B17) were inoculated into the MSM. Also, a mixture of these bacterial isolates (1ml of each isolate) was inoculated into the same type of medium. Hence, the flasks were incubated at 30°C, 150 rpm, pH 7.5 for 7 days (El-Sheshtawy *et al*., 2017). The remaining crude oil after treatment were extracted from different microcosms and gravimetric analysis was also performed.

12) **Synergistic remediation of oil using iron nanoparticles and degrading bacteria:** The bacterial strains was inoculated into MSM (100 ml) in a 250 ml Erlenmeyer flask. The cultures were incubated in a temperature controlled shaker incubator at 150 rpm at 30°C for 7 days, using (1g) of petroleum oil as a sole carbon source and adding (0.1 g) of nanoparticles. A sample without an inoculum was taken as a control. Hence, the flasks were incubated at 30°C, 150 rpm, pH 7.5 for seven days (Haddad *et al*., 2008). The residual crude oil samples were extracted from different microcosms and analyzed after seven days. The used crude oil as sole carbon source should be analyzed before and after inoculation and incubation to record its composition in order to investigate the metabolic activity of the cultured bacteria and the behavior of the strains in the presence of petroleum components especially by GC method was employed to determine in the residual constituent after cultivation of bacteria and NPs.

STATISTICAL ANALYSIS

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Data were unloaded through a statistical package for Social Sciences Statistical Program IBM SPSS V. 25 Statistical Package. Package and through it was using the following test: ANOVA test to study difference between samples.

RESULTS AND DISCUSSION

The production of nanoparticles had received great attraction in the last decades due to their wide range of applications. In this investigation, we wish to report on the production and characterization of nanoparticles from bacterial strains isolated from oil polluted water samples. Also, the role of nanoparticles for enhancing the biodegradation of crude oil is investigated.

1) Physico-chemical properties of water samples

The different physico-chemical parameters of the water samples obtained from 1 and 2sites at the oil refinery company were determined and listed in Table 1. The results obtained indicate that the sample of site 2 has higher electrical conductivity than that of site 1 water sample, and also high total dissolved solid, density and salinity.

Table(1): Physico-chemical characteristics of collected water samples

Properties	Water sample (site 1)	Water sample (site 2)
Density at 60 F	1.00455 g/ml	1.00340 g/ml
Specific gravity	1.00555	1.00439
pHat25 oC	7.107	7.749
Conductivity	6.25 ms/cm at17.5oC	15.75 ms/cm at 17.5oC
Conductivity	0.625 x 10-2 mohs/cm at17.5oC	1.575 x 10-2 mohs/cm at17.5oC

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Salinity (as NaCl)	2230 mg/l	4180 mg/l
Total Dissolved Solids (T.D.S.)	2320 mg/l	4200 mg/l

2) Isolation and screening of biodegrader bacterial isolates : Hydrocarbon degrading bacteria were first isolated almost a century ago. Head *et al*, (2006) reported that about 79 bacterial genera that can use hydrocarbons as the sole source of carbon and energy, as well as, 9 cyanobacterial genera, 103 fungal genera and 14 algal genera that are known to degrade or transform hydrocarbons.

This present study revealed that the bacterial population count at 8.5X105and 4X107CFU/mL using (LB) medium of oil-polluted water samples 1 and 2 respectively.

Additionally, the bacterial count on MSM medium at 15X107 and 9X105 CFU/mL of oil-polluted water samples 1 and 2 respectively after 7 days of incubation period. On the other hand, the bacterial count on BHM medium at 9X106 and 7X106 CFU/mL of oil-polluted water samples 1 and 2 respectively after7 days of incubation period. From these results the bacterial count of the sample (1) was higher than sample (2) when using different types of media (LB, MSM and BHM). As mentioned before the results of physio-chemical parameters of the water samples indicated that higher electrical conductivity, total dissolved solids, but 1 more than 2 and salinity of second water sample than first sample. Therefore, the second water sample is less suitable for the bacterial growth than first sample. Cai *et al*., (2015) reported

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that it widely believed that oil is a harsh habitat for microbes because of its high toxicity and hydrophobicity.

Seventeen bacterial isolates (B1-B17) were detected from oil-contaminated water samples by different culture media and their morphological characterizations are listed in (Table 2). In water sample (1)the bacterial isolates B11and B5 are considered as predominant isolateson BHM media. While, In water sample (2)the bacterial isolates B1,B11 andB8are the most predominant isolateson BHM media. The bacterial isolate B13 is considered as predominant isolate on LB and MSM media. So, B1, B5, B8, B11and B13 are the most predominant isolatesondifferent types of media.El-Sheshtawy *et al*., (2021) identified two bacterial species showing the highest growth rate on MSM medium.

Table(2): Morphological characteristic of the bacterial isolates' colonies

Isolate	characteristics						
	Media	**color**	**edge**	**elevation**	**shape**	**transparency**	**abundance**
B1	BHM2	creamy	irregular	flat	Egg shape with highlight	transparent	predominant
B2	BHM2	creamy	zigzag	flat	big	transparent	moderate
B3	BHM2	yellow	entire	flat	Big eye shape	opaque	moderate
B4	BHM2	Simon	entire	raised	circle	opaque	moderate
B5	BHM1	Green pigment	irregular	raised	big		predominant
B6	BHM1	Light creamy	entire	raised	Egg shape	transparent	moderate
B7	BHM2	Light creamy	irregular	raised	big	opaque	predominant
B8	BHM2	creamy	White highlight	flat	big	opaque	predominant
B9	BHM2	Dark creamy	irregular	flat		transparent	rare
B10	BHM1	Light yellow	entire	raised	Moderate size circle	opaque	moderate
B11	BHM1,	Creamy	entire	flat	Big	opaque	predominant

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	BHM2	Green pigment			irregular		
B12	LB1	Dark creamy	entire	raised	big	opaque	moderate
B13	LB2,MSM2	Light creamy	Egg shape entire	raised	Moderate size Egg shape	Opaque shine	predominant
B14	MSM1,MSM2, BHM2,BHM1, LB1	white	entire	raised	small	opaque	moderate
B15	LB2, MSM2	Light creamy	entire	raised	Small circle	Shine opaque	moderate
B16	LB1	Light creamy	entire	flat	Big circle	0paque	moderate
B17	MSM1,MSM2	creamy	Zigzag entire	raised	mucous	Shine opaque	moderate

Abundance*: Abundant, Moderate and Rare colony count on the growth media

CFU/100 ml**: Colony Forming Unit /100 ml MSM

Testing the ability of bacterial isolates for the production of nanoparticles

In the present study, the screening of bacterial isolates for producing nanoparticles and the selection of the highest nanoparticles producer was investigated.

Extracellular biosynthesis of Fe nanoparticles from Fe3+ solution was investigated using different bacterial isolates. Biosynthesis refers to the phenomena which take place by biological routes or through enzymatic reactions. These eco-friendly processes, referred to as green technology, can be used to achieve better metal nanoparticles using microbial cells (Mandal *et al*. 2006). Table (3) and Fig. 1 summarizes the amount of extracellular magnetic nanoparticles produced in this study. The bacterial isolates (B5 and B8) have high production of nanoparticles.

The extracellular biological synthesis of metal nanoparticles by the

investigated microbial strains resulted in the formation of NPs of different sizes, which might be attributed to the existence of different types of enzymes excreted by these organisms through the biosynthesis processes as previously reported by Ovais *et al*. (2018).

The data for dynamic light scattering (DLS) were collected, and the results are shown in Fig. 2. The DLS results show that the smallest particle size among the prepared magnetic nanoparticles was achieved by B5 and B8 (73.22 and 71.21nm, respectively). The results revealed that iron nanoparticles possessed the smallest size nanoparticles by B8, which is characterized not only by its high ability to produce nano-metals, but also by producing the smallest particle size compared with the other investigated bacterial isolates synthesizing nanoparticles. Using dynamic light scattering analysis, Ghorbani (2013) found that the size of the silver nanoparticles produced by S. typhimurium was 87nm. Therefore, the (B8) bacterial isolate was selected as having a high bacterial production of magnetic nanoparticles for genetic identification and its effect on the crude oil biodegradation process.

Table(3): ANOVA test to the difference between types of bacterial isolates according to gravimetric analysis for the production of nanoparticles with Fe precursor (g)

Different microorganisms	Mean	SD	SE	F	P-Value
B1	0.020	0.010	0.006	52.494	> 0.001 H.S
B2	0.020	0.000	0.000		

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Different microorganisms	Mean	SD	SE	F	P-Value
B3	0.033	0.006	0.003		
B4	0.057	0.006	0.003		
B5	0.073	0.006	0.003		
B6	0.030	0.000	0.000		
B7	0.013	0.006	0.003		
B8	0.057	0.006	0.003		
B9	0.013	0.006	0.003		
B10	0.027	0.006	0.003		
B11	0.017	0.006	0.003		
B12	0.030	0.000	0.000		
B13	0.017	0.006	0.003		
B14	0.010	0.000	0.000		
B15	0.002	0.000	0.000		
B16	0.003	0.000	0.000		
B17	0.004	0.001	0.001		
Total	0.025	0.020	0.003		

SD: Standard Deviation SE: Standard Error

P-value: The moral sign of statistical results (significant and non significant)

F: The value of ANOVA statistical analysis

** H.S: Highly Significant at P-value less than (0.01)

Table(3) show there is a significant difference at level (0.01) according to gravimetric analysis for the production of nanoparticles with Fe precursor (g) where (F) value was (52.494) which significant at level (0.01) in the direction of (B5) with mean ± SD (0.073 ± 0.006) which the highest mean.

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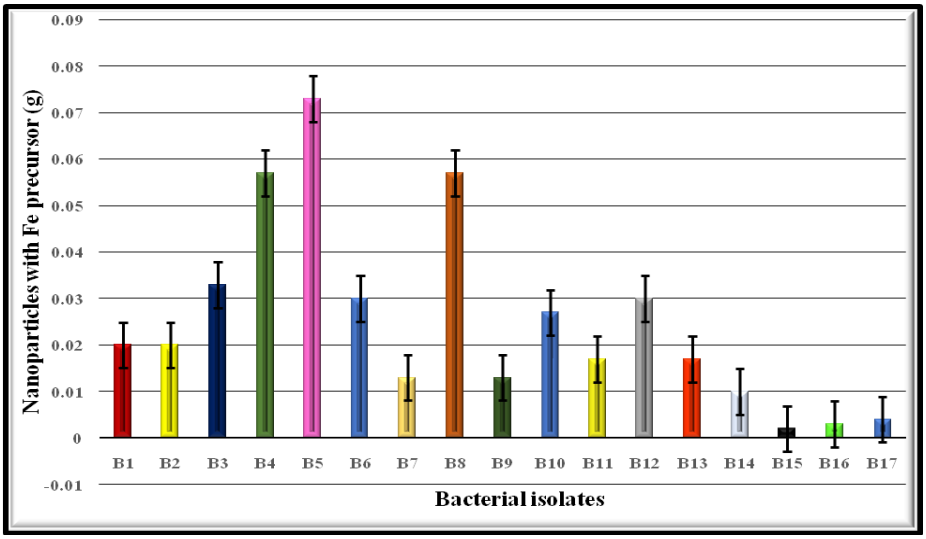

Fig(1): Gravimetric analysis for the production of nanoparticles with Fe precursor (g) by different types of bacterial isolates

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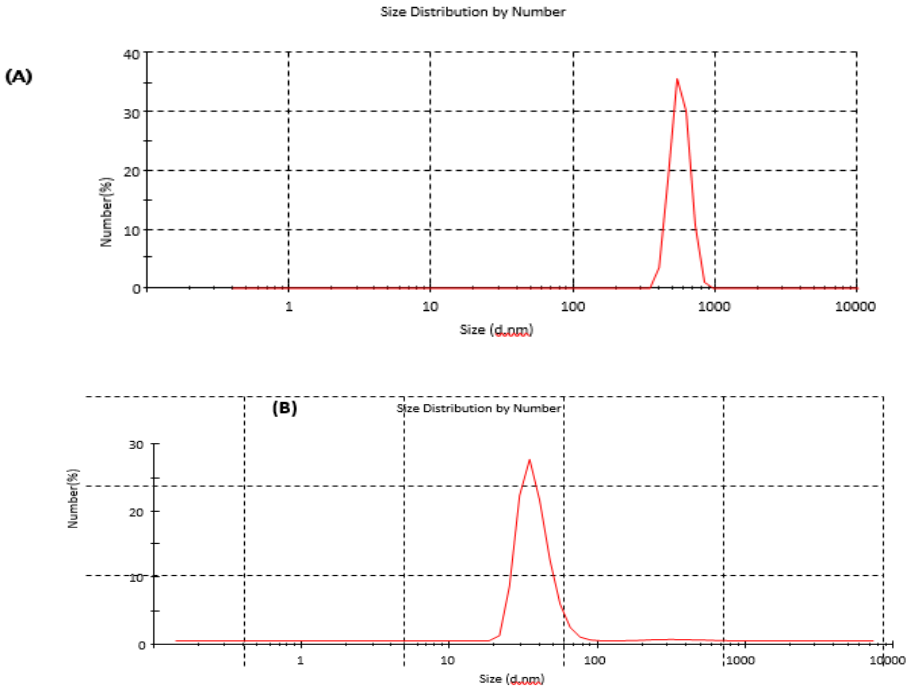

Fig(2): Magnetic nanoparticles size (A) Control, (B)produce by bacterial isolate (B8) (C) produce by bacterial isolate (B5) using dynamic light scattering analysis

3) Phylogenic analysis of the selected oil degrading bacteria: The bacterial isolate is a Gram-negative, rod-shaped under light microscope. The bacterial isolate B8 is identified by16S rRNA as Pseudomonas aeruginosa ATCC 10145with a similarity of 99.53 %. Using MEGA software. A sequence of Pseudomonas aeruginosa isolated in this study was matched with other species from BLAST analysis related to the Pseudomonas

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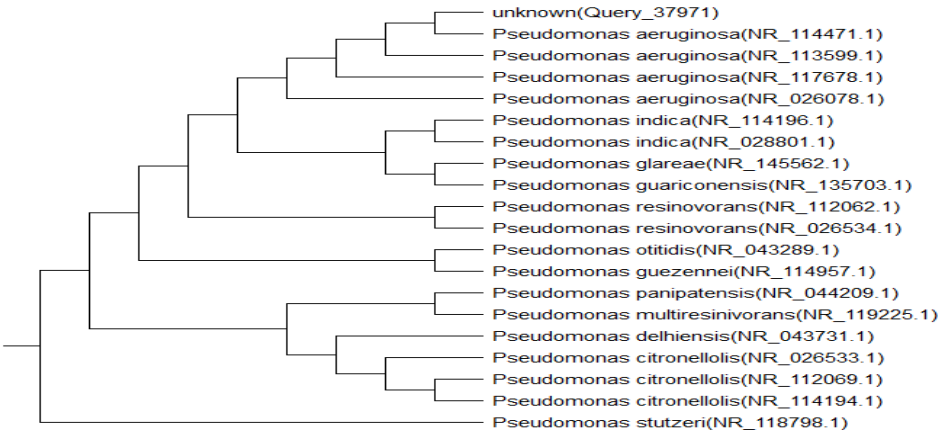
species. Fig. (3) illustrates the phylogenetic tree was reconstructed from Pseudomonas aeruginosa and closely related bacterial strains by the Neighbor-joining method of the 16S rRNA gene.

In the present study, it is evident that the Pseudomonas aeruginosa can produce magnetic iron oxide nanoparticles. Khan *et al*., (2019) reported that the biosynthesized magnetic nanoparticles can be used in many applications, including magnetic resonance imaging, diagnostics, and therapeutics (i.e., magnetic hyperthermia). Green synthesis of iron nanoparticles has accumulated an ultimate interest over the last few years to their distinctive properties, application in various fields of science and technology (Devatha *et al.*, 2018).



Fig(3): A phylogenetic tree of Pseudomonas aeruginosa ATCC 10145evolutionary relationships to other strains, taken from the NCBI database and the phylogenetic tree done by the Mega X program

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4) Gravimetric analysis of the degraded crude oil: The percentage biodegradation of crude oil by different bacterial isolates (B1toB17) was estimated by gravimetric analysis and the results are listed in (Table 4 and Fig. 4). The results reveal that the bacterial strains degraded the crude oil in the range from 18% to 95% after 14 days of incubation period. The microcosms containing the bacterial isolates (B8, B9, B11 and B13) separately gave higher percentage of degradation than other bacterial isolates. Verma *et al*. (2006) stated that, the oily sludge degradation capacity of Pseudomonas sp. SV 17, represents approximately 60% of saturates and aromatics fractions after 5days.El-Sheshtawy *et al*., (2014) showed that the bacterial strains degraded in the range from 30% to 50% of the crude oil after 7days.

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Table(4): ANOVA test to the difference between types of bacterial isolates according to residual of crude oil after biodegradation by different bacterial isolates as pure and their mixture of percentage degradation

Different microorganisms	Mean	SD	SE	F	P-Value
B1	58.00	1.00	0.58	337.44	> 0.001 H.S
B2	66.00	0.00	0.00		
B3	18.33	0.58	0.33		
B4	89.00	0.00	0.00		
B5	77.00	1.00	0.58		
B6	21.33	0.58	0.33		
B7	91.00	0.00	0.00		
B8	92.33	0.58	0.33		
B9	92.67	0.58	0.33		
B10	70.00	0.00	0.00		
B11	92.00	1.00	0.58		
B12	50.00	10.00	5.77		
B13	92.00	0.00	0.00		
B14	88.67	0.58	0.33		
B15	89.33	1.15	0.67		
B16	30.00	0.00	0.00		
B17	63.67	0.58	0.33		
Bacterial consortium	95.00	1.00	0.58		
Total	70.91	25.48	3.47		

SD: Standard Deviation SE: Standard Error

P-value: The moral sign of statistical results (significant and non significant)

F: The value of ANOVA statistical analysis

** H.S: Highly Significant at P-value less than (0.01)

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Table(4): show there is a significant difference at level (0.01) according to residual of crude oil after biodegradation by different bacterial isolates as pure and their mixture of percentage degradation where (F) value was (337.44) which significant at level (0.01) in the direction of (Bacterial consortium) with mean ± SD (95.0 ± 1.0) which the highest mean.

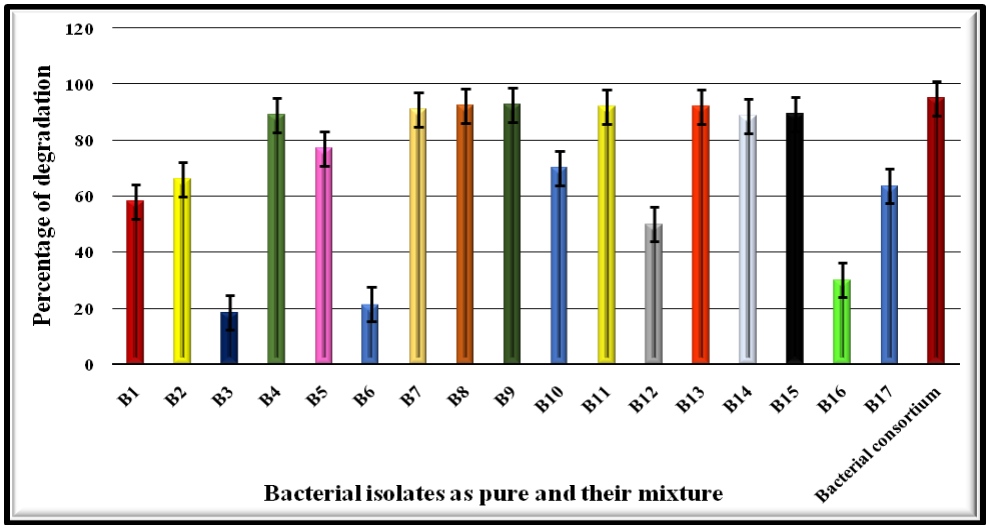

Fig.(4): Gravimetric analysis of oil biodegradation by bacterial isolates of 14 days incubation with pure or consortium culture starting with 10g/l crude oil

5) Gas chromatographic analysis: The first step in the aerobic degradation of alkanes by bacteria is catalyzed by oxygenases. These enzymes, which introduce oxygen atoms derived from molecular oxygen into the alkane substrate, play an important role in oil bioremediation and in the co-metabolic degradation of compounds (Vomberg and Klinner, 2000). In the present study, the biodegradation of crude oil was analyzed after 7 days of

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incubation period using the GC for aliphatic compounds. The data obtained show that the percentage degradation ranged from 16.76 % to 97.85 %. Additionally, the highest percentage degradation was determined in microcosms containing (B4, B7, B8, B9, B11, B13, B14 and the mixture of bacterial isolates about 90.58, 93.35, 97.85, 95.85, 96.59, 94.17, 97.07 %, and 97.7 respectively).

The percentages of residual n-paraffins and iso-paraffins present in crude oil after biodegradation were determined and compared with the control sample of Table 5. It was found that the best biodegradation of short-chain total paraffins was observed in microcosm containing B11 bacterial stain (Table 5 and Fig. 5). Additionally, the increasing of the percentage residual of the n- and iso-paraffins was detected in microcosm of the medium chain length (C21–C29) and long chain length (C30-C38). We believe that the increasing of the percentage in any compound may be attributed consuming of other high molecular weight compounds leading up to a proportional accumulation of such hydrocarbon compound on the low molecular weight compounds (El-Sheshtawy *et al*., 2015).

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Table(5): Percentages of residual of n- and iso-paraffins samples after bacterial degradation using GC chromatography

Bacterial isolate	**Short C12-C20**			**middleC21-C29**			**longC30-C38**		
	nor	iso	total	nor	iso	total	Nor	iso	total
control	27.28	22.25	49.53	22.45	20.67	43.12	7.35	0	7.35
1	17.75	5.21	22.96	47.38	7.51	54.89	19.93	2.22	22.15
2	28.2	9.03	37.23	42.24	7.74	49.98	12.25	0.54	12.79
3	26.41	5.86	32.27	44.78	5.35	50.13	16.23	1.37	17.6
4	23.78	4.93	28.71	49.07	5.34	54.41	16.45	0.43	16.88
5	14.42	3.29	17.71	41.45	21.84	63.29	15.13	3.87	19.00
6	18.75	8.90	27.65	39.95	12.37	52.32	18.65	1.38	20.03
7	28.29	5.55	33.84	43.64	0.00	43.64	22.52	0.00	22.52
8	25.74	3.24	28.98	54.41	0.72	55.13	15.62	0.27	15.89
9	26.24	5.99	32.23	40.67	13.27	53.94	13.8	0.03	13.83
10	22.94	3.89	26.83	51.91	2.37	54.28	17.52	1.37	18.89
11	10.6	0.00	10.6	56.88	11.21	68.09	19.2	2.11	21.31
12	19.02	4.71	23.73	42.11	13.86	55.97	19.62	0.68	20.3
13	9.25	8.33	17.58	57.07	12.26	69.33	13.09	0	13.09
14	18.96	0	18.96	67.81	5.46	73.27	7.77	0	7.77
15	29.35	4.	34.25	51.94	1.27	53.21	12.54	0	12.54
16	21.3	0	21.3	46.7	8.97	55.67	18.82	4.21	23.03
17	24.42	10.4	34.82	42.61	4.47	47.08	17.89	0.21	18.1

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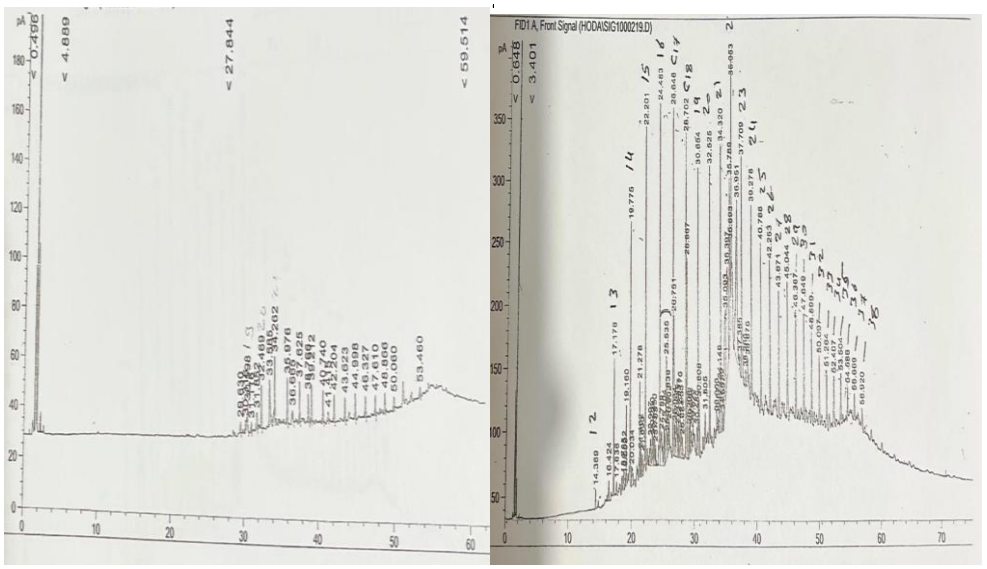

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Fig(5): Chromatogram of extract crude oil sample: (A) before sample as a control (b) as a representative treated crude sample by bacterial strain (B11)

6) Biodegradation of crude oil in the presence of magnetic bio-nanoparticles

a) Gas Chromatographic analysis: Magnetic NPs were synthesized from Fe2+ by P. aeruginosa. The iron NPs were obtained extracellular at pH7.0

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after 27h. The percentage biodegradation was determined with/ without of bio-nanoparticles is shown in Tables (6 and 7), Figs. (6 and 7).

The biodegradation process was accelerated by reducing the time of incubation from 14 days to 7 days with magnetic nanoparticles. El-Sheshtawy *et al*., (2021) investigated the maximum degradation efficiency of 93.32% after 3 days of incubation.

It was suspected that whether in the presence of nanoparticles either have no effect on the percentage of biodegradation process or enhanced it. In microcosms containing magnetic nanoparticles with (B2, B6, B8, B9, B10, B11, B14 and B15) the nanoparticles were found to have no effect on the biodegradation process.

On the other hand, the enhancement of the biodegradation process was observed in microcosms containing B3, B4, B5, B7, B12, B13, B16, B17, and bacterial consortium. The highest biodegradation of crude oil was observed in microcosm containing nanoparticles and bacterial consortium of different isolates after 7 days. The bacterium consortium is required to complete biodegradation of oil pollutants because the hydrocarbon mixtures vary markedly in volatility, solubility, and susceptibility to degradation, and the necessary enzymes needed cannot be found in a single organism (Adebusoye *et al*., 2007). This concept agrees with Bordenave *et al*., (2007); Al-Saleh *et al*., (2009) started that the individual microorganisms metabolize only a limited range of hydrocarbon substrates and crude oil isa complex mixture of

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compounds. So the biodegradation of it requires mixture of different bacterial groups to degrade a wider range of hydrocarbons. Zhang *et al*., (2010) reported that the percentage biodegradation of crude oil about 87.5% by a consortium of seven strains after 7 days.

The results in our hand indicate that, the presence of magnetic nanoparticles helps the bacterial isolates to highly consume the different molecular weights hydrocarbons which seems to be newly and valuable biodegradation trend. Hydrocarbons differ in their susceptibility to microbial attack and in the past, they had generally been ranked in the following order of decreasing susceptibility: n-alkanes > branched alkanes > low molecular weight aromatics > cycloalkanes (Paudyn *et al*., 2008). From the above results, it can be concluded that the presence of nanoparticles that provide efficient surface properties, can enhance the ability of the bacterial strains in terms of improving the biodegradation process. However, so far, very limited studies have been reported on nanoparticles effect on the microbiological reaction rates (Zhang *et al*., 2011)

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Table(6): ANOVA test to the difference between types of bacterial isolates according to percentage's degradation of crude oil by bacterial isolates without bio-nanoparticles using GC

Different microorganisms	Mean	SD	SE	F	P-Value
Control	0.00	0.00	0.00	15351944.49	> 0.001 H.S
B1	57.233	0.058	0.033		
B2	65.720	0.000	0.000		
B3	16.770	0.010	0.006		
B4	90.590	0.010	0.006		
B5	78.900	0.000	0.000		
B6	20.243	0.006	0.003		
B7	93.350	0.000	0.000		
B8	97.850	0.000	0.000		
B9	97.853	0.006	0.003		
B10	68.943	0.006	0.003		
B11	96.590	0.000	0.000		
B12	48.230	0.010	0.006		
B13	94.173	0.006	0.003		
B14	97.070	0.000	0.000		
B15	94.363	0.006	0.003		
B16	28.143	0.006	0.003		
B17	61.590	0.000	0.000		
Bacterial consortium	96.700	0.000	0.000		
Total	68.648	31.553	4.179		

SD: Standard Deviation SE: Standard Error

P-value: The moral sign of statistical results (significant and non significant)

F: The value of ANOVA statistical analysis

** H.S: Highly Significant at P-value less than (0.01)

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Table (6) show there is a significant difference at level (0.01) according to Residual of crude oil after biodegradation by different bacterial isolates as pure and their mixture of percentage of degradation% where (F) value was (15351944.49) which significant at level (0.01) in the direction of (Bacterial consortium) with mean ± SD (96.7 ± 0.0) which the highest mean.

Table(7): ANOVA test to the difference between types of bacterial isolates according to Percentage's degradation of crude oil by bacterial isolates with bio-nanoparticles using GC

Different microorganisms	Mean	SD	SE	F	P-Value
Control	0.00	0.00	0.00	63193.99	> 0.001 H.S
B1	60.57	0.64	0.367		
B2	65.25	0.00	0.000		
B3	30.07	0.06	0.033		
B4	94.22	0.00	0.000		
B5	88.63	0.06	0.033		
B6	21.33	0.58	0.333		
B7	96.07	0.15	0.088		
B8	97.20	0.10	0.058		
B9	95.00	0.00	0.000		
B10	67.91	0.00	0.000		
B11	96.50	0.10	0.058		
B12	53.83	0.06	0.033		
B13	96.00	0.00	0.000		
B14	97.89	0.00	0.000		
B15	94.00	0.00	0.000		
B16	58.63	0.01	0.006		
B17	70.88	0.00	0.000		
Bacterial consortium	98.91	0.01	0.006		
Total	72.78	29.07	3.85		

SD : Standard Deviation SE : Standard Error

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P-value: The moral sign of statistical results (significant and non significant)

F: The value of ANOVA statistical analysis

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Table (7) show there is a significant difference at level (0.01) according to Residual of crude oil after biodegradation by different bacterial isolates as pure and their mixture of percentage of degradation% with NPs where (F) value was (63193.99) which significant at level (0.01) in the direction of (Bacterial consortium) with mean ± SD (98.91 ± 0.01) which the highest mean.

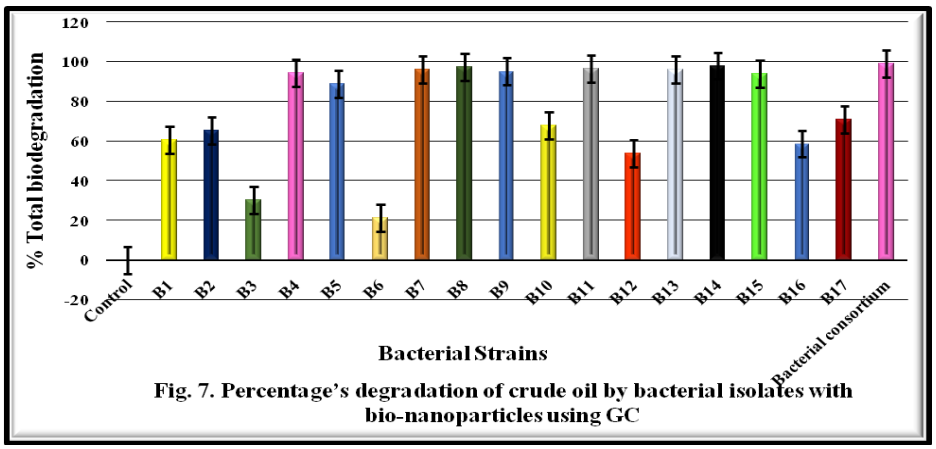

Fig. 7. Percentage's degradation of crude oil by bacterial isolates with bio-nanoparticles using GC

CONCLUSIONS

Petroleum contaminants are considered as one of the critical problems that face global nature, which causes the decline of environmental health. The bioremediation technology was simple and effective in this study to minimize

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the hazards of toxic materials. Nanoparticles seem to be new and valuable for the biodegradation trend. In the present study, the biosynthesized magnetic iron nanoparticles using different bacterial isolates were prepared and characterized. The smallest particle size was produced by bacterial isolate (B8) compared with other bacterial synthesized nanoparticles. This isolate was identified by 16S rRNA gene sequence analysis into Pseudomonas aeruginosa ATCC 10145with a similarity of 99.53 %. The individual bacterial strains have biodegradation abilities less slightly than their combination (consortium). The main reason for this is that petroleum hydrocarbons have wide range of volatility, solubility, and susceptibility to degradation. Therefore, the necessary enzymes needed for biodegradation cannot be found in a single organism. The microcosm containing mixed bacterial culture could carry out a maximum degradation at 89.9% for the studied crude oil after 7 days of incubation period in the presence of the magnetic bio-nanoparticles. Furthermore, the nanoparticles can enhance the bacterial consortium strains' ability to improve the biodegradation of crude oil. As mixed bacterial culture can efficiently degrade the crude oil contaminates of the water samples in sites in Alexandria, Egypt, by the Oil Refinery Company.

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التخليق الحيوي لجسيمات النانونية المغناطيسية عن طريق البكتريا المحللة للبترول وتقييم قدرتها على إزالة الملوثات البترولية

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المستخلص

التلوث البيئي من قبل الهيدروكربون النفطي هو واحد من الشواغل الهامة في العالم المعاصر.هدفت هذه الدراسة إلى تقييم تعزيز التحلل الحيوي لزيت البترول باستخدام الجسيمات النانوية المخلقة بيولوجيا. تم عزل سبعة عشر بكتيريا محللة للنفط عن عينات مياه ملوثة بالنفط الخاممن شركة تكرير النفط – الإسكندرية، مصر. تم التخليق الحيوي للجسيمات النانوية المغناطيسية بواسطة عزلات بكتيرية مختلفة. وتم فحص اختيار أعلى منتج للجسيمات النانوية. تم تحديد التخليق الحيوي للجسيمات النانوية ودورها في عملية التحلل البيولوجي. ومع ذلك، أظهرت النتائج أن أحد الأنواع البكتيرية أعطت أعلى إنتاج للجسيمات النانوية. ثم تم تحليل الجسيمات النانوية المركبة باستخدام تشتت الضوء الديناميكي (DLS) لتحديد حجم الجسيمات النانوية عند ٧١,٢١ نانومتر. تم تعريف هذه العزله من خلال تحليل تسلسل الجينات 16S rRNAالي Pseudomonas aeruginosa ATCC 10145 مع نسبة تشابه ٩٩,٥٣ ٪. تم دراسة عملية التحلل الحيوي للنفط الخام مع البكتيريا النقية ومع خليط من البكتيريا المحللة للزيت الخام مع / بدون جسيمات نانوية حيوية. تم استخراج النفط المتبقي بعد المعالجة الحيوية. بعد ١٤ يوما من فترة الحضانة، تم تحديد الحد الأقصى لتدهور النفط الخام عند ٩٦,٧٪ مع خليط من العزلات البكتيرية من خلال تحليل GC دون الجسيمات النانوية الحيوية.يزيدالمحفز الجسيمات النانوية من معدلات التفاعل الميكروبيولوجي عن طريق تحفيز نشاط الميكروبات أثناء عملية التحلل الحيوي. تم زيادة النسبة المئوية للتحلل الحيوي مع اتحاد البكتيريا والجسيمات الحيوية النانوية المغناطيسية إلى ٩٨,٩٪ بعد ٧ أيام من الحضانة. ومع هذه الكفاءة الممتازة للتحلل البيولوجي، أشارت هذه النتائج إلى أن الاتحاد البكتيري مع الجسيمات النانوية الحيوية يمكن تطبيقة صناعيا في التحلل البيولوجي للمواقع الملوثة بالنفط.

الكلمات الرئيسية: التحلل الحيوي، اللتخليق الحيوي للجسيمات النانوية، البكتيريا، النفط الخام

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