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Crude oil removal via isolated cyanobacteria in presence of linear alkyl benzene sulfonates

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ABSTRACT

This study was carried out to evaluate the ability of two cyanobacterial isolates, *Anabaena flose-aquae* and *Westiellopsis prolifica*, to degrade Iraqi crude oil in batch cultures with the addition of linear alkyl benzene sulfonate. The degree of oil removal was measured after a 15-d culture period. According to biomass and gas-chromatography data, axenic cultures of both strains were able to degrade crude oil, showing complete removal of some hydrocarbon compounds during the 15-d exposure period. The strains produced biosurfactants at a final concentration of 2–3.1 g/L, which was further evidence of their oil-degrading capabilities.

Keywords: Oil removal; Crude oil; Cyanobacteria; Biodegradation; Batch culture

1. Introduction

With increasing petroleum exploration and petrochemical activities around the world, several oil spills are reported on an average day. Spills can occur in oceans, estuaries, rivers, lakes, or on land, where they can affect living organisms and place individuals, species and communities at risk of smothering, hydrocarbon toxicity, hypothermia, and chronic long-term effects. Some hydrocarbon components are considered to be carcinogenic and neurotoxic organic pollutants [1].

The severity of the impact of an oil spill depends on a variety of factors, including characteristics of the oil itself and natural conditions such as water temperature and weather, which influence the behavior of oil in aquatic environments. Different habitats also have differing sensitivities to oils [2].

Production and transport of crude oil are the main sources of these pollutants; oil refineries also discharge wastewater with high hydrocarbon content. In addition, natural seepage from the ground and anthropogenic activities other than petrochemistry are also can be sources of dangerous wastes [3]. To protect water and other resources from damage, safeguard them for public enjoyment, and ensure the survival of numerous species, cleanup of affected shorelines has become a significant part of oil spill response [4].

Remediation of oil-contaminated environments can be achieved by either biological or physicochemical methods. One of the most common classes of chemicals used in responding to oil spills is dispersing agents, which are chemicals that contain surfactants, or compounds that act to break liquids such as oil into small droplets within the water column where they are subjected to natural processes that help to break them down further. However, the attendant negative consequences of the physicochemical approach are now directing greater consideration to the exploitation of the biological alternatives [5].

Biodegradation of hydrocarbons compounds by natural organisms has considered the main process acting in the degradation of hydrocarbon that polluted the environments [6]. This mechanism is not a new concept and it has been extensively studied in controlled conditions [7] and in open field experiments [8], but it has acquired a new significance as an increasingly effective and potentially inexpensive cleanup technology.

It is recognized that many microorganisms have the ability to use hydrocarbons as an energy source and that

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this is highly dependent on the (i) chemical nature of the compounds within the petroleum mix (ii) environmental factors [9].

Bacteria and fungi that have the ability to degradation of Hydrocarbon are widely distributed in marine, freshwater, and soil habitats. There are also more evidences of cyanobacteria consumption of crude oil directly [10]. However, in the study of microorganisms that can metabolize these compounds, the rate of metabolism occasionally override the rate of mass transfer from the surrounding environment to the cells, which leads not only to restriction of growth rates but also to loss of a toxic effects by these hydrocarbons [11,12].

On the other hand, it is reported that, in contrast to chemical dispersants material, which cause environmental damages after application for decline of spilled oil in aquatic systems, bio-surfactants from freshwater or soil microorganisms are less toxic and are completely bio-degradable [13].

In Iraq, despite the best efforts of response teams to contain oil spilled in aquatic systems, little investigation has been attempted concerning the remediation of petroleum-contaminated freshwater courses by either physico-chemical or biological methods. The present work aims to evaluate the potential role of two freshwater cyanobacterial strains, *Anabeana flose-aquae* and *Westiellopsis prolifica*, in the biodegradation of crude oil and their potential application for bioremediation in situ.

2. Materials and methods

2.1. Culture conditions of cyanobacteria

Axenic cultures of the cyanobacteria *Anabaena flos-aquae* var. *gracilis* Klebahn (Nostocales) and *Westiellopsis prolifica* and (Stigonematales) were isolated from the Tigris River with Baghdad city and used in all experiments, culture medium Chu-No. 10 were used throughout this work [14]. Both strains were incubated at 25°C temperature and ±2500 Lux in an illuminated incubator under a light/dark system of 18/6 h for the duration of the experiments. Optimal phytonutrient concentrations (5 mg/L phosphate; 10 mg/L nitrate and N:P ratio are 1:10 mg/L) were used to gain high growth rates and short doubling time. The medium of phosphate-buffered was adjusted to pH 7 by using NaOH. Then, the medium was placed in 1000-mL polycarbonate flasks and autoclaved. Patterson's method was applied to obtain axenic cultures [15]. The stock cultures were regularly re-cultivated and inoculated into the experimental systems at the logarithmic phase.

2.2. Crude Oil

Medium crude oil (API gravity=32.1/Basrah type) was obtained from Al-Dura oil refinery. The oil was added to the growth cultures without any treatment. Conical flasks (1000 mL) were used as incubation reactors. Each flask was inoculated with cyanobacterial culture at the exponential phase of the growth and 1 mL of the crude oil. Control and treatment cultures were performed in triplicate. For the controls, cyanobacteria were incubated in culture medium without oil.

2.3. Growth measurement

Chlorophyll-*a* concentration was used as an indicator of cyanobacterial growth, and was measured daily throughout the exposure period according to Miller et al. (1978).

$$\text{Chlorophyll-}a\left(\mu\text{g/ml}\right) = 11.9 \times (2.43\left(D_b - D_a\right) \times \left(V / L\right) \quad (1)$$

where D_b = Optical density for Chl-*a* extract before adding of (2N HCl at 665, 750 nm); D_a = optical density for Chl-*a* extract after adding of (2N HCl at 665, 750 nm); V = volume of solvent; L = cuvette length (photocell)/(cm)

2.4. Estimation of biosurfactants produced by cyanobacteria

Three containers, each containing 1000 mL of sterile No. Chu-10 culture medium, were inoculated with isolated cyanobacteria at the logarithmic phase and 1 mL of crude oil was added. The containers were incubated in an illuminated incubator at 25°C with regular shaking for 14 d. The amount of surfactant produced was estimated by centrifuging samples at 6000 rpm for 30 min. The clear supernatant was removed carefully using a syringe. The supernatant was acidified to pH 2 and kept for 24 h at 4°C. The supernatant was transferred to a separating funnel, then chloroform and methanol solvent were added to extract the biosurfactant, and the aqueous phase was discarded. Finally, the extract was dried in an oven at 45°C. The dried extract was weighed to find the amount of biosurfactant [16].

2.5. Detection of the degradation of crude oil

Growth culture media were analyzed after the 15-d exposure period to determine the residual concentrations of crude oil components. The control consisted of culture medium and 1 mL crude oil without cyanobacteria or linear alkyl benzene sulfonate (LAS). All samples were tested by gas chromatography (Shimadzu, model 2014) using a CPSIL5-CB capillary chromatography column. Peak areas were calculated using the equation below to estimate the amount of hydrocarbons that's remain after cyanobacterial degradation:

$$\text{Area} = \text{Base}/2 \times \text{height} \quad (2)$$

$$\text{Remaining percentage of hydrocarbons} = \left(\text{Sample area}\right)/\left(\text{Total area}\right) \times 100 \quad (3)$$

2.6. Chemical dispersant (Surfactant)

Linear alkyl benzene sulfonate was provided by the Ministry of Manufacture/Avicenna Co. Ltd., Iraq. It was supplied as an aqueous solution of sodium salt with an average molecular weight of 320 g/mol and a minimum purity of 96.5%. Two concentrations of LAS (Linear alkyl benzene sulfonate) were prepared (0.5 and 1 mg/L) and added to the incubation reactors to test the influence of dispersing agent in the crude oil biodegradation.

2.7. Statistics

Total crude oil residues were correlated with the growth of both species of cyanobacteria (as Chlorophyll-*a* content) using product moment correlation analysis. Mean crude oil removals by both strains were compared using a t-test. Differences were considered significant at a P-value less than or equal to 0.05.

3. Results and discussion

3.1. Cyanobacterial biomass in the presence of crude oil and LAS

Growth curves of both cyanobacteria showed similar behavior in response to crude oil in the presence of LAS during an exposure period of 15 d (Figs. 1 and 2). Although both strains underwent a prolonged lag phase, extending to the 6th day, this was followed by a rapid increase in the growth rate of *Anabaena flos-aquae*and a more gradual increase in the case of *Westiellopsis prolifica*, both reaching a peak at the end of the exposure period. Production of chlorophyll-*a* was used as an indicator of cyanobacterial growth with time, and reflects the ability of the microorganisms to adapt to and degrade crude oil compounds [17]. This is consistent with the findings of [18] who found that *Cyclotellacryptica* was stimulated by North Sea crude oil and its paraffin. Similar results were reported by [18], who reported that the cyanobacteria *Microcoleus chthonoplastes* and *Phormidium corium* grew better in the presence of crude oil, indicating that hydrocarbons may have been used. Our data show positive effects of selected concentrations of LAS (0.5 and 1 mg/l) on the growth of cyanobacteria and on oil removal. These findings agree with the explanation proposed by [20] who reported that several microbes were able to degrade oil better in the presence of dispersant, and could further degrade certain components of these chemicals. The extended lag period seen in this work agrees with the results obtained by [21], which saw a similar prolonged lag phase following the treatment of algal culture with crude oil, followed by high biomass production. It became clear that biomass needs to reach a threshold value, above which the enzyme systems needed to carry through the degradation process were produced. Although the addition of nutrients is necessary to enhance the biodegradation of oil pollutants [22], the extent of biodegradation can be limited by excessive levels of alternate carbon substrates or surfactants [23], which might explain the limited growth of *W. prolifica*in the presence of 1 mg/L LAS as well as its greater sensitivity to oil compared with *A. flos-aquae*.

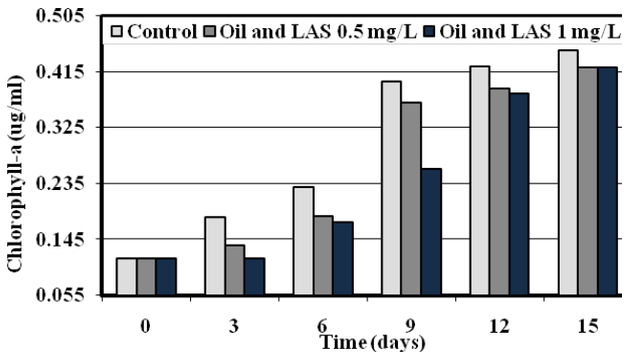

Fig. 1. Growth response of *Anabaena flos-aquae* to crude oil in the presence of LAS.

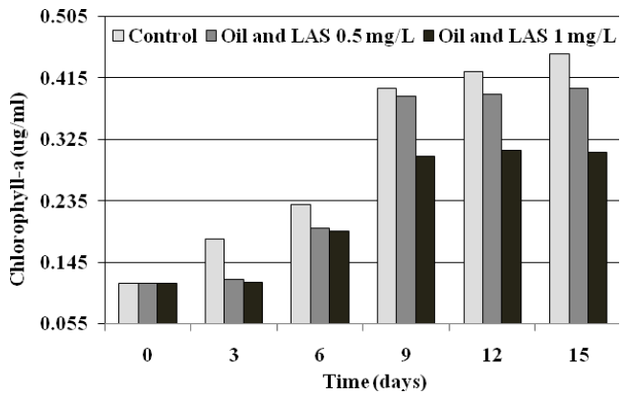

Fig. 2. Growth response of *Westiellopsis prolifica* to crude oil in the presence of LAS.

3.2. Biosurfactants produced by cyanobacteria

The use and uptake of water insoluble substances such as petroleum alkanes in crude oil need a particular physiological adaptation of the microorganisms such as the synthesis of specific amphiphilic molecules, which called biosurfactants. Hence, to facilitate hydrocarbon uptake through the hydrophilic outer membrane, several microorganisms produce extracellular surface active agents [24]. These include compounds that have low molecular weight such as triacylglycerols, phospholipids and fatty acids [25]. In this study, the amount of biosurfactants produced by *A. flos-aquae* and *W. prolifica* reached 3.1 and 2 g/L, respectively. These results are comparable to those of [26] who reported that the amount of emulsions produced by *Bacillus subtilis* reached 2 and 1.47 g/L, compared with which the strains tested in this study are efficient producers of biosurfactants in a hydrocarbon-rich medium.

3.3. Estimation of oil removal

Gas chromatography resolved 43 separate components in the control mixture (Table 1) while the samples treated with both cyanobacterial strains contained only 26 components after 15 d of incubation (Tables 2 and 3). This decrease in the number of peaks and reduction in peak area for some materials in the treatments compared with the control samples clearly indicate that both *A. flos-aquae and W. prolifica* are good hydrocarbon degraders as well as demonstrating a positive effect of adding LAS to enhance the biodegradation. These results are in good agreement with [27,28] that showed that phenanthrene was mineralized better by some microbes when dispersant (Corexit 9500) was added. About 89% of Maya heavy crude oil was reported to be biodegraded by axenic cultures of *Burkholderia cepacia* RQ1 in shake flasks within 15 d [29].

Table 1
Numbers of chemical composition of crude oil in the undegraded control sample

Peak	Ret. time (min.)	Area (cm^3)	Height (mm)	Area %
1	0.506	19.927	1161	0.0018
2	2.818	5251.5	432	0.0005
3	3.427	121.12	18.409	0.0126
4	3.709	11.134	424.28	1.1644
5	5.699	279.405	31439.8	29.218
6	7.138	4606.2	5292	0.0004
7	8.269	1365.3	350.1	0.0001
8	10.164	9.6012	307.8	0.0009
9	12.579	1991.7	511.0	0.0001
10	13.905	1622.7	567.9	0.0001
11	14.242	1919.7	358.2	0.0001
12	14.351	2714.4	628.2	0.0002
13	14.451	2050.5	585.9	0.0001
14	16.997	87.312	1281.6	0.0009
15	17.082	4175.1	1116.9	0.0004
16	19.725	140.50	3122.1	0.0146
17	21.918	43.964	1.056	4.5975
18	22.365	31.89	9.28	3.3350
19	23.826	179.060	36.24	18.725
20	26.296	49.169	8379	0.0513
21	27.930	23.547	877.920	2.4624
22	28.600	470.782	106.495	0.0492
23	29.235	5.473	140.541	0.5724
24	29.752	10.101	244.974	1.0563
25	31.347	17.625	233.893	1.8431
26	31.696	4.247	221.870	0.4441
27	32.776	157.748	6.657	16.496
28	34.893	12.333	308.514	1.2897
29	35.350	20.92	452.459	2.1915
30	35.914	12.006	272.230	1.3020
31	37.098	9.613	186.460	1.0053
32	37.845	7.628	133.190	0.7977
33	38.949	3.844	99.439	0.4020
34	39.724	13.504	252.265	1.4121
35	40.677	364.581	72.586	0.0381
36	48.921	1.845	69.868	0.1929
37	41.489	5.450	138.777	0.5699
38	43.789	960.296	24.965	0.1004
39	45.898	4.302	100.437	0.4499
40	48.470	40.005	3833.1	0.0041
41	49.423	1.763	27.234	0.1845
42	52.467	1412.1	549.0	0.0018
43	52.585	3353.4	450.9	0.0003
Total		811.322	48.354	100.00

Table 2
Numbers of chemical composition of crude oil in growth culture inoculatedwith *Anabaena flos-aquae*in the presence of 0.5 mg/L LAS

Peak	Ret. time (min.)	Area (cm^3)	Height (mm)	Area %
1	0.043	1212	494	0.0028
2	2.978	1203	390	0.0028
3	3.255	10.853	1850	0.0249
4	3.712	7180	1138	0.0165
5	4.059	4244	693	0.0097
6	5.215	1.922	82.427	4.4090
7	5.667	34.695	3.854	79.554
8	6.910	2038	585	0.0047
9	7.261	53.400	2130	0.1224
10	7.551	12919	3761	0.0296
11	10.845	1012	468	0.0023
12	13.328	8683	995	0.0199
13	13.440	2330	860	0.0053
14	18.698	1780	723	0.0041
15	19.250	30.285	1858	0.0694
16	19.356	4202	1190	0.0096
17	21.816	1.159	17.327	2.6588
18	22.353	38.286	4276	0.0878
19	22.587	1934	710	0.0044
20	24.834	104.246	3824	0.2390
21	26.617	659.437	14.323	1.5120
22	28.479	67.382	3817	0.1545
23	30.590	2.062	125.156	4.7300
24	32.097	985.026	21.266	2.2586
25	32.653	689.069	33.689	1.5800
26	36.493	1.084	7773	2.4875
Total		87.623	37.515	100.00

4. Conclusions

Two strains of cyanobacteria were capable of removing crude oil by degrading certain compounds of the oil hydrocarbons. Attenuation was partly facilitated by the addition of LAS as a dispersal agent that increased the bioavailability of oil. Application of traditional microbiological methods with modern genome-based technologies as well as other biochemical approaches will provide an excellent opportunity for the scientific community to predict microbial involvement in major oil spills in future.

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Table 3
Numbers of chemical composition of crude oil in growth culture inoculated with *Westiellopsis prolifica* in the presence of 0.5 mg/L LAS

Peak	Ret. time (min.)	Area (cm^3)	Height (mm)	Area %
1	0.0387	1090.8	444.6	0.0025
2	2.6802	1082.7	351	0.0025
3	2.9295	9.767	1665	0.0224
4	3.3408	6462	1024.2	0.0148
5	3.6531	3819.6	623.7	0.0087
6	4.6935	1.730	74.184	3.9681
7	5.1003	31.225	3.469	71.598
8	6.219	1834.2	526.5	0.0042
9	6.5349	48.06	1917	0.1101
10	6.7959	11627.1	3384.9	0.0266
11	9.7605	910.8	421.2	0.0020
12	11.995	7814.7	895.5	0.0179
13	12.096	2097	774.0	0.0047
14	16.828	1902	650.7	0.0036
15	17.325	27.256	1672.2	0.0624
16	17.420	3781.8	1071	0.0086
17	19.634	1.435	15.5943	2.3929
18	20.117	34.457	3848.4	0.0790
19	20.328	1740.6	639.0	0.00396
20	22.350	93.821	3441.6	0.2151
21	23.955	593.493	12.8907	1.3608
22	25.631	60.643	3435.3	0.1390
23	27.531	1.856	112.640	4.257
24	28.887	886.523	19.1394	2.0327
25	29.387	620.162	30.3201	1.422
26	32.843	1.516	6995.7	2.2387
Total		88.414794	34.050	100.00

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