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Biodegradation of crude oil by some cyanobacteria under heterotrophic conditions

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ABSTRACT

Petroleum hydrocarbons are one of the most common groups of persistent organic contaminants in the environment. The degradation process of the oil takes place by microorganisms to remove it from the environment. The cyanobacteria *Nostoc punctiforme* and *Spirulina platensis* are used in this study to investigate their ability to grow and degrade different concentrations of crude oil under heterotrophic conditions. It was found that *S. platensis* can grow at different concentrations of crude oil (0.5, 1, 1.5, and 2%). No growth was obtained with *N. punctiforme* incubated with crude oil concentrations (0.5, 1, 1.5, and 2%) until 11 days, after this period the growth progressively increased, especially with 2% crude oil. Chlorophyll *a*, contents of *S. platensis*, decreased with increasing incubation period and approximately unchanged with increasing concentration of crude oil. High carotenoids contents in *S. platensis* was obtained after 7 and 11 days of incubation with different concentrations of oil except at 1.5% oil. Increase in chlorophyll *a* and carotenoids was observed in *N. punctiforme*, incubated with crude oil at different concentration and incubation period. The analyses of crude oil residue by GC–MS showed that Decane ($C_{10}H_{22}$), Pentacosane ($\mathbf{C_{25}H_{52}}$), Hexacosane ($\mathbf{C_{26}H_{54}}$), Octacosane ($C_{28}H_{58}$), Nonacosane ($\mathbf{C_{29}H_{60}}$) totally removed from the medium by cyanobacteria. Aromatic compounds increased compared to the blank. Overall, our results indicate that *S. platensis* and *N. punctiforme* can grow heterotrophically, and biotransfer aliphatic compounds to aromatic compounds.

Keywords: Biodegradation; Heterotrophic algae; Crude oil; *Nostoc punctiforme*; *Spirulina platensis*

1. Introduction

The main sources of hydrocarbon pollution are the spills and leaks of petroleum products [1]. Bioremediation is cheaper technology than other remediation technologies. Bioremediation uses plants and microorganisms to clean up pollutants in the environment [2]. Numerous microorganisms, including bacteria, fungi, and yeasts are known for their ability to degrade hydrocarbons [3,4]. Talaie et al. [5] reported that pure culture of *Pseudomonas aeruginosa*, which we isolated from the oil contaminated soils, could degrade floating crude oil with high removal efficiency (90%). Some species of algae are capable of heterotrophic growth on organic carbon sources [6]. Cyanobacterial

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polysaccharides play a major role in the emulsification of the oil, actually breaking the oil into small droplets that are subsequently attacked by the heterotrophs [7]. Anderson and Mcintosh [8] surveyed 38 cyanobacterial strains for their ability to grow photoheterotrophically and chemoheterotrophically. The biodegradation of organic pollutants by algae is to encourage the algal cell to grow in the presence of the pollutant. Cerniglia et al. [9] observed nine cyanobacteria, five green algae, one red alga, one brown alga, and two diatoms could oxidize naphthalene. Chaillan et al. [10] reported that cyanobacterial mats are ubiquitous in tropical petroleum-polluted environments. They form a high biodiversity microbial consortium that contains efficient hydrocarbons degraders. Raghukumar et al. [11] reported that mixed cultures of the three cyanobacterial species (*Oscillatoria salina*, *Plectonema terebrans*, and *Aphanocapsa* sp.) removed over 40% of the crude oil. Additionally, these cultures formed excellent cyanobacterial mats when grown in mixed cultures, and thus have the potential for use in mitigating oil pollution on seashores, either individually or in combination. Radwan and Al-Hasan [12] observed that the biodegradation activity in cyanobacterial cultures could be attributed to the metabolism of contaminating bacteria present in the non-axenic cultures. This work aims at studying the potential of the cyanobacteria *Spirulina platensis* and *Nostoc punctiforme* to grow under heterotrophic condition using crude oil as sole carbon source and its ability for crude oil biodegradation under the laboratory conditions.

2. Materials and methods

2.1. Isolation and identification of cyanobacteria

Isolation and purification of cyanobacteria were done according to the methods described by Rippka [13]. Briefly, *S. platensis* was isolated after repeated light migrations on solid medium [14]. *N. punctiforme* (Kuz.) was isolated after repeated light migrations on BG11, medium [15] from oil-contaminated soils near Tanta City, Egypt.

2.2. Algae cultivation with crude oil

Crude oil obtained from Cairo Oil Refinery Company at Tanta City, was added to 250 ml Erlenmeyer flasks containing 100 ml, Zarrouk medium for *S. platensis* and pH of the medium was adjusted to 10 and BG11, medium for *N. punctiforme* (Kuz.) at pH 7. An inoculum of algal culture was added to flasks containing crude oil concentrations (0, 0.5, 1.0, 1.5, 2%). The Erlenmeyer flasks were incubated at 25 ± 1°C on constant shaking at 80 rpm on dark condition. All experiments were done in three replicates.

2.3. Assessment of algal growth

The biomass of cyanobacteria was determined daily by measuring the optical density of the algal suspension at 750 nm by using Unico UV-2000 spectrophotometer.

2.4. Pigments estimation

A known volume of algal culture was centrifuged at 8000 rpm for 10 min and the pellet was extracted with known volume of ethyl alcohol and kept in water bath for 30 min at 60°C, and then centrifuged again. Absorbance of the pooled extracts was measured on Unico UV-2000 spectrophotometer at 650, 665, and 452 nm. Calculations were made according to the formulae described by Senger [16] for chlorophyll *a* and carotenoids.

2.5. Determination of biodegradation activity of the algae

Biodegradation of crude oil was analyzed by using GC–MS HP 6,890 gas carrier helium (1 ml/min). Capillary Column 30 m × 0.25 mm ID × 0.25 μm film and the temperature programming was 70–290°C, 5/15 min.

3. Results and discussion

3.1. Estimation of algal growth

Results in Fig. 1 show the *S. platensis* growth heterotrophically using different concentrations of

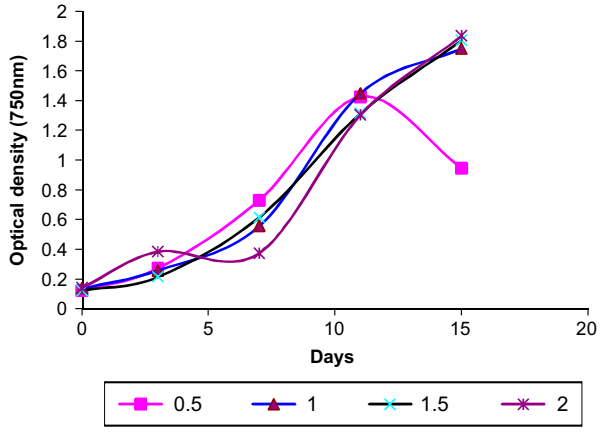

Fig. 1. Effects of different concentrations of crude oil on growth *S. platensis* measured as optical density (750 nm).

crude oil as sole carbon source (0.5, 1, 1.5, and 2%). The highest amount of growth was obtained when increasing concentrations of crude oil from 0.5 to 2%. The highest growth of *S. platensis* obtained after 15 days of incubation with 2% crude oil. These results are in agreement with that obtained by Chen and Zhang [17]. Zhang et al. [18,19], stated that *Spirulina* sp. has been found to utilize organic carbon substrates for heterotrophic and mixotrophic (photoheterotrophic) growth. Marquez et al. [20,21], found that the growth on glucose supplemented medium was much better than under photoautotrophic growth conditions.

It is evident from Fig. 2, that growth of *N. punctiforme* was low with different concentrations of crude oil (0.5, 1, 1.5, 2%) until 10 days of incubation. After this period the algal growth was progressively increased at concentrations 0.5 and 2%. After 3 days of incubations, the highest growth was attained at 0.5% followed by 1, 1.5, and 2%, respectively. The increase in the growth of the algae after days of treatment with the crude oil at lower concentration (0.5%) as compared to at higher concentrations (1, 1.5, and 2%) could be explained by the presence of toxic compounds resulted from biodegradation of low levels of crude oil and the ability of the algae to use these degraded compounds as mitogenic source for their growth. At higher concentrations of the crude oil, however, high amount of toxic compounds could be produced after oil biodegradation resulting in potential toxicity to the algae and thus limit its optimal growth. Because these high concentrations of the oil (1 and 1.5) were still able to enhance the growth of the algae, it could be suggested that the *N. punctiforme* developed a certain mechanism for the adaptation and tolerance toward these high levels of the crude oil. These mechanisms need further investigation. These results agree with Chow et al. [22], who indicated that chemical composition or even the metabolites of cyanobacteria

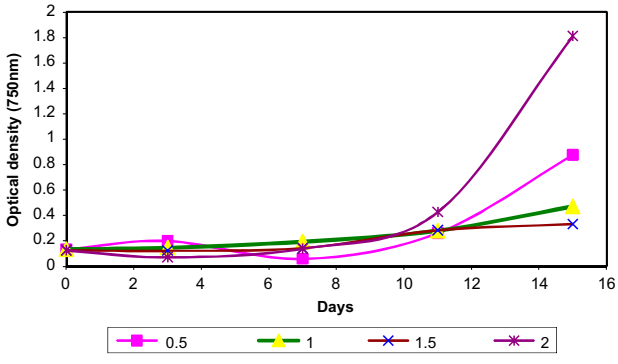

Fig. 2. Effects of different concentrations of crude oil on growth of *N. punctiforme* (Kuz.) measured as optical density (750 nm).

could significantly change as they adapted to the dark heterotrophic condition. Huang and Chow [23] denoted that, several strains of *N. punctiforme* were found to grow very well heterotrophically in the dark. Summers et al. [24], reported that *N. punctiforme* can grow in continual darkness as a respiratory heterotroph when supplied with sucrose, glucose or fructose, although the rate is less than half of the photoautotrophic rate.

3.2. Estimation of pigments

Results in Table 1 show the effect of crude oil on chlorophyll *a* and carotenoids (μg/ml) content under heterotrophic conditions of *S. platensis*, after 15 days of incubation. These results indicated that, chlorophyll *a*, decreased with increasing incubation period. The reduction in chlorophyll content may be the result of inhibition of chlorophyll biosynthesis brought about by inhibition of α-aminolevulinic acid dehydrogenase and protochlorophyllide reductase [25]. Villarejo et al. [26], reported that presence of organic carbon can alter both the photosynthetic and heterotrophic metabolism of *Chlorella* [27]. Production of photosynthetic pigments decreases as compared with the amounts present in the absence of organic carbon source. In other words carotenoids content of *S. platensis* increased from 1st to 7th days however, in *N. punctiforme* increased from 11th to 15th days.

Increase in carotenoids content and decrease in amount of chlorophyll *a* may be due to adaptation strategy against absence of light and use crude oil as sole carbon source.

According to Table 2. Chlorophyll *a* and carotenoid content in *N. punctiforme* increased with some minor fluctuations increasing incubation period and also with increasing crude oil concentrations. The maximum amount of chlorophyll *a* in *N. punctiforme* at 2% crude oil was 2.27 ± 0.907 μg/ml. Carotenoids content of *N. punctiforme* was 1.44 ± 0.820 μg/ml at 2% crude oil. Chow et al. [22], reported minor change of pigments and macromolecular contents of *Nostoc* H N 520 and *Nostoc* H N 701 grown in dark heterotrophically. Sundaram and Soumya [25], confirmed that organic stress affects on pigments content (chlorophyll *a*, carotenoid and phycocyanins) in cyanobacteria.

3.3. Biodegradation activity of crude oil by the blue green algae

The results obtained by GC–MS analyses (Table 3 and 4) show that, the amount of aliphatic and aromatic compounds residual varies with varying initial concentration and type of algae. Aliphatic compounds present in crude oil concentrations (0.5, 1, and 1.5%)

Table 1
Effect of different concentrations of crude oil on chlorophyll *a* and carotenoids (μg/ml) content of *S. platensis*. Results are mean of 3 replicates (±standard error of the mean)

	Crude oil conc.%	3rd day	7th day	11th day	15th day
Chlorophyll *a* (μg/ml)	0.5	2.21 ± 0.34	1.32 ± 0.16	1.22 ± 0.54	0.31 ± 0.009
	1.0	2.75 ± 0.24	1.18 ± 0.04	1.23 ± 0.53	0.64 ± 0.19
	1.5	2.50 ± 0.31	1.61 ± 0.27	1.89 ± 0.14	0.99 ± 0.03
	2.0	2.06 ± 0.17	1.66 ± 0.29	1.67 ± 0.81	0.80 ± 0.21
Carotenoids (μg/ml)	0.5	0.44 ± 0.14	1.44 ± 0.51	0.97 ± 0.45	0.72 ± 0.22
	1.0	0.70 ± 0.02	1.69 ± 0.06	1.69 ± 0.17	1.03 ± 0.16
	1.5	0.64 ± 0.16	1.23 ± 0.23	1.12 ± 0.06	1.77 ± 0.28
	2.0	0.49 ± 0.09	1.60 ± 0.18	1.22 ± 0.36	1.51 ± 0.42

Table 2
Effect of different concentrations of crude oil on chlorophyll *a* and carotenoids (μg/ml) content of *N. punctiforme* (Kuz.). Results are mean of three replicates (±standard error of the mean)

	Crude oil conc.%	3rd day	7th day	11th day	15th day
Chlorophyll *a* (μg/ml)	0.5	0.25 ± 0.037	0.42 ± 0.55	0.73 ± 0.049	1.87 ± 0.403
	1.0	0.95 ± 0.556	0.84 ± 0.421	0.63 ± 0.2065	2.01 ± 0.214
	1.5	0.09 ± 0.071	1.05 ± 0.11	1.23 ± 0.3875	1.93 ± 0.003
	2.0	0.94 ± 0.8305	1.10 ± 0.42	1.22 ± 0.5345	2.27 ± 0.907
Carotenoids (μg/ml)	0.5	0.32 ± 0.123	0.47 ± 0.05	0.54 ± 0.095	1.08 ± 0.047
	1.0	0.47 ± 0.194	0.45 ± 0.32	0.48 ± 0.110	1.12 ± 0.371
	1.5	0.12 ± 0.0479	0.68 ± 0.304	0.7 ± 0.045	1.16 ± 0.011
	2.0	0.75 ± 0.606	0.82 ± 0.54	0.86 ± 0.368	1.44 ± 0.820

were completely disappeared when incubated with *S. platensis*, (Tables 3 and 4) except tetracosane, the residual concentration was 0.45% compared to blank concentration 9.87% in case of concentration 0.5%. Aliphatic compounds were not disappeared when incubated with *S. platensis* grown at 2% crude oil. The aliphatic compounds were Tridecane, Tetradecane, Pentadecane, Hexadecane, Heptadecane, Tricosane, and Tetracosane.

In case of *N. punctiforme* aliphatic compounds completely disappeared at concentration 1%. Decane ($C_{10}H_{22}$), Pentacosane ($C_{25}H_{52}$), Hexacosane ($C_{26}H_{54}$), Octacosane ($C_{28}H_{58}$), Nonacosane ($C_{29}H_{60}$) totally removed from all concentrations of crude oil in both of *S. platensis* and *N. punctiforme* (Table 3). Sorkhoh et al. [28], demonstrated that cyanobacterial mat has a strong potential in hydrocarbon degradation. Prince et al. [29]; Oudot [30], mentioned high biodegradation of crude oil by cyanobacterial mat. Some residual compounds increase in comparison to the blank; this may be due to the formation of intermediate compounds during biotransformation or biodegradation processes when blue-green algae were used. The amount of aromatic compounds remaining in crude oil after 15 days of incubation with liquid cultures of *N. punctiforme* and *S. platensis* were increased progressively in comparison with blank as shown in Table 4 except Benzene, decyl ($C_{16}H_{26}$), and Anthracene, -Dimethyl ($C_{16}H_{14}$) are completely removed when different concentrations of crude oil were incubated with S. *platensis*. Wang and Fingas [31], demonstrated that aromatic fraction contained is a greater concentration due to resistant to microbial degradation. It can be speculated that aliphatic compounds degraded by algae to two units, acetate and malonate that is capable of being folded and formed aromatic ring through number of sequential steps, the whole sequence of reactions is carried out by an enzyme complex secreted by cyanobacteria, which converts acetyl-CoA and malonyl-CoA into the final product. These enzyme complexes may combine polyketide synthase and polyketide cyclase activities [32]. Goto et al. [33], reported that cyanobacteria were shown to frequently encode LanM type enzymes, i.e. bifunctional enzymes catalyzing both dehydration and cyclization reactions. Harada [34]; Voloshko et al. [35];

Table 3
Aliphatic compounds remaining in crude oil residual after 15 days of incubation with liquid cultures of *N. punctiforme* (Kuz.) and *S. platensis* compared to blank

Compounds	Molecular formula	Crude oil%								
		Nostoc punctiforme (Kuz.)					*Spirulina platensis*			
		Blank	0.5	1	1.5	2	0.5	1	1.5	2
Decane	$C_{10}H_{22}$	0.96	ND	ND	ND	ND	ND	ND	ND	ND
Dodecane	$C_{12}H_{26}$	1.66	ND	ND	2.98	2.59	ND	ND	ND	ND
Tridecane	$C_{13}H_{28}$	2.34	I.7 6	ND	3.1	6.43	ND	ND	ND	3.02
Tetradecane	$C_{14}H_{30}$	0.22	5.92	ND	1. 61	2.46	ND	ND	ND	3.66
Pentadecane	$C_{15}H_{32}$	2.5	ND	ND	4.34	1.82	ND	ND	ND	3.95
Hexadecane	$C_{16}H_{34}$	2.65	2.28	ND	1.81	2.09	ND	ND	ND	2.78
Heptadecane	$C_{17}H_{36}$	1.95	ND	ND	1.01	4.41	ND	ND	ND	2.72
Octadecane	$C_{18}H_{38}$	1.48	ND	ND	2. 3 2	ND	ND	ND	ND	ND
Nonadecane	$C_{19}H_{40}$	8.81	ND	ND	1. 86	2.06	ND	ND	ND	ND
Docosane	$C_{22}H_{46}$	14.62	1. 80	ND	8.9	0.95	ND	ND	ND	ND
Tricosane	$C_{23}H_{84}$	6.82	1.78	ND	3.76	1.43	ND	ND	ND	12.66
Tetracosane	$C_{24}H_{50}$	9.87	3.04	ND	I .79	3.25	0.45	ND	ND	6.1
Pentacosane	$C_{25}H_{52}$	8.8	ND	ND	ND	ND	ND	ND	ND	ND
Hexacosane	$C_{26}H_{54}$	6.03	ND	ND	ND	ND	ND	ND	ND	ND
Octacosane	$C_{28}H_{58}$	10.19	ND	ND	ND	ND	ND	ND	ND	ND
Nonacosane	$C_{29}H_{60}$	3.36	ND	ND	ND	ND	ND	ND	ND	ND

Table 4
Aromatic compounds remaining in crude oil after 15 days of incubation with liquid cultures of *N. punctiforme* (Kuz.) and *S. platensis* compared to blank

Compounds	Molecular formula	Crude oil%								
		Nostoc punctiforme (Kuz.)					*Spirulina platensis*			
		Blank	0.5	1	1.5	2	0.5	1	1.5	2
Naphthalene, 1-methyl	$C_{11}H_{10}$	0.96	4.95	7.02	8.11	5.33	1.24	ND	6.64	1.68
Dimethylnaphthalene	$C_{12}H_{12}$	6.6	23.58	19.5	10.81	11.82	4.82	ND	32.13	17.29
Trimethylnaphthalene	$C_{13}H_{14}$	4.68	21.96	28.45	8.02	14.72	20.72	ND	27.86	20.93
p-Phenyltoluene	$C_{13}H_{12}$	0.32	ND	2.35	1.56	ND	ND	ND	1.72	ND
Phenanthrene, 1-methyl	$C_{15}H_{12}$	0.45	6.18	1. 39	5.94	1.93	ND	ND	2.01	1.72
Anthracene, -methyl	$C_{15}H_{12}$	1.0	I.12	5.71	ND	0.69	8.26	ND	5.46	7.37
Dimethylphenanthrene	$C_{16}H_{14}$	2.45	8.82	4.21	2.59	5.65	9.46	ND	ND	6.51
Benzene, decyl	$C_{16}H_{26}$	0.92	ND	ND	ND	1.5	ND	ND	ND	ND
Anthracene, -dimethyl	$C_{16}H_{14}$	0.61	ND	4.83	ND	0.9	ND	ND	ND	ND

Rawat and Bhargava [36]; Kurmayer [37], demonstrated that cyanobacteria produce various cyclic peptides.

GC analyses confirmed that complete removal of crude oil is possible with 1% incubation of *S. platensis*; this may be because the culture was contaminated with heterotrophic bacteria that degraded crude oil completely (Table 3). Chaillan et al. [10], reported that cyanobacterial strain form a high-biodiversity microbial consortium that contains efficient hydrocarbons degraders.

4. Conclusion

This study demonstrated that *N. punctiforme* and *S. platensis* are able to grow under heterotrophic condition using crude oil as sole carbon sources. Both algae can degrade aliphatic compounds contents of crude oil.

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