Biodegradation of crude oil enhancing by using nanoliposome

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Abstract: The present study is to replace the chemical surfactants that used for treatment the oil spill with alternate substrates. These substrates such as industries wastes which contain high level of phospholipids e.g. waste of soybean oil can be used in preparation of nano-liposome. Moreover, the use of waste contributes towards a reduction in environmental pollution and the economic valuation of such products. The nano-liposome and nano-surfactant associated nano-liposome was tested to enhance the biodegradation process by applying essential nutrients for bacterial growth.The percentage biodegradation of crude oil by six bacterial strains were estimated by gravimetric analysis. The bacterial strains were degraded the crude oil in range from (35 to 60 %) after 7 days of incubation period. The medium containing mixed bacterial consortium (*Pseudomonas xanthomarina, Pseudomonas stutzeri* and *Bacillus subtilis spizizenii*) in the presence of nano-liposome as natural surfactant gave the higher percentage of degradation at 60 %.On the other hand, the presences of nano-tween80 associated nano-liposome was enhanced the biodegradation process of n-paraffins more than iso-paraffins by some microorganisms. On contrary, the percentage degradation of iso-paraffins increased by other bacterial isolates when using the nanoliposome only.
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1. Introduction

The escape of oil into the environment has increased gradually in quantity and has become more objectionable causing oil pollution (**Holman *et al.*, 1999**). The struggle pollution has become one of the most important problems for man to solve. Pollution by oil appears in different forms, as oil slicks on the surface of water, surface films, hydrocarbons dissolved and dispersed in water and tar balls.

Oil pollution is a sever global environmental problem causing a number of adverse negative impacts on human health, fisheries, agricultures, tourism, ecosystems and eventually the national income. Petroleum hydrocarbons are ubiquitous contaminants in the aquatic environment because of both human activities and natural processes (**Yasser, 2004**).

The three principal classes of hydrocarbons founds in crude oil are alkanes (paraffins), cycloalkanes (naphthenes), and aromatics, Trace quantities of alkenes (olefins), which are unsaturated chain compounds, may also be found in crude oil, and Non hydrocarbon petroleum constituents ***(*Arun *et al.*, 2011*)*** also found as sulphur, nitrogen, porphyrins, oxygen, resins, asphaltenes and trace metals.

The fate of most petroleum substances in the marine environment is ultimately defined by their transformation and degradation due to microbial activity. About a hundred known species of bacteria and fungi are able to use oil components to sustain their growth and metabolism. In pristine areas, their proportions usually do not exceed 0.1-1.0% of the total abundance of heterotrophic bacterial communities. In areas polluted by oil, however, this portion increases to 1-10 % (**Atlas, 1993).** Biodegradation of oil is one of the most important processes involved in weathering and the eventual removal of petroleum from the environment (**Prince *et al.*, 2003)**. Biodegradation of hydrocarbons by natural populations of microorganisms (such as many species of bacteria, fungi, and yeasts) represents one of primary mechanisms by which petroleum and other hydrocarbon pollutants are eliminated from the environment **(Leathy and Colwell, 1990; Prince, 1993**).

The resistance to biodegradation of aromatics increases with ring numbers and their molecular weights (**Balba *et al.*, 1998**). Polyaromatics hydrocarbons (PAHs) composed of four or more rings tend to be more resistance to biodegradation as they adsorb into sediment and soil, since they are hydrophobic hydrocarbons (**Jones, 1998**). Although these components of oil are less biodegradable than the saturate components, it has been demonstrated that with biostimulation the rate of degradation can be increased by 40% (**Jones, 1998**).The existence of

PAHs in the environment has caused great environment concerns (**Arun *et al.,* 2011**).

The chemical dispersion is found to be one of the most common used methods for oil pollution treatment. The use of dispersants is becoming more popular because of the failure of more conventional techniques to clean up spills such as those from the Argo Merchant. The ability to evenly distribute the dispersant is questionable, and the cost is enormous. Chemical Dispersants are liquid blends of surfactants (surface active agents) and solvents, designed to hasten breakup of oil slicks into fine droplets that disperse naturally in the sea. An important feature of dispersants is the ability to break water-in-oil emulsions that form naturally as the oil slick (**Lewis *et al.,* 1995; Fiocco and Lessard, 1997**).

The surfactant molecules in oil spill dispersants can be classified according to their charges into non-ionic surfactants (tween 80), ionic surfactants and amphoteric or Zwitter ionic. The presence of surfactants enhanced the biodegradation of phenanthrene due to its increased solubility, compared with its biodegradation in the absence of surfactant.

Generally, nonionic surfactants are less toxic to microorganisms than ionic surfactants. The negatively charged surface of bacterial cells makes the cells more sensitive to the introduction of charged surfactants, especially positively charged cationic surfactants. The toxicity of a surfactant is also dependent on its molecular structure. Toxicity decreased with increasing hydrophilicity (HLB) of the surfactants. The increasing order of toxicity of the surfactants is followed by nonionic surfactants (Tween 80) < anionic surfactants < cationic surfactants. The previous studies indicate that nonionic surfactants are better choices for enhancing the biodegradation of PAHs, due to their low cytotoxicity. Polyoxyethylenesorbitan surfactants (Tween series) have been shown to possess low toxicity **(Zheng and Obbard, 2001; Jin *et al.,* 2007; Cheng *et al.,* 2008)**. For the same head group and similar molecular structure, the toxicity to the bacteria is affected by the chain length of the hydrophilic moiety. Generally, surfactant toxicity becomes lower as the chain length increases (i.e. an increasing hydrophilicity or HLB) **(Jin *et al.,* 2007)**.

Liposome and nano-liposome are amphipathic surfactants have emulsification activity, can be manufactured by using safe ingredients obtained from natural sources, such as egg, soy, or milk (**Thompson, 2007**). Nano-liposome may contain, in addition to phospholipids, other molecules such as cholesterol.

The complexation between polymer (as tween 80) and liposome PC/Chol is a way to increase the long-term stability of liposomes, as well as inhibit liposome fusion (**Trubetskoy and Torchilin, 1995; Barenholz, 2001; Hwang *et al.,* 2001**).

Liposome is not widely used by industries due to its high production cost and use of expensive substances.The aim of the present study is to reduce the production cost; by the use of waste soybean which contain high levels of natural phospholipids (PC) necessary for production of liposome. Moreover, the use of waste contributes towards a reduction in environmental pollution and the economic valuation of such products.Treatment of oil spill on water by applying nano-liposomes and nano-surfactant (nano-tween 80) associated liposome to enhance biodegradation process by applying essential nutrients for bacterial growth.

2. Material and Methods

All chemical reagent (Tween 80, Soybean [L-α phosphotidylcholine (PC)] and cholesterol) (99% pure) were donation from Sigma Aldrich Company.

2.1. Crude oil

An Arabian medium crude oil used for this study was acquired from western desert. Their specifications are listed in the result and discussion part.

2.2. Bacterial strains

Bacillus licheniformis ATCC 10716, *Pseudomonas aeruginosa* ATCC-10145, *Rhodococcus erythropolis* ATCC 13260 were supplied by the microbial resources center (MIRCEN), Faculty of Agriculture, Ain Shams University, Cairo, Egypt.

Pseudomonas xanthomarina KMM 1447 and *Pseudomonas stutzeri* ATCC 17588 bacterial isolates isolated from at Gemsa Bay (Red Sea).

Bacillus subtilis spizizenii DSM=15029 bacterial isolates isolated from sediment contaminated by crude oil in Suez Oil Processing Company (SOPC), Egypt.

2.3. Physico –chemical characteristics of the tested crude oil

The general physico-chemical characteristics of the crude oil; has been determined according to the ASTM, IP and UOP standard methods. The density of the crude oil was determined according to the IP 190 capillary stoppered pyknometer method. Also, the Kinematic viscosity and pour point of the crude oil were determined according to the ASTM D445-IP71glass capillary viscometer method and ASTM D9-IP 15 methods respectively. The sulfur content was determined by the quartz tube method according to the ASTM-D1551 and IP 63 methods. Wax content of the four crude oils was determined according to UOP 46-85. Molecular weight of crude oil and its fractions was determined according to

ASTM-D 2505. The carbon residue content of the crude oil was determined according to the ASTM D524-IP 14 Rams bottom carbon residue method.

2.4. Fractionation of crude oil into their components hydrocarbon types and classes

Crude oils were fractionated into their components, hydrocarbon types and classes, namely asphaltene, resins, saturates, mono-, di-, poly aromatics.

The hydrocarbon classes were characterization by determining their refractive index as shown in (Table 1).

Table 1. Refractive index (R.I.) of hydrocarbons

Hydrocarbon	R.I. at 20°C
Saturate	<1.48
Monoaromatics	1.48-1.53
Diaromatic	1.53-1.59
Polyaromatic	>1.59
Resin	Dark

2.5. Preparation of liposome by Reverse phase evaporation

2.5.1. Liposome was prepared by modified reverse phase evaporation (mREV) (Andreas and Karola, 2010)

Liposome PC/Chol (2:1) were dissolved in chloroform: ethanol (Sigma Aldrich chemical company) (2:1) and transferred into suitable conical flask. The flask was then connected to rotary evaporator and water bath with temperature at 50-60°C under aspirate vacuum to ensure total removal of solvent. When the thin film layer formed in the wall of the flask, temperature decreases gradually to 30°C. The thin film layer formed was flushed with nitrogen gas for 5 min, and maintained overnight under vacuum to remove trace of solvent.

The thin film was re-suspended in 10ml of buffer (pH 7) in deionized water by using gentle sonication for 20min. at room temperature until the lipid film was completely hydrated. Liposome which carried different molar proportions of PC/Chol (1:0, 1:1, and 2:1) was studied.

2.5.2. Preparation of polymer associated liposome

The complexation between polymer (tween 80) and liposome has been studied as way to increase the long-term stability of liposome. Grafting hydrophilic polymer onto the head groups of phospholipids, or the addition of water soluble polymer containing several hydrophobic groups has been shown to increase circulation time, as well as to inhibit liposome fusion (**Trubetskoy and Torchilin, 1995; Barenholz, 2001; Hwang *et al.*, 2001**).

The polymer was dissolved in deionized water were added to liposome suspension previously prepared by ratio (1:1). The PC/cholesterol ratio was the same in all samples (2:1). The mixture was sonicated using high speed for 10 min.

2.5.3. Preparation nano-liposome

Nanoliposome was prepared by modified reverse phase evaporation (mREV). The PC-Cholesterol liposome and polymer associated PC-cholesterol liposome suspension were sonicated by probe-type sonicator at pulse on for 5 seconds and pulse off 10 second for 3 min (**Hamid *et al.*, 2011**).

In the final steps, the nano-liposome sample was stored in sterilized bottle in the dark at room temperature.

2.6. Characterization of liposome

2.6.1. Transition electron microscopy (TEM)

The morphology of nanoliposome was studied by TEM. Each sample was fixed on a Formvar cupper-coated carbon grid using a solution containing 2% uranyl acetate to enhance image quality (**Monique *et al.*, 2007**). The excess staining solution was removed with filter paper in 30s at room temperature.

Images were acquired using a transmission electron microscope (JEOL, JEM-2100, HRTEM) operated at an accelerating voltage of 80KV to avoid fusion of liposome.

2.6.2. Dynamic light scattering and Zeta potential

The 'liposome' mean diameter, size distribution, and zeta potential were measured with dynamic light scattering (DLS) "NANO ZS", Malvern Zetasizer equipment (Worcestershire, UK), at 25°C, using a He-Ne laser of 633 nm, size range (0.3nm -10 µm), Zeta potential range (3.8nm-100 µm)**(Yuan Tong *et al.*, 2010; Ana *et al.*, 2011).**

2.7. Crude oil degradation trial

The ability of different bacterial strains to degrade crude oil under aerobic condition was evaluated. 100 ml of mineral salt medium (MSM) into 250 ml flasks supplemented with 2 % crude oil was prepared (**Haghighat *et al.*, 2008**). The pure bacterial strains, (2 ml, 2 x 10^7 CFU/ml) were inoculated into the MSM. Mineral salt medium (MSM) was used for biodegradation process. The medium contained (g/l): Na_2HPO_4 2.0, KH_2PO_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 carbon source and 1ml of a trace element solution. Trace element solution contained $ZnSO_4.7H_2O$, 525 mg/l, $MnSO_4.4H_2O$, 200 mg/l, $CuSO_4.5H_2O$ 705 mg/l, $Na_2MnO_4.2H_2O$, 15 mg/l, $CoCl_2.6H_2O$, 200mg/l, H_3BO_3, 15 mg/l, $NiSO_4.6H_2O$, 27mg/l

Also, mixture of the known bacterial strains and mixture of the isolated bacterial strains (1ml of each isolate) were inoculated into the same type of medium. Hence, the flasks were incubated at 30°C, 150 rpm, pH 7.5 for 7 days. The remaining crude oil

samples were extracted from different microcosm and gravimetric analysis was also determined.

2.8. Biodegradation of crude oil in the presence of nano-liposome and nano-tween80-associated nano-liposome separately

The crude oil (2g) was added to the mineral salt medium MSM (100 ml) in a 500 ml Erlenmeyer flask containing as a sole carbon source and 0.1 g of different nano-surfactants separately. The flasks were autoclaved at 120°C for 20 min. The bacterial strains (2 ml of inoculum) were inoculated into MSM (100 ml). The cultures were incubated on a temperature controlled shaker incubator at 150 rpm at 30°C for 7 days. A sample without inoculum was taken as a control (**Haghighat *et al.*, 2008**). The crude oil samples were extracted from different microcosm and were gravimetrically analyzed.

2.9. Extraction of crude oil after treatment by bacterial strain and gravimetric estimation

At the end of incubation period , seven days, the polluted bacterial broth (100ml) was thoroughly shaken with carbon tetrachloride (50 ml 3 times) in a separating funnel and the three fractions were collected in case of crude oil sample. The collected organic layer was dried over anhydrous sodium sulphate. The solvent was removed on a rotary evaporator until a constant weight (**Khalil, 2007**). The oil sample was accurately weighed, percentage of the biodegraded oil was calculated and alterations in its chemical composition were studied by chromatographic analysis (GC) (**Zakaria, 1998; El-Sheshtawy, 2011).**

2.10. Analysis of extracted crude oil samples

2.10.1. Gas Chromatography (GC)

Extracted oil has been studied by GC which were monitored using Agilent 6890 plus, Gas chromatograph attached to computerized system with chemstation software condition of operation according to the standard test method IP 318/ 75 (**Institute of Petroleum, 1995**). The component separation was completed on HP-1 capillary column (100 % methyl silicone siloxane, 30 m length, 0.35 mm internal diameter and 0.25 mm thickness film). For a typical chromatogram, a 0.5 µl crude oil sample was introduced into a splitter injector which was previously heated at 350°C. The oven temperature was programmed 100-320°C at a fixed rate of 3°C/min. The nitrogen (oxygen-free) was used as a carrier gas with a flow rate of 2 ml/min. A mixture of pure n-paraffin was used as standard. The peak area of each resolved component (consisting of either n- and iso-paraffin) is determined individually. While, the unresolved complex mixtures (humps) composed of non n-paraffins presumably mainly cycloparaffins and aromatics with long side chains, were determined only as a total.

3. Results and Discussion

3.1. Physico-chemical characterization of crude oil.

An Arabian heavy crude oil was used for this study. This crude have a high specific gravity of 0.9348 at 15.6^{o}C, low API gravity of 15.16 at 15.6^{o}C, viscosity 141.23cSt at 60^{o}C, a pour point 20^{o}C, low wax content 2.6%, and high asphaltene 8.8% as shown in (Table 2). Its characterization was conducted to more closely approximate the weathered state of crude oil after a spill occurs.

Table 2. physico-chemical properties of the studied crude oil

Experiments analysis	Method	Result
Density g/ml, at 15.56^{o}C Specific gravity API gravity	ASTMD-1298	0.9639 0.93 15.16
Kinematic Viscosity, cSt at 60^{o}C	ASTMD-445	141.23
Pour point, ^{o}C	ASTMD-97	20
Total Sulphur, wt.%	ASTMD-4294	3.2
Molecular weight	-	249.37
Wax Content, wt.%	UOP-64	2.6
Carbon residue, wt.,%	ASTMD-189	12
Asphaltene Content , wt.%	IP-143	8.8
Ash content, wt.%	ASTMD-482	0.95
Hydrogen %	Elemental analysis	10.63
Oxygen %		3.04
Carbon%		80.55

3.2. Gas chromatography analysis of crude oil sample

The GC analysis of crude oil exhibits many peaks over hump; these peaks represent the paraffinic hydrocarbon (iso- and n-alkane). The hump represents the heavy non eluted compounds which unresolved complex mixture (UCM). Each crude oil has iso-components beside the normal ones starting from C_{12} to max carbon number C_{27}.

The hydrocarbon distribution of the saturated fraction of the crude oil as given in (Table 3 and Fig.1) showed that there is a gradual increasedin paraffinic hydrocarbons percentages by increased of carbon number up to C_{18} followed by continuous decrease up to C_{47}. This trend is characteristic for all saturated fractions of crude oil.

3.3. Characterization of Liposome

3.3.1. Transition electron microscopy TEM

Transmission electron microscopy (TEM) is often used to determine size and morphological characterization of liposome (**Maza *et al.*, 1998**)..

Many TEM studies on liposome also indicated that the particle size, vesicle shape and lamellarity of liposome may be different due to the process of preparation (**Satya *et al.*, 1982; Sułkowski *et al.*, 2005; EunChul *et al.*, 2007; Chetanachan *et al.*, 2008; Shimizu and Yoshitaka, 2011;**).

In this study, was focused only on thin film method by using modified reverse phase evaporation (mREV) to produce nanoliposomal particles of much smaller unilamellar.

Figs (2) show TEM micrographs of Pc/Chol liposome and nano-tween80 associated liposome. It is clear that all vesicles have similar geometric (spherical in shape) and differ only in size. The average size of PC/Chol vesicles was >100nm while for nanotween80-liposome was <100nm. The reduction of size of particle is due to repulsion force from charges on particles of liposome and this is particles of liposome and this is agreement with result obtained by (Eun-Chul) (**Eun Chul *et al.*, 2007).**

Table3. Hydrocarbon distribution of saturated fraction of crude oil

Carbon No.	n-alkane	iso-alkane	Carbon No.	n-alkane	iso-alkane	Carbon No.	n-alkane	iso-alkane
C_{12}	0.39	-	C_{25}	3.54	0.7	C_{38}	0.18	-
C_{13}	1.29	0.34	C_{26}	3.27	0.85	C_{39}	0.13	-
C_{14}	3.15	2.27	C_{27}	3.18	0.62	C_{40}	0.11	-
C_{15}	4.21	2.91	C_{28}	2.61	0.5	C_{41}	10.00	-
C_{16}	4.68	3.1	C_{29}	2.46	0.19	C_{42}	0.08	-
C_{17}	4.73	4.03	C_{30}	2.02	0.53			
C_{18}	4.73	3.26	C_{31}	1.66	0.88			
C_{19}	4.27	1.81	C_{32}	1.15	0.85			
C_{20}	4.25	0.95	C_{33}	0.91	0.51			
C_{21}	4.05	1.25	C_{34}	0.75	0.36			
C_{22}	3.99	1.42	C_{35}	0.62	0.13			
C_{23}	3.75	1.11	C_{36}	0.36	0.07			
C_{24}	3.78	0.63	C_{37}	0.30	-			

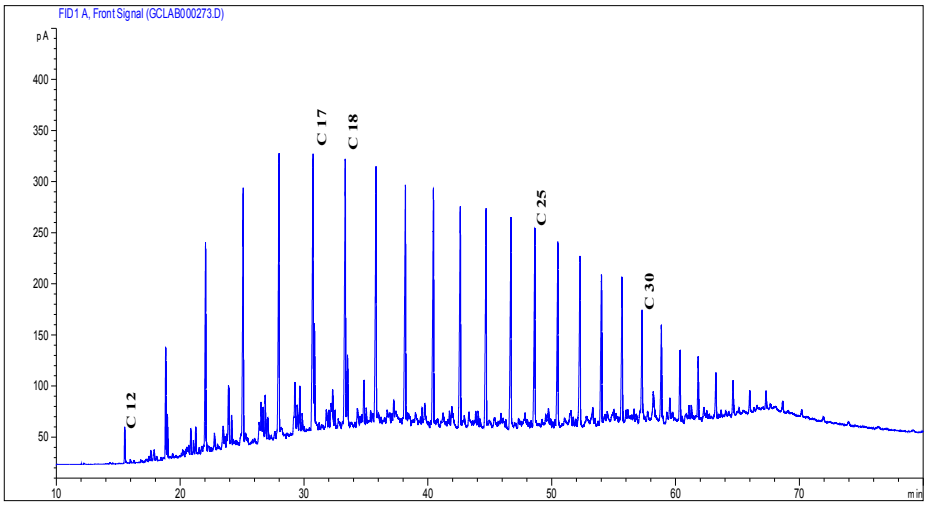

Fig (1): GC of crude oil

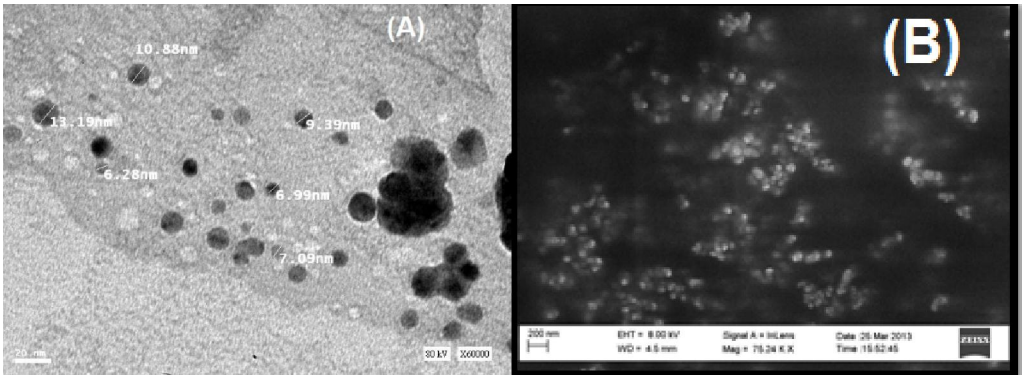

Fig. 2: TEM micrographs of: (A) nanoliposome (B) nano-tween80 liposome

TEM images do not lead readily to evaluation of particle size of liposomes. We also characterized the liposomes by dynamic light scattering (DLS) to obtain true distribution of particle size.

3.3.2. Zeta potential and differential light scattering

Zeta potential is the charge at particles mobile surface and is used to determine the degree of flocculation or deflocculation in nano-systems and to make prediction regarding the stability of the colloidal dispersion. Its nature may be negative or positive depending on type of surfactants and lipid structure. A high zeta potential (+,-) indicates that the system shall be deflocculated as for aggregation particles have to overcome electrostatic energy barriers (**Kuldeep *et al.*, 2012**).

The PC/Chol liposome has negative surface charge which reduces aggregation and increased stability which show clearly from (Fig.2A).

When used nano-tween80liposome which has high dispersion efficiency (90.44%). The average particle size reduce from (130.9 to 14.40nm) which help in dispersion process of oil spill and also zeta potential reduce from (-14 to -7.04) as shown in (Table 4 and Figs. 3, 4) which were consistent with results obtained from TEM and efficiency is supported by the finding of Shimizu **(Vieral *et al.*, 1996; Shimizu *et al.*, 2011).**

Table 4. Zeta potential and particle size of nanoliposome and nano-tween80liposome

Sample	Z-average Diameter (nm)	Zeta potential	Polydispersity index (pdI)	Quality of result
Nano-liposome	130.9	-14	0.588	Good
Nano-tween80liposome	14.40	-7.04	0.366	Good

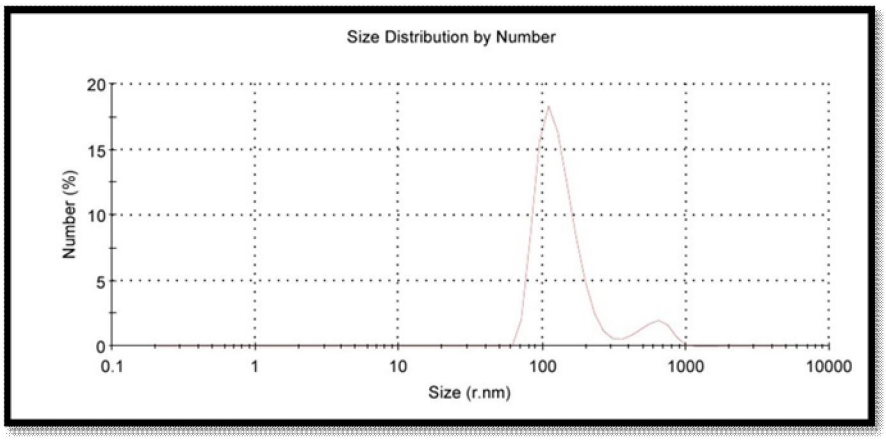

Fig.3: Size distribution and zeta potential of nano-liposome

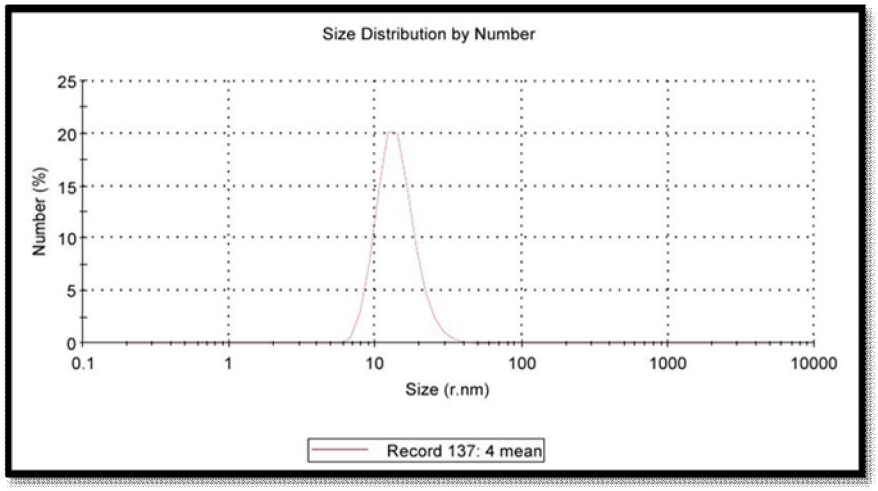

Fig.4: Size distribution and zeta potential of nano-tween80liposome

3.4. Biodegradation of crude oil by different bacterial strains

The aim of this experiment tested of some bacterial strains for biodegradation of crude oil sample in the presences of the natural surfactant (nano-liposome) and nanotween80-associated nano-liposome aiming to enhancing the biodegradation process.Totally six pure bacterial cultures able to grow in mineral salts medium with 2 % crude as carbon source were identified as (*Bacillus licheniformis, Rhodococcus erythropolis, Pseudomonas aeruginosa, Pseudomonas xanthomarina, Pseudomonas stutzeri* and *Bacillus subtilis spizizenii*). These biodegrading bacterial strains were used as a pure and mixed bacterial consortium for testing the biodegradation process.

3.4.1. Gravimetric analysis

The percentage biodegradation of crude oil by six bacterial strains was estimated by gravimetric analysis and listed in (Tables 5, 6). The Tables showed that the bacterial strains degraded in the range from (35 to 60 %) of the crude oil with the initial concentration of (2 g) in mineral salt medium MSM (100 ml), after 7 days of incubation period. The medium containing mixed bacterial consortium (*Pseudomonas xanthomarina, Pseudomonas stutzeri* and *Bacillus subtilis spizizenii*) in presence of nanoliposome as natural surfactant gave higher the percentage of degradation at 60 % after 7 days. Crude oil contains many kinds of hydrocarbons which are fractioned into saturated and aromatic hydrocarbons, resins and asphaltenes by silica gel chromatography **(Rosini, 1960).** A number of compounds slightly degradable by microorganisms are contained in the fraction of aromatic hydrocarbons. Metagenomic analyses by many researchers have showed that only a few enzymes which degrade aromatic hydrocarbons are produced in most microorganisms in natural environments **(Kubato *et al.*, 2005; Uchiyama *et al.*, 2005).** Leahy and Colwell **(Leahy and Colwell, 1990)** reported that mixed populations with overall broad enzymatic capacities are required to degrade complex mixtures of hydrocarbons such as crude oil or diesel fuel. Such mixed cultures display metabolic versatility and superiority to pure cultures **(Hamme *et al.*, 2000).** Therefore, a microbialconsortium containing a number of microorganisms which synthesize the degradative enzymes for different parts of the decomposition pathway is considered to be well suited to the degradation of aromatic hydrocarbons. Microorganisms not directly involved in the degradation process also probably play a role by producing micronutrients or surface-active agents for the solubilization of aromatic hydrocarbons **(Bharathi and Vasudevan, 2001; Ozaki *et al.*, 2007)**. **Sugiura *et al.*, (1997)** reported that biodegradation caused by mixed cultures was more effective than that caused by pure cultures mainly due to the complexity of oil products. Various organisms have the capability of degrading various forms of hydrocarbons and thus when a consortium of these microbes is applied to degrade various forms of hydrocarbons in a single source like crude oil, the total degradation is more effective.

Table 5. Percentage biodegradation of petroleum hydrocarbons after treatment with different bacterial strains using nano-liposome as surfactant

Sample	Weight of residual crude oil (g/l)	Biodegradation percentage* (%)
Control	2.0	0
***Bacillus licheniformis* (B.l.)**		
Crude oil + (B.l.)	1.2	40
Crude oil + (B.l.) + nano-liposome	1.1	45
***Rhodococcus erythropolis* (R.e.)**		
Crude oil + *(R.e.)*	1.3	35
Crude oil + *R.e.*+nanoliposome	1.2	40
***Pseudomonas aeruginosa* (P.a.)**		
Crude oil + (P.a.)	1.2	40
Crude oil + (P.a.)+nanoliposome	1.1	45
Mixed culture ($M.C._1$) (*B.l.* + R.e. + *P.a.*)		
Crude oil + (M.C.1)	1.3	35
Crude oil + (M.C.1)+nanoliposome	0.9	55
***Pseudomonas xanthomarina* KMM 1447(P.x.)**		
Crude oil + *(*P.x.)	1.3	35
Crude oil + (P.x.).+nanoliposome	1.1	45
***Pseudomonas stutzeri* ATCC 17588 (P.s.)**		
Crude oil + (P.s.)	1.3	35
Crude oil + (P.s.) + nanoliposome	1.2	40
***Bacillus subtilis spizizenii* DSM=15029 (B.s.)**		
Crude oil + (B.s.)	1.3	35
Crude oil + (B.s.) + nanoliposome	0.9	55
Mixed culture ($M.C._2$) (P.x. + P.s. + B.s.)		
Crude oil + ($M.C._2$)	1.2	40
Crude oil + ($M.C._2$)+nanoliposome	0.8	60

* Biodegradation percentage = Weight of original oil – wt. of residual / wt. of original oil * 100

Table 6. Percentage biodegradation of petroleum hydrocarbons after treatment with different bacterial strains using nano-tween80liposme as mixture surfactants

Sample	Weight of residual crude oil (g/l)	Biodegradation percentage (%)
Control	2.0	0
***Bacillus licheniformis* (B.l.)**		
Crude oil + (B.l.)	1.3	35
Crude oil + (B.l.) + nano-tween80liposome	1.3	35
***Rhodococcus erythropolis* (R.e.)**		
Crude oil + (R.e.)	1.2	40
Crude oil + R.e.+nano-tween80liposome	1.3	35
***pseudomonas aeruginosa* (P.a.)**		
Crude oil + (P.a.)	1.3	35
Crude oil + (P.a.)+ nano-tween80liposome	1.1	45
Mixed culture ($M.C._1$) (B.l. + *R.e.* + P.a.)		
Crude oil + ($M.C._1$)	1.3	35
Crude oil + ($M.C._1$)+ nano-tween80liposome	1.2	40
***Pseudomonas xanthomarina* KMM 1447(P.x.)**		
Crude oil + *(*P.x.)	1.3	35
Crude oil + (P.x.)+nano-tween80liposome	1.1	45
***Pseudomonas stutzeri* ATCC 17588 (P.s.)**		
Crude oil + (P.s.)	1.3	35
Crude oil + (P.s.) + nano-tween80liposome	1.2	40
***Bacillus subtilis spizizenii* DSM=15029 (B.s.)**		
Crude oil + (B.s.)	1.3	35
Crude oil + (B.s.) + nano-tween80liposome	1.1	45
Mixed culture ($M.C._2$) (P.x. + P.s. + B.s.)		
Crude oil + ($M.C._2$)	1.3	35
Crude oil + ($M.C._2$)+ nano-tween80liposome	1.2	40

3.4.2. 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 this study, the biodegradation of crude oil was analyzed after fifteen days of incubation period using the GC for aliphatic compounds. Tables (7, 8) showed the results of GC of the residual crude oil samples of different microcosms, and the control sample (crude oil sample without treatment by microorganisms).

The percentage of residual n-paraffins and iso-paraffins present in crude oil after biodegradation was calculated by comparing with the undegraded control **(**Table 7). The obtained data showed better degradation of n-paraffins than iso-paraffins in all microcosms containing the known bacterial strains (*Bacillus licheniformis, Rhodococcus erythropolis* and *Pseudomonas aeruginosa)*. While in microcosm containing these known bacterial strains and the natural surfactant (nano-liposme**)**, the degradation of n-paraffins higher than iso-paraffin took place. On the other hand, the best biodegradation of iso-paraffins was observed in microcosm containing different bacterial isolates (*Pseudomonas xanthomarina, Pseudomonas stutzeri* and *Bacillus subtilis spizizenii*). While, when used the nano-liposome for enhanced the biodegradation process. The better biodegradation of iso-paraffins was observed in microcosmcontaining mixed bacterial consortium of isolates and surfactant (Fig.6). From the above results, can be concluded that, the used of nano-liposome for enhanced biodegradation process of n-paraffins more than iso-paraffins was observed by some microorganisms. On the other hand, the higher percentage degradation of iso-paraffins was also obtained in culture media containing other bacterial isolate 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 **(Mohamed *et al.*, 2006; Paudyn *et al.*, 2008).**

Table 7. Distribution of carbon number in gas chromatogram of residual crude oil after treatment by different bacterial strains using nanoliposme as surfactant

Sample	Percentage residual of total paraffins after bio-treatment	
	n-paraffins	iso-paraffins
Control	82.47	17.53
***Bacillus licheniformis* (B.l.)**		
Crude oil + (B.l.)	75.27	23.60
Crude oil + (B.l.) + nano-liposome	73.50	26.50
***Rhodococcus erythropolis* (R.e.)**		
Crude oil + *(R.e.)*	079.1	25.17
Crude oil + *R.e.*+nano-liposome	78.12	21.88
***Pseudomonas aeruginosa*(P.a.)**		
Crude oil + (P.a.)	77.28	22.17
Crude oil + (P.a.) + nano-liposome	70.64	29.43
Mixed culture ($M.C._1$) (B.l. + *R.e.* + P.a.)		
Crude oil + ($M.C._1$)	71.19	28.81
Crude oil + ($M.C._1$) + nano-liposome	69.90	24.41
***Pseudomonas xanthomarina* KMM 1447(P.x.)**		
Crude oil + (P.x.)	83.33	16.4
Crude oil + (P.x.)+ nano-liposome	75.02	.9842
***Pseudomonas stutzeri* ATCC 17588 (P.s.)**		
Crude oil + (P.s.)	90.19	9.81
Crude oil + (P.s.) + nano-liposome	86.64	13.36
***Bacillus subtilis spizizenii* DSM=15029 (B.s.)**		
Crude oil + (B.s.)	95.54	4.46
Crude oil + (B.s.) + nano-liposome	79.55	20.45
Mixed culture ($M.C._2$) (P.x. + P.s. + B.s.)		
Crude oil + ($M.C._2$)	86.82	13.18
Crude oil + ($M.C._2$) + nano-liposome	91.44	8.56

The data in Table (7) showed also that, the better degradation of n-paraffins than iso-paraffins in all microcosms containing the known bacterial strains (*Bacillus licheniformis, Rhodococcus erythropolis and Pseudomonas aeruginosa)*. While, the higher degradation of n-paraffins than iso-paraffins was observed when added the mixture of different nano-surfactant into culture medium. On the other hand, the best biodegradation of iso-paraffins was observed in microcosms containing different bacterial isolates (*Pseudomonas xanthomarina, Pseudomonas stutzeri* and *Bacillus subtilis spizizenii*). But, when using these bacterial isolates and mixture of two different nano-surfactants were observed that, no enhanced the biodegradation process of iso-paraffins.

So, it can be concluded that, the presences of nano-liposome and mixture of nano-liposome and nanotween80 was enhanced the biodegradation process of n-paraffins more than iso-paraffins by some microorganisms. On contrary, the percentage degradation of iso-paraffins increased by other bacterial isolates when using the nano-liposome only. It can be attributed to, the natural surfactant (nano-liposome) has positively effect on the growth rate of tested microorganisms by growing on petroleum usually produce potent emulsifiers and these surfactants help to degrade petroleum **(Rosenberg, 1993).** Similarly, several researches have reported that the use of surfactants have enhanced degradation of crude oil **(Balba *et al.*, 2002; Urum *et al.*, 2003)**. In our study, the degradation at nanotween-80 with nano-liposome decreased at a higher rate compared to the degradation at nano-liposome. Some researchers reported that synthetic surfactants were effective on biodegradation of diesel oil and cell growth was inhibited at high concentrations of tween 80 **(Lee *et al.*, 2006)**.

Table 8. Distribution of carbon number in gas chromatogram of residual crude oil after treatment by different bacterial strains using nano-tween80liposome as mixture of surfactant

Sample	Percentage residual of total paraffins after bio-treatment	
	n-paraffins	iso-paraffins
Control	82.47	17.53
***Bacillus licheniformis* (B.l.)**		
Crude oil + (B.*l.*)	75.27	23.60
Crude oil + (B.*l.*) + nano-tween80liposome	74.29	25.71
***Rhodococcus erythropolis* (R.e.)**		
Crude oil + (R.e)	79.1	25.17
Crude oil + (R.e) + nano-tween80liposome	70.00	30.00
***Pseudomonas aeruginosa* (P.a.)**		
Crude oil + (P.a.)	77.28	22.17
Crude oil + (P.a.)+ nano-tween80liposome	73.00	27.00
Mixed culture ($M.C._1$) (B.l. + *R.e.* + P.a.)		
Crude oil + ($M.C._1$)	71.19	28.81
Crude oil + ($M.C._1$) + nano-tween80liposome	75.12	24.88
***Pseudomonas xanthomarina* KMM 1447(P.x.)**		
Crude oil + (P.x.)	83.33	16.4
Crude oil + (P.x.)+ nano-tween80iposome	82.19	17.81
***Pseudomonas stutzeri* ATCC 17588 (P.s.)**		
Crude oil + (P.s.)	90.19	9.81
Crude oil + (P.s.) + nano-tween80liposome	85.06	14.94
***Bacillus subtilisspizizenii* DSM=15029 (B.s.)**		
Crude oil + (B.s.)	95.54	4.46
Crude oil + (B.s.) + nano-tween80liposome	73.06	26.94
Mixed culture ($M.C._2$) (P.x. + P.s. + B.s.)		
Crude oil + (M.C.2)	86.82	13.18
Crude oil + (M.C.2) + nano-tween80liposome	81.98	18.02

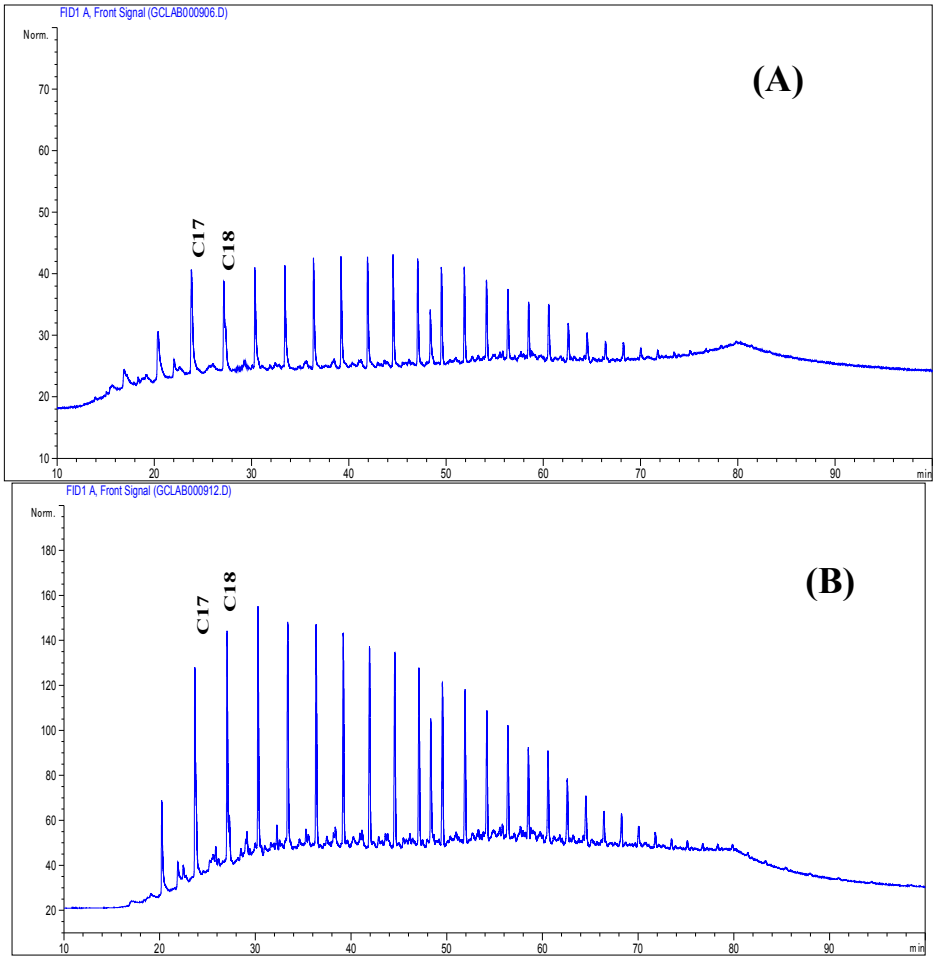

Fig. 6. G C chromatogram: (A) mixture of (P.x. + P.s. + B.s.) with nano-liposome (B) mixture of (P.x. + P.s. + B.s.) without nano-liposome.

Conclusion

Oil pollution is a severglobal environmental problem causing a number of adverse negative impacts on human health, fisheries, agricultures, tourism, ecosystems and eventually the national income. Petroleum hydrocarbons are ubiquitous contaminants in the aquatic environment because of both human activities and natural processes. All research models seek to describe the key physical and chemical processes that transport the oil and in the sea.

Our study is carried on an "Arabian heavy crude oil". We noticed that the crude have a high specific gravity at 15.6°C, low API gravity at 15°C, high viscosity at 60°C, high pour point, and high asphaltene.Its characterization was conducted to more closely approximate the weathered state of crude oil after a spill occurs. The oil component of the crude oil was further separated into hydrocarbon types by applying the alumina column chromatography.

Using (mREV) were produced nanoliposome of natural soybean phospholipids of (20-100 nm), in diameter, which were stable to storage at room temperature resisting aggregation or fusion for long time, and can be used in safety in treatment of oil spill.

The polymer (tween 80) associated Pc/Chol liposome, the ratio is (1:1) which gave good result in dispersing oil spill testing that is a way to increase the long-term stability of liposomes, as well as inhibit liposome fusion.

The presences of nano-liposome and mixture of nano-liposome and nano-tween 80 was enhanced the biodegradation process of n-paraffins more than iso-paraffins by some microorganisms. On contrary, the percentage degradation of iso-paraffins increased by other bacterial isolates when using the nano-liposome only can be attributed to, the natural surfactant (nano-liposome) has positively effect on the growth rate of tested microorganisms by growing on petroleum usually produce potent emulsifiers and these surfactants help to degrade petroleum.

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