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Evaluation of Genotoxicity and Mutagenicity Induced by Crude Oil Contaminated Water Before and After Biodegradation.

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ABSTRACT

Crude oil and its derivatives are the most prevalent environmental pollutants to all biological systems and these products have severe impacts in the plant and animal ecosystem including human health. The present study aimed to treatment crude oil contaminated water and reduces genotoxicity induced by crude oil throughout biodegradation. Isolation of bacterial strain able to degrade crude oil was obtained and identified as *Enterobacter sp*. OCS1with 99% identity based on partial sequenced 16S rRNA analysis. This isolate was able to produce a biosurfactant during the course of hydrocarbon degradation. High biodegradation efficiency of heavy crude oil was observed after 14 days. It was 87.2% and 97.7% for heavy crude oil A and B, respectively. The current results indicated that the exposure of mice to crude oil, any of the two types under study, showed genotoxicity represented by increased the frequency of micronucleated cells in bone marrow and sperm abnormalities. In addition, they exhibited cytotoxicity as indicated by the decrease in polychromatic erythrocytes ration, sperm count, motility and sperm viability. However, treatment of crude oil contaminated water by isolated bacteria OCS1 resulted in a significant improvement in all tested parameters. The study suggests application of the OCS1 as an appropriate candidate for bioremediation of crude oil contaminants.
Key words: Genotoxicity, Crude oil, Biodegradation.

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INTRODUCTION

Particularly, crude oil spills into environments was observed during exploitation, refining and transportation. Accidental crude oil spills and frequent illegal disposal of oily waste into the sea result in various damage to marine life that is being affected by oil spills due to the presence of toxic organic compounds that affect human health [1].

Aromatic and Aliphatic hydrocarbons are the two fractions of crude oil that are reported for their health hazardous nature [2]. Crude oil exposure may cause damage to liver, lungs, intestines, kidneys and other internal organs [3-4]. Polycyclic aromatic hydrocarbons (PAH) may lead to cancer, nausea, Inhalation leads to headache, respiratory irritation, while dizziness, Toluene, Benzene, Eethyl benzene and Xylene cause mutations, cancers, nervous disorders, birth defects and liver disease, irregular heartbeats, depression etc. [1, 5-7]. Critical environmental and health defects of Crude oil pollution has been paid for implementing and developing innovative technology for cleaning up this contaminant. There are various methods for treating petroleum contaminated sites such as Chemical and Mechanical methods, but these methods are expensive and generally have limited effectiveness. On the other hand, biodegradation is the promising technology for the treatment of such petroleum pollutant areas since it will result in to complete mineralization and is cost-efficient. Biodegradation, which may refer to mineralization of hydrocarbon contaminants into water, carbon dioxide, cell protein, and inorganic compounds or conversion of complex organic pollutants to other simpler organic compounds by biodegradation agents such as microorganisms [8-10].

Various species of *Alcaligenes*, *Bacillus*, *Corynebacterium* and *Pseudomonas* have been observed to estimate petroleum oil degradation by producing biosurfactant [11-13].

Crude oil consist of a mixture of liquid hydrocarbon compounds, these hydrocarbon compounds are genotoxic substances that induce carcinogenic lesions in laboratory animals **[**14-15]. Other studies, indicated that it induced a clastogenic properties which commonly causes breaks of DNA, chromosomal damage and sister chromatid exchanges [3,16].

Monitoring the genotoxic effects of environmental contaminants including those derived from petroleum oil; required methodological instrument acting as a biomarker tools. The micronucleus (MN) test, one of those most frequently used in environmental genotoxicity researches, has served as an index of cytogenetic damage for over 30 years [17]**.** This is a sensitive, powerful indicator and fast test to detect genomic alterations due to clastogenic effects and impairments of mitotic spindle. The MN test was originally developed for the analysis of chemical genotoxicity in mammals [18-19]**.**

In recent studies, the attention is focused on isolation of bacteria strain which able to degrade crude oil and identification this strain based on 16S rRNA. In addition, the attentions are focused on the biosurfactant-production with hydrocarbon degrading microorganisms. Micronucleus test, reflecting genotoxic effects, is used as biomarker of early pollution. The genotoxic impact of the water contaminated with crude oil was assessed by studying the presence of micronucleus induction in the bone marrow, as an example for somatic cells, and sperm abnormalities, as an example for germ cells of mice drinking with this water and the possible bioremediation with isolated bacteria.

EXPERIMENTAL METHODS

Culture enrichment

Crude oil-contaminated sediments from Jeddah, KSA were collected and enriched in mineral salt media (MSM) containing 0.5% crude oil as the sole carbon source and incubated on a rotary shaker at 150 rpm and 37°C. The enriched cultures were purified and each isolate was subsequently inoculated in separate flask of the medium with the same substrate at the rate of 0.5% every 5 days. These enriched cultures were used for the degradation studies. The isolate with highest degradation was chosen for our study.

Characterization and identification of the isolate

The isolate was identified on the basis of their cultural, cellular morphology and biochemical characteristics using API 20E system (Biomerieux ,A.A.; France)[20].

Amplification of 16S rRNA Gene and Sequence Determination.

The genomic DNA was isolated from strain OCS1 according to the method described by Hesham [21] . the sequences in the 16S rRNA databases have been generated with the most common primer pairs 27F and 1492R. the reaction mixture volume of 25 μL containing 10 mM tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, each dNTP at 0.2 mM concentration, 1.25 IU of Taq polymerase, each primer at a concentration of 0.2 mM, and 1 μL of the DNA template. PCR was carried out with the following cycling parameters: 5 min of denaturation at 95 °C, followed by 30 cycles of 30 s at 95 °C (denaturing), 30 s at 56 °C (annealing), and 90 s at 72 °C (elongation), with a final extension at 72 °C for 7 min. Ten μL of the amplified mixture was then analyzed using 1.0% agarose gel electrophoresis. The gel was stained with staining of ethidium bromide, visualized under UV light, and photographed. Product of the correct size was purified and sequenced using an ABI automated sequencer.

Sequence Alignment and Phylogenetic Analysis

Sequences of inserts were compared to the 16S rRNA gene sequences of the OCS1 isolate in GenBank using BLAST software. To determine the taxonomic position of the OCS1 isolate, a phylogenetic tree was constructed with aligned using CLUSTAL W (1.81).

Evaluation of crude oil biodegradation

Biodegradation of hydrocarbon was assayed by inoculating 250 ml flasks containing 50 ml MSM medium with fresh bacterial culture. Crude oil was added as a sole carbon and energy source at a concentration of 0.5% (v/v). Biodegradation was monitored by determination of Biosurfactant, bacterial growth at OD 600 and residual hydrocarbon at 2 days interval.

Gravimetric Analysis.

The n-hexane was used to extract of the residual crude oil left in MSM at specified time interval and diluting with dichloromethane (CH_2Cl_2). The optical density (OD) of the crude oil extract against the blank at 420 nm was recorded on a Shimadzu UV(1800 UV–vis absorption spectrophotometer, Tokyo, Japan) [22].

Detection Cell-Surface Properties.

Phale et al. [23] study the production of extracellular biosurfactant by bacteria. This production was analyzed by monitoring the ability of biosurfactant to stabilize 1-naphthaldehyde in water emulsion. Five Millie of bacterial growth was centrifuged at 3000 rpm. The supernatant was used as a biosurfactant source. The reaction mixture (5 mL) contained 200 μL supernatant, 3.8 mL phosphate buffer (50 mM, pH 7.5), and 1mL of 1% of 1-naphthaldehyde in water emulsion (after sonicated for 1 min). The reaction mixture was vortexes for 1 min and incubated at room temperature for 5 h. The absorbance due to stability of emulsion was detected at 660 nm against control. One unit is defined as the amount of biosurfactants required to obtain an increase in absorbance of 1.0 OD unit.

Cell surface hydrophobicity determination

Cell surface hydrophobicity of Isolate was accessed by the bacterial adhesion to hydrocarbons assay [24]. Briefly, strain was grown in 30 mL MSM containing 0.5% (w/v) crude oil for 14 days in an orbital shaker at 37° C and 150 rpm. Cells were collected by centrifugation. Subsequently, an aliquot of 500 µL crude oil was added to the 5.0 mL microbial cell suspension and was vortexes for 2 min. The OD at 600 nm was taken.

BATH expressed as adherence of cell (%) to crude oil is calculated as follows:

BATH= {1- (A600 of the aqueous phase/A600 of the initial cell suspension)} X 100.

Effect of temperature on biodegradation

To study the effect of temperature on crude oil degradation, various incubation temperatures were studied. The conical flasks containing 100 ml of MSM supplemented with 0.5% crude oil were inoculated with 10^{-6} CFU/ml of isolate and incubated in a rotary shaker at 150 rpm and pH 7.0. Finally, samples were removed for analysis of crude oil degradation.

Effect of pH on biodegradation

The effect of pH was detected by incubating the culture at optimum temperature with various pHs(4-9) for 14 days. The pH of MSM media with crude oil (0.5%) was maintained using 1M NaOH and 1N HCl. To maintain the pH, phosphate buffer (pH 7 and 8), citrate phosphate buffer (pH 4– 6) and carbonate bicarbonate buffer (pH 9) were used [25]. The conical flasks containing 100 ml of MSM supplemented with 0.5% crude oil were inoculated with 10^{-6} CFU/ml of isolate and incubated in a rotary shaker at 150 rpm. Samples were removed for analysis of crude oil degradation. The optimum pH for maximum oil degradation was detected.

Effect of Inoculum concentration on biodegradation

To deteminate the inoculum concentration effect on crude oil degradation, different inoculum concentrations (10^{-3}, 10^{-4}, 10^{-5}, 10^{-6} and 10^{-7}) were inculcated in MSM. The conical flasks containing 100 ml of MSM supplemented with 0.5% crude oil were inoculated with isolate and incubated in a rotary shaker at 150 rpm, 37°C and pH 7.0. Crude oil degradation was analyzed.

Evaluation of genotoxicity

Animals were divided randomly into thirteen equal groups (5 animal / group). The first group was drinking with normal tap water daily and served as control. Animals of the second and third group were drinking with water contaminated with heavy crude oil (A) and (B) daily for ten consecutive days. Animals of the fourth and fifth group were drinking with water contaminated with heavy crude oil (A) and (B) after treated with isolated bacteria. After the last treatment (24h), animals were euthanized by cervical dislocation and samples of bone marrow were collected for the micronucleus assay and samples of epididymis were collected for sperm count, motility and viability and sperm abnormalities determinations.

Micronucleus assay

The micronucleus test was performed on bone marrow according to the method described by Salamone et al. [26]. Femurs were removed from the animals and a drop of bone marrow was placed on another drop of fetal calf serum on one end of a clean glass slide. Cells were spread with the aid of another slide and left to dry. Two slides were obtained per animal and stained with 5% Giemsa stain before microscopic examination. A total of two Thousand polychromatic erythrocytes (PCEs) were detected per mouse, and the frequency of micronucleated PCEs (Mn-PCEs) were detected. The ratio of PCEs to normochromatic erythrocytes (NCEs) was calculated for the determination of the cytotoxicity in bone marrow.

Sperm Abnormalities Analysis

After euthanized animal by cervical dislocation the epididymides (vas deferens, free of fats and other tissues) from each side of testis in each mouse (either control or treated) were removed and sperm were collected as quickly as possible. The content was thoroughly shaken, filtered through a silken cloth and dropped on grease-free clean slide to evaluate the movement and swimming activity of sperm to determine the motility of the sperm using microscope. Spermatozoa were counted using heamocytometer and sperm viability was evaluated under a light microscope after sperm stained with eosin nigrosin. For morphological abnormalities, a drop of a homogenate smeared on a cleaned slide was allowed to air drying and stained by Eosin Yellow to examine sperm head and tail abnormalities. For each animal, 1000 sperms were examined according to the criteria of Jeong et al. [27].

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Statistical Analysis

All data were statistically analyzed using analysis of variance (ANOVA). The significance of the differences among treatment groups was analyzed by Waller–Duncan k-ratio [28]. All statements of significance were based on probability of *P≤0.05*.

RESULTS

Isolation and identification of bacteria

The crude oil micro-flora enriched on MSM medium supplemented with crude oil as the sole carbon following several successive transfers in the enrichment medium. An enrichment culture was inoculated in MSM containing crude oil as a sole carbon and energy source for the isolation of crude oil degrading bacteria from crude oil contaminated soil collected from different sites in Jeddah, Saudi Arabia. A total of 13 bacterial strains were isolated. isolates were then tested for its ability to grow in MSM medium with the addition of 0.5% crude oil.

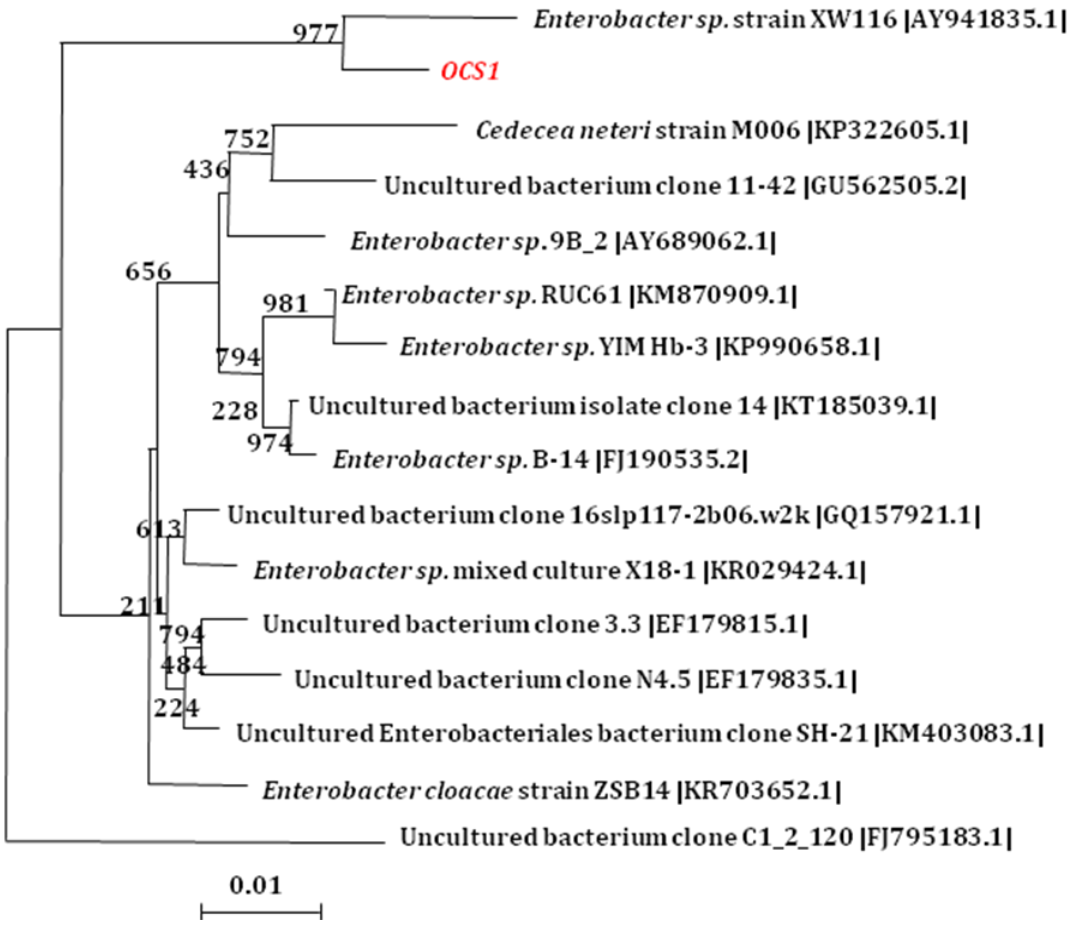

Figure (1): Phylogenetic tree for bacterial isolate based on nucleotide sequences of 16S r-RNA genes was constructed by neighbor-joining method. The scale bar shows the genetic distance. The number presented next to each node shows the percentage bootstrap value of 1000 replicates. The Uncultured bacterium clone Cl_2_120 treated as the out-group. The GenBank accession numbers of the bacteria are presented in parentheses.

Measure microbial hydrocarbon degrading ability by monitoring a color change, detect the highest degradable isolate that was designated OCS1. This strain degraded ~ 0.5% (v/v) of heavy crude oils (A and B) in less than 14 days.

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This isolate was aerobic, Gram-negative, rod shaped, motile and utilized glucose. Moreover this isolate was catalase and citrate test positive also reduced nitrate to nitrite. Spore forming, oxidase, indole, Methyl red, Voges Proskauer (VP), urease test and blood hemolysine were negative.

The degradable isolate was identified as *Enterobacter* sp. OCS1 with a GenBank accession number [LC114001] through the comparison of 16S rRNA gene sequence (Fig.1). The closest reference strain (99% identity) was *Enterobacter* sp. strain XW116 with a GenBank accession number AY941835.1.

Temperature, inoculums concentration and pH were adjustable factors. Change in the biodegradation efficiency was observed with the last factors.

Effect of temperature on biodegradation

Figure 2 (A and B) displayed an increase in biodegradation efficiency by increase the temperature from 25°C - 37°C in crude oil concentration 0.5%, shacking condition (150 rpm) and pH 7 for 14 days. The optimum temperature of biodegradation efficiency for both crude oils was 37°C, after that it was decrease by increase the temperature.

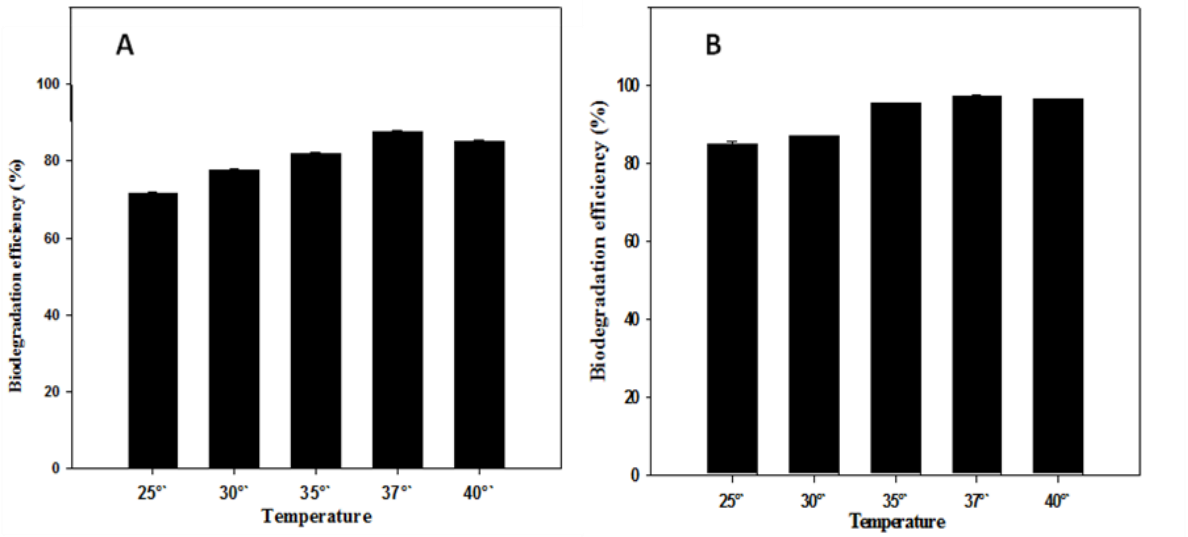

Figure (2): Effect of temperature in biodegradation efficiency of heavy crude oil (A) and (B) at concentration 0.5%, shacking condition (150 rpm) and pH 7 for 14 days.

Effect of pH on biodegradation

Effect of pH on the biodegradation efficiency was detected in figure 3 (A and B). The best pH of biodegradation efficiency displayed at pH 7 and weak basic pH 8 in heavy crude oil (A) and (B) at crude oil concentration 0.5%, 37°C and shacking condition (150rpm) for 14 days. The biodegradation decreased in acidic pH5 and basic pH 9 in both crude oils**.**

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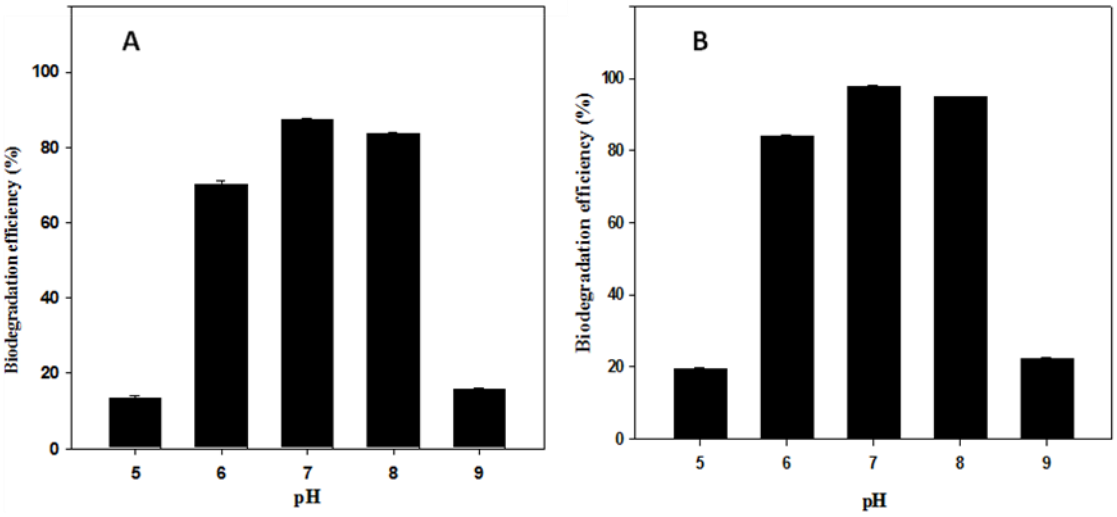

Figure (3): Effect of pH in biodegradation efficiency of heavy crude oil (A) and (B) at concentration 0.5%, shacking condition (150 rpm) and 37°C for 14 days.

Effect of inoculum concentration on biodegradation

By increasing the inoculum concentration, the biodegradation efficiency increased. Table (1) showed the high biodegradation at inoculum 10^{-7} for heavy crude oil (0.5 %) at pH 7, 37°C and shacking condition (150 rpm). Increases of the inoculum concentration have a positive effect.
All variables had significant influences on oil biodegradation, with temperature, pH and inoculum concentration having positive effects.

Among the biodegradation conditions studies, the optimum conditions for biodegradation of both crude oil were a pH at 7, inoculums concentration of 10^{-7} and temperature at 37 °C.

Table 1: Residual crude oil and Biodgradation efficiency (%) of the heavy crude oil Type (A) and (B) after biodegradation by the bacterial isolate at different inoculum concentrations, crude oil concentration 0.5%, shacking condition (150 rpm), pH 7 and 37°C for 14 days.

Type of crude oil	CFU/ml	Original conc. of crude oil (g/L)	Residue crude oil (g/L)	Biodegradation Efficiency (%)
Heavy crude oil (A)	10^{-3}	5	2.864 ± 0.1066	42.71% ± 0.3996
	10^{-4}	5	2.078 ± 0.0548	58.44% ± 0.2884
	10^{-5}	5	1.045 ± 0.0525	79.10% ± 0.3785
	10^{-6}	5	0.794 ± 0.0080	84.11% ± 0.4986
	10^{-7}	5	0.640 ± 0.0101	87.20% ± 0.4358
Heavy crude oil (B)	10^{-3}	5	2.650 ± 0.0321	47.00% ± 0.4041
	10^{-4}	5	1.793 ± 0.0136	64.14% ± 0.1527
	10^{-5}	5	0.695 ± 0.0125	86.10% ± 0.4041
	10^{-6}	5	0.356 ± 0.0072	92.88% ± 0.4618
	10^{-7}	5	0.115 ± 0.0032	97.70% ± 0.4725

The biodegradation of crude oil under optimum conditions

The endurance of microorganisms in hydrocarbons supplemented medium is an important factor in determining the biodegradation of hydrocarbons liquid phase. Since the strain OCS1 was isolated from a

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petroleum contaminated soil, it could easily survive in the oil-contaminated aqueous medium. Figure 4A and 4B, observed the bacterial growth, biosurfactant production, and crude oil biodegradation for both heavy crude oils at 37°C and pH 7, after 14 day under shaking condition (150 rpm). *Enterobacter sp.* OCS1 could be utilized crude oil hydrocarbon as the sole carbon and energy source. The bacterial growth of strain OCS1 increased gradually and reached the maximum after14 days in both heavy crude oils.

As illustrated in Figure 4A and 4B, strain OCS1 exhibited a high biodegradation efficiency (~ 87.20%; ~ 97.70%) of heavy crude oil (A) and heavy crude oil (B), respectively, at optimum condition.

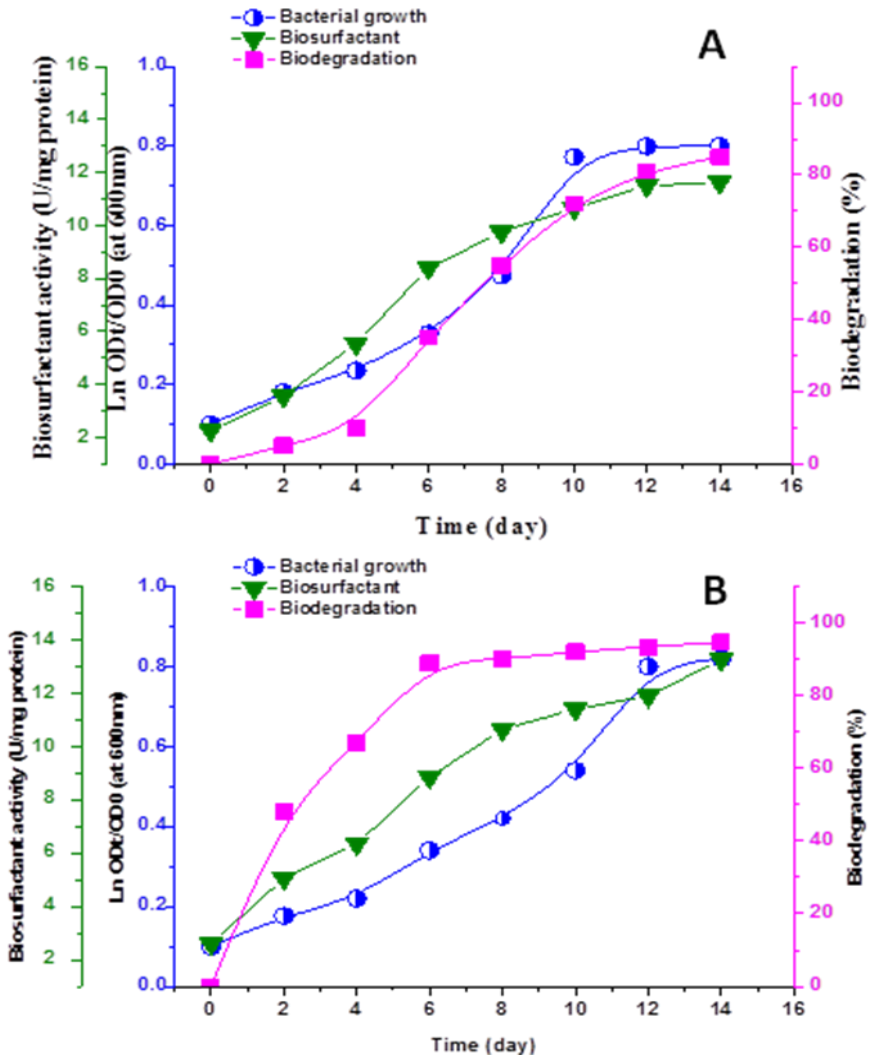

Figure 4: Typical time course profiles of the heavy crude oil degradation by *Enterobacter sp.* OCS1, cultivated in: (A) 100 mL MSM and 0.5±0.01 g heavy crude oil(A); (B) 100 mL MSM and 0.5±0.01 g heavy crude oil type (B), incubation was kept at 37°C and pH 7under shacking condition (150 rpm) for 14 days.

Production of biosurfactant in the medium by the culture and affinity of cells towards crude oil were studied. Figure 4A and 4B showed the biosutfactant emulsification activity which increased gradually to reach the maximum values at exponential phase (Figure 4A and 4B). After 14 days, there were the highest bacterial adhesion to hydrocarbons (BATH) which were 58%; 60% in case of heavy crude oil (A) and (B), respectively . The result of our study demonstrated high ability of isolated aerobic heterotrophic bacteria, *Enterobacter sp.* OCS1 to degrade total petroleum hydrocarbon. In the recent study, biosurfactant production was studied with 0.5 % (v/v) crude oil as the sole source of carbon and the strain exhibited a high yield of biosurfactant. The

kinetic growth curve of *Enterobacter sp.* OCS1 and the biosurfactant activity curve with upward trends indicated a parallel relationship between biosurfactant activity and bacteria growth.

Micronucleus Investigation

In micronucleus test, (Table 2) represent the percentage of micronucleated polychromatic erythrocytes (Mn-PCEs) in all groups managed and the polychromatic erythrocytes in proportion to normochromatic erythrocytes (PCEs/NCEs), all forms of these cells shown in Fig. 5. Table 2 showed that rates of Mn-PCEs in control untreated group within the accepted spontaneous range of the normal (3.6 ± 0.31), while treatment with the water contaminated with heavy crude oil (A) and (B) caused cytotoxicity and genotoxicity in cells of bone marrow represented in significant increase (*P<0.05*) in number of Mn-PCEs and lead to significant reduction in rate of PCEs/NCEs in all treated groups.

Table 2: Effect of different types of heavy crude oil contaminated water on the induction of micronuclei and the ratio of polychromatic to normochromatic erythrocytes before and after bacterial degradations.

Treatments	Mn-PCEs (Mean ± S.E.)	PCEs % (Mean ± S.E.)	NCEs % (Mean ± S.E.)	PCEs / NCEs (Mean ± S.E.)
1	3.60 ±0.31 ^{f}	54.0 ± 2.56 ^{a}	46.0 ± 2.55 ^{i}	1.17 ± 0.020 ^{a}
2	24.0±1.15 ^{a}	38.0 ± 1.46 ^{i}	62.0 ± 1.46 ^{a}	0.62 ± 0.014 ^{h}
3	16.0±1.15 ^{b}	40.6 ± 1.15 ^{hi}	59.4 ± 1.11^{ab}	0.68 ± 0.032^{gh}
4	15.0 ±1.20 ^{b}	40.7 ± 1.14 ^{hi}	59.3 ± 1.55^{ab}	0.68 ± 0.030^{gh}
5	8.00 ± 0.61^{de}	46.0 ± 1.16 ^{def}	54.0 ± 1.20^{def}	0.85 ± 0.040^{ef}

1: Control, 2: Heavy crude oil (A), 3: Heavy crude oil(B), 4: Heavy crude oil(A) treated with bacteria, 5: Heavy crude oil(B)treated with bacteria. Five rats were used in each group. Means with different superscripts (a, b, c, d) between groups in the same column are significantly different at *P<0.05*.

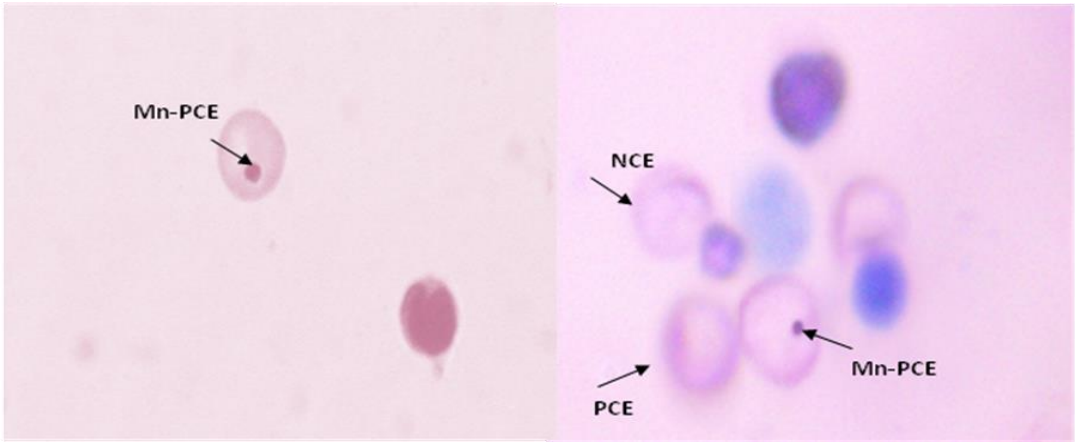

Figure (5): Showing different forms of bone marrow cells; Normal erythrocyte (NCE); Normal polychromatic erythrocyte (PCE) and micro-nucleated erythrocyte (Mn-PCE).

While treatment of the crude oil contaminated water with the *Enterobacter* sp. OCS1 bacteria gave significant marked recovery in frequency of Mn-PCEs and improvement in percentage of PCEs/NCEs in the treated group compared to groups managed with crude oil contaminated water only.

The percentage of the reduction of Mn-PCE in groups treated with *Enterobacter sp.*OCS1 bacteria was illustrated in Figure (6). The maximum inhibition percent was shown in groups treated with bacteria reach to 44 % and 64 % in heavy crude oil (A) and (B) treated groups.

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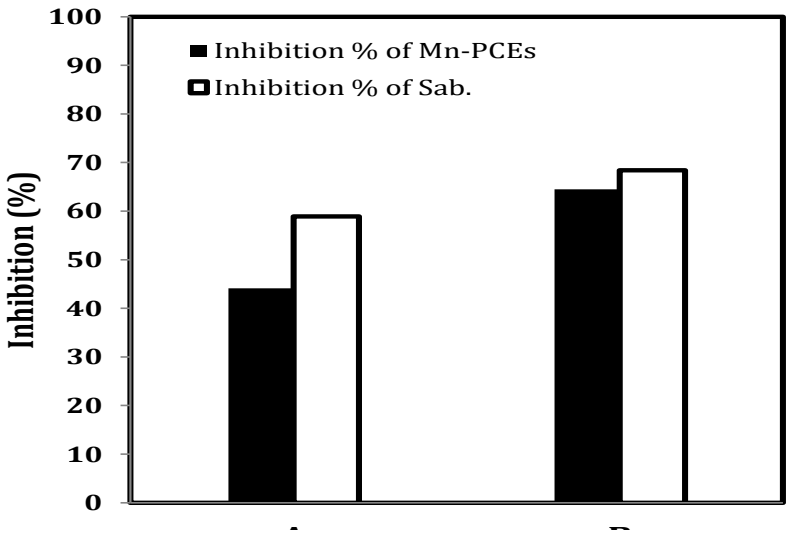

Figure (6): Histogram showing the inhibition percent of micro-nucleated polychromatic erythrocytes (Mn-PCE) and sperm abnormalities (Sab) in different treatments groups.

Sperm Abnormalities Assessment

The findings of the current study revealed that animals treated with water contaminated with (A) and (B) heavy crude oil, provoked marked deteriorations in epididymal functions as represented by reduced sperm count, motility and viability as well as, increased sperm head and tail abnormalities in the epididymal sperm of the treated mice (Tables 3, 4 and Figure 7).

Table 3: Effect of different types of heavy crude oil treated water on the epididymal sperm count, viability and total abnormality percentage before and after bacterial degradations.

	Treatments	Sperm count x 10^6	Sperm Viability (%)	Sperm motility (%)	Total Abnormal sperms
1		35.0 ± 1.15 ^{a}	91.0 ± 0.57 ^{a}	90.0 ± 1.56 ^{a}	5.3 ± 0.88 ^{g}
2		20.0 ± 1.11 ^{f}	60.0 ± 1.10 ^{gh}	55.0 ± 1.16 ^{h}	42.6 ± 1.46 ^{a}
3		24.0 ± 0.89 ^{de}	62.0 ± 0.61 ^{gh}	58.0 ± 1.20 ^{g}	36.0 ± 1.01 ^{b}
4		25.0 ± 1.50 ^{de}	68.0 ± 1.10 ^{f}	70.0 ± 1.14 ^{f}	20.6 ± 1.5 ^{d}
5		29.0 ± 0.61 ^{bc}	72.0 ± 1.51 ^{e}	75.0 ± 1.16 ^{e}	15.0 ± 1.2 ^{e}

1: Control, 2: Heavy crude oil (A), 3: Heavy crude oil (B), 4: Heavy crude oil (A) treated with bacteria, 5: Heavy crude oil(B)treated with bacteria. Means with different superscripts (a, b, c, d, e, f,g & h) between groups in the same column are significantly different at *P<0.05*.

Table 4: Effect of different types of heavy crude oil treated water on the epididymal sperm abnormality before and after bacterial degradations

Treat-ments	Tail Abnorma-lity	Head Abnormalities					Total Abnorma-lities
		Amorphous	Banana	Without-hock	Big head	Small head	
1	1.6 ± 0.33 ^{e}	2.0 ± 0.07 ^{de}	0.33 ± 0.25 ^{e}	1.3 ± 0.24 ^{de}	0	0	5.3 ± 0.80 ^{g}
2	11.3± 0.66 ^{a}	8.0 ± 0.25 ^{a}	8.3 ± 0.31 ^{a}	6.4 ± 0.30 ^{ab}	4.3 ± 0.45 ^{a}	4.3 ± 0.30 ^{b}	42.6± 1.45 ^{a}
3	9.7 ± 0.34 ^{ab}	5.6 ± 0.41 ^{b}	5.4 ± 0.25 ^{bc}	6.3 ± 0.34 ^{ab}	3.3 ± 0.30 ^{b}	5.6 ± 0.88 ^{a}	36.0 ± 1.1 ^{b}
4	6.3 ± 0.66 ^{c}	3.7 ± 0.61 ^{c}	3.2 ± 0.30 ^{c}	3.0 ± 0.57 ^{c}	3.0 ± 0.57 ^{b}	1.6 ± 0.33 ^{d}	20.0 ± 1.20 ^{d}
5	4.3 ± 0.33 ^{cd}	2.3 ± 0.42 ^{d}	2.33 ± 0.34 ^{c}	2.3 ± 0.35 ^{cd}	1.7 ± 0.35 ^{c}	2.0 ± 0.02 ^{d}	15.0± 1.20 ^{e}

1: Control, 2: Heavy crude oil (A), 3: Heavy crude oil (B), 4: Heavy crude oil(A)with bacteria, 5: Heavy crude oil(B)with bacteria. Means with different superscripts (a, b and c) between groups in the same column are significantly different at *P<0.05*.

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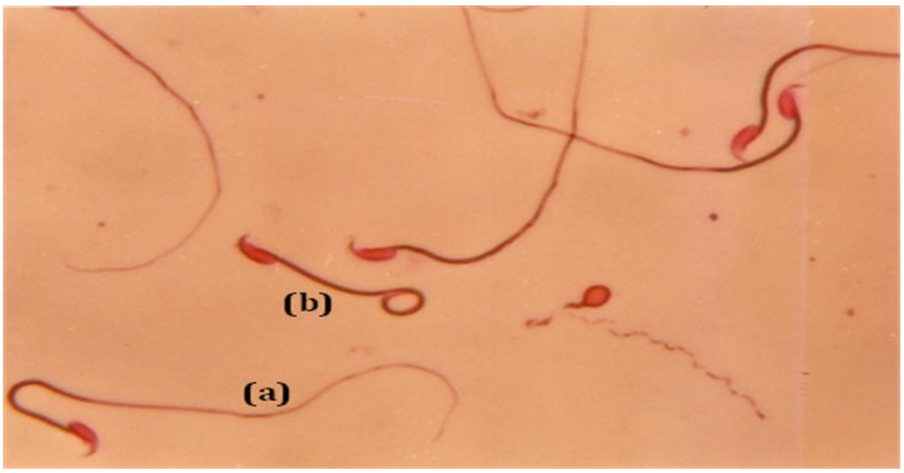

Figure (7): Showing normal and different forms of sperm abnormalities; (a) normal sperm, (b) banana head with coiled tail.

The present study indicated that treatment of crude oil contaminated water with *Enterobacter sp.* OCS1 bacteria significantly reduced the genotoxic and cytotoxic effect induced by crude oil as represented by a pronounced improvement in the epididymal function as shown by the increased sperm count, enhanced the percentage of viability and motility and decreased the incidence of sperm morphology aberrations (sperm head and tail abnormalities).

The percentage of the reduction of sperm abnormalities in groups treated with *Enterobacter sp.* OCS1 bacteria was illustrated in Figure (6). The maximum inhibition percent was shown in groups treated with bacteria reach to 59 % and 69 % in (A) and (B) heavy crude oil treated groups**.** Table (4) represents the mean of different abnormalities forms of sperm head and tail.

DISCUSSION

Biodegradation of petroleum hydrocarbons by aerobic heterotrophic bacteria in saline aquatic environment has been studied by other researchers. Biodegradation of hydrocarbon was studied by using aerobic bacteria as reported by Ichor et al. [29]. The result of our study demonstrated the ability of the aerobic *Enterobacter sp.* OCS1 isolate was able to degrade total petroleum hydrocarbon. This result was agreement with Obayori et al. [30] who studied the biodegrading ability of hydrocarbons (PAHs) by *Enterobacter cloacae*.

In this study, the increase of temperature from 25 to 37°C led to increase in biodegradation of crude oil and the optimum temperature was observed at 37°C. The positive effect of temperature on biodegradation rate of oil could be explained by increases in the solubility of hydrocarbon and the enzymatic activity of microorganisms. However, above 40°C, the membrane becomes more susceptible to hydrocarbon toxicity [31-32]. Moreover, a very high increase in temperature causes a decrease in enzymatic activity of microorganism and therefore decreases the hydrocarbon biodegradation rate. Increases in the inoculum concentration have a positive effect as reported by Ferreira et al. [31]. The optimum pH of biodegradation in recent study was observed at pH 7 and this was agreement with the previous study which reported, that the optimum pH for biodegradation of hydrocarbons was around 6-8 [33]. At optimum condition, the isolate OCS1 exhibited high Biodegradation of crude oil and high production of Biosurfactant. Various studies have reported on biodegradation of crude oil hydrocarbons in both terrestrial and marine environment under oxic conditions [34]. Crude-oil substrate could induce high biosurfactant production as reported by Zhang et al. [35]. In this study, biosurfactant production and bacteria growth indicated a parallel relationship as reported by Domingues et al. [36] and Kumari et al. [37]. The present investigation was also carried out to explore the genotoxicity and cytotoxic effects induced by crude oil contaminated water and investigated the possible bioremediation and degradation role of *Enterobacter sp.* OCS1 bacteria. It well be evident that the exposures of humans and animal to petroleum hydrocarbons, which is increasing in terms of the environmental levels, and application to body, have genotoxic and cytotoxic effects [38].

Crude oil as genotoxins can bind to DNA and trigger off a damaging biological changes, such as an impaired enzyme function or general metabolism, cytotoxicity, reproduction disturbances, immunotoxicity, growth inhibition, or carcinogenesis [39]. In the present study, treatment of mice with heavy crude oil contaminated water (A and B) for ten consecutive days induced genotoxicity in bone marrow cells by increasing the frequency of micronucleated cells as assessment by the micronucleus test. In this concern, Delunardo et al. [40] and Santos et al**.** [41] indicated that crude oil can provoked genotoxic damages as demonstrated by the induction of micronuclei in H. reidi erythrocytes at different time periods revealed that this test is efficient for detecting the genotoxicity of the compounds present in crude oil, mainly in the interval between 48 and 268 h of exposure, the highest amount of damage was observed during an exposure of 196 h. The micronucleus (MN) test, based on the presence of micronuclei that presents in actively dividing cell populations, has served as an index of cytogenetic damage [41-43**].** Micronuclei are produced in all cell types after irregular division when a chromosome fragment or a whole chromosome is not lost during the anaphase, but is delayed with respect to the chromosomes rest, constituting a small secondary nucleus in an interphase**.** The carcinogenic and mutagenic effects of aromatic hydrocarbon have been extensively investigated in mammalian and other animal cell systems **[**4, 14, 44-45]**.** In these concern Hassan et al. [14] proved that some polycyclic aromatic compounds are genotoxic substances that induce carcinogenic lesions in laboratory animals and significantly increased the induction of micronucleus in bone marrow cells. Also, Eskandari et al. [44] confirmed that the elevation of genotoxicity in mussels after a ten day exposure to crude oil is an obvious response to the genotoxic substances of crude oil.

Sperm abnormalities assessment is another biological marker indicating the cytogenetic damage induced by the exposure to the crude oil-contaminated water. The currently results clearly demonstrated that treatment of crude oil-contaminated water markedly decreases sperm count, motility, viability and increases the incidence of sperm abnormalities indicating a pronounced cytotoxic effect of the crude oil. These results confirmed well with the previous reports of Ramesh et al. [46] and Hassan et al. [14] who investigated that the exposure to polycyclic aromatic hydrocarbon induced epididymal deleterious dysfunction and provoked marked testicular cytotoxicity. Savitha and Panneerselvam [47] reported that the reduction in the sperm count often results due to the interference in the spermatogenesis and the elimination of sperm cells at different stages of development and increase apoptosis at specific stages of germinal cycle [48]**.** Hence, the decrease in epididymal sperm count observed in crude oil-treated mice might reflect the spermatogenic cell death. Polycyclic hydrocarbon decrease ATP which may serve as an energy source for sperm motility, and inhibit energy metabolism that may be one of the limiting factors responsible for loss of sperm motility as reported by Savitha and Panneerselvam [47]**.**

CONCLUSION

The present study indicated that, crude oil-induced genotoxic and cytotoxic effects on bone marrow and epididymal sperm of mice. In this response, *Enterobacter sp.* OCS1 was able to grow on the two types of crude oils and produce Biosurfactant. Therefore, all of these positive effects manifest that *Enterobacter sp*. OCS1 has great potential to be applied in heavy crude oil biodegradation and protect the genotoxicity and/or cytotoxicity induced.

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