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B.A. PANAH[1,2], F. NAJAFI[1], N. SOLTANI[2], R.A. KH. NEJAD[1, 3], S. BABAEI[1,2]

[1]Faculty of Biological Sci., Kharazmi Univ., Tehran, Iran
[2]Department of Petroleum Microbilogy, ACECR, Res. Inst. of Applied Sci., Tehran, Iran
[3]Department of Biology, Sci. and Res. Branch, Islamic Azad Univ., Tehran, Iran

samarehbabaei@yahoo.com

BIODEGRADATION ABILITY AND PHYSIOLOGICAL RESPONSES OF CYANOBACTERIUM *LEPTOLYNGBYA* SP. ISC 25 UNDER NAPHTHALENE TREATMENT

Cyanobacteria (*Cyanoprokaryota*) have gained a lot of attention in recent years because of their potential applications in biotechnology. In this study the cyanobacterium *Leptolyngbya* sp. ISC 25 was identified as tolerating and effectively degrading naphthalene as a toxic compound in the environment. The cyanobacterium was treated with different concentrations of naphthalene. Physiological responses such as survival, Chlorophyll *a* content, photosynthesis rate and ammonium excretion were investigated in logarithmic phase of growth curve. The biodegradation ability of the cyanobacterium was measured by GC and GC/MS analysis. Results indicated that chlorophyll *a* concentration decreased with naphthalene increasing and was approximately zero in the presence of 1 % naphthalene, Phycobiliproteins content enhanced up to 0.2 % of naphthalene, but at higher concentrations decreased significantly. Photosynthesis rate and ammonium excretion decreased in all treatments. Results of GC analysis confirmed the degradation of naphthalene by *Leptolyngbya* sp. ISC 25 in comparison with control (without the cyanobacterium). The results of GC/MS analysis identified the products of naphthalene degradation by *Leptolyngbya* sp. ISC 25. Totally lower concentrations of naphthalene is not lethal for cyanobacterium *Leptolyngbya* sp. and this strain can biodegrade naphthalene to 2(4H)-benzofuranone-tetrahydro-trimethyl mainly.

K e y w o r d s: ammonium excretion, biodegradation, chlorophyll *a*, *Leptolyngbya*, naphthalene, photosynthesis.

Introduction

Polycyclic Aromatic Hydrocarbons (PAHs) are a group of compounds that are made of two or more aromatic rings. PAHs are released into the environment through contamination by crude oil or refinery products (Kumar et al., 2009). Environmental Protection Agency (EPA) has identified 16 unsubstituted PAHs as priority pollutants. Eight of these PAHs are considered to be possible carcinogens, and because of their dispersion in the environment and possible exposure to human, have been an important subject for investigations (Menzie et al., 1992). Carcinogenic PAH compounds are released into the environment by natural and anthropogenic sources and are usually present in food, air, water and soil (Menzie et al., 1992). Naphthalene is a major PAH component in the water soluble fraction of crude and certain fuel oils.

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A wide variety of cyanobacteria can oxidize aromatic hydrocarbons under photoautotrophic growth conditions. In fact, microbial activity is known as the most important and effective functions to remove PAHs (Agbozu and Opuene, 2009; Atlas and Bragg, 2009; Cerniglia et al., 1980a; Nwuche and Ugoji, 2008). The first investigation about the ability of cyanobacterium in oxidation of PAHs was accomplished by Ellis (1977). But the knowledge about the potential ability of cyanobacteria to degrade oil residues is still very limited. Cultures of *Microcoleus chthonoplastes* Fl. Dan. and *Phormidium corium* (Agardh) Gomont were able to degrade n-alkanes (Hasan et al., 1994). *Oscillatoria* sp. and *Agmenellum quadruplicatum* oxidized naphthalene to 1-naphthol (Cerniglia et al., 1980b). *Phormidium tenue* (Menegh.) Gomont could be used for bioremediation of naphthalene and anthracene on polluted seashores (Kumar et al., 2009).

Besides, these toxic compounds affect the physiological activities of cyanobacteria. *Cyanobacteria* are essential constituents of aquatic ecosystem since they are the first trophic level in the food chains and they are the major organisms providing oxygen and organic substances to other life forms (Kong et al., 2011). So, these microorganisms and their physiological activities in presence of pollutions are very important to researchers. The effect of naphthalene on the growth and photosynthetic activity of cyanobacteria were investigated in some studies (Gaur and Singh, 1990; Kabli, 1998). Kumar et al. (2009) investigated the toxic effect of naphthalene on *Ph. tenue* in polluted seashore.

The present study was undertaken (i) to evaluate the biodegradation ability of the cyanobacterium *Leptolyngbya* sp. ISC 25, and (ii) to elucidate the physiological responses of this species under the naphthalene stress.

Materials and Methods

Isolation of Cyanobacterium

The cyanobacterial strain, *Leptolyngbya* sp., was isolated from soil in a recent study (Soltani et al., 2012). DNA sequence analysis of phylogeny with that in the National Center for Biotechnology Information (NCBI) database further confirmed the species. Ribosomal RNA gene of the axenic strain was sequenced and deposited in NCBI under the access ion no. of GU138681.

Culture condition

The cyanobacterium was subcultured on BG11 medium. Temperature was maintained at 30±1 ºC. Cultures were bubbled with air (flow rate, 200 mL · min^{-1}) under constant light intensity of 60 μmol photon m^{-2} s^{-1} supplied by three fluorescent tubes. The organisms were transferred into carbonless BG11 medium in logarithmic phase of growth. Naphthalene was dissolved in acetone (at 0.1 g · L^{-1}) and added to these cultures at various concentrations (0.05; 0.2; 0.4; 0.6 and 1 %) and the cultures were incubated as described above.

Analytical methods

The growth of cyanobacterium, was estimated as the increase in dry weight (Leganés et al., 1987). Chlorophyll was extracted using 90 % aqueous methanol and measured spectrophotometrically at 665 nm (Marker, 2006). Phycobiliproteins were extracted after osmotic shock and analyzed spectrophotometrically at 750, 652, 615 and 562 nm (Marker, 2006). Ammonium release test was performed according to phenol method and analyzed spectrophotometrically at 630 nm, as described by APHA-AWWA-WPCF (1985).

Photosynthesis

O_2 evolution was measured with a Clark-type O_2 electrode in 5 min. Two mL of suspension were placed in a temperature controlled cuvette (25 ºC) and illuminated at desired condition (Dodds, 1989).

Degradation of naphthalene by cyanobacterium

Degradation of naphthalene representing aromatic compounds used at 0.05 % was analyzed by GC and GC/MS. A control (cyanobacterium in medium without naphthalene) was maintained for every experiment.

GC and GC/MS

The GC analysis was accomplished by GC-15A system. A Rtx-5MS Capillary column (30 m × 0.25 mm × 0.25 μm) was used with H_2 as carrier gas (1 mL · min^{-1}). The oven program was 50−26 ºC at 10 ºC min^{-1}. For the GC/MS analysis, a HP5-MS capillary column (30 m × 0.25 mm) was used with helium as carrier gas (1 mL · min^{-1}). The oven program was 60−100 ºC at 10 ºC min^{-1} then 150−295 ºC at 4 ºC min^{-1}. The injection port and detector were at 100–290 ºC and 298 ºC respectively.

Statistical analysis

Data are means and standard deviation of at least 3 replicates. SPSS Windows ver.15 software was used for examination of statistical significance of the differences.

Results and Discussion

Growth rate was evaluated via measuring the dry weight of the cyanobacterial biomass. Some results are presented according to the specific growth rate (Table 1). They showed that the growth of cyanobacterium *Leptolyngbya* sp. ISC 25 was affected by naphthalene. Biomass production was enhanced in the presence of 0.05 and 0.2 % naphthalene, but the rate of growth was slower than that in control. In fact, the cyanobacterium can tolerate naphthalene in these concentrations especially in 0.05 %. Maybe the cyanobacterium utilizes naphthalene as a carbon source for its growth. But with increase in naphthalene concentration, the growth of the cyanobacterium decreased significantly, until its death at 0.05 and 0.2 % concentration. The toxicity effects of aromatic hydrocarbons such as naphthalene on the growth of cyanobacteria have been studied in previous studies (Gaur and Singh, 1990).

Chlorophyll *a* content decreased with increasing concentration of naphthalene and in 1 % of naphthalene, it was approximately zero (Table 2) which has been confirmed in previous studies (Amotz et al., 1982; Hasan et al., 1994). This reduction can be attributed to the inhibition of chlorophyll synthesis fallowed by obstruction of α-aminolevulinic acid and proto-chlorophyllide reductase activity (Ouzounidou, 1995).

Table 1

The specific growth rate of *Leptolyngbya* sp. ISC 25 in the presence of different concentration of naphthalene

Treatment naphthalene (%)	SGR (d^{-1})
control	0.183 ± 0.0028^{a}
0.05	0.024 ± 0.0041^{b}
0.2	0.021 ± 0.0012^{b}
0.4	0.0033±0.017-
0.6	-0.018 ± 0.0041^{c}
1	-0.020 ± 0.0005^{c}

N o t e . Here and in Table 2. The data are the values of three experiments ± SE. SGR – specific growth rate.

Table 2

The effect of naphthalene treatment on pigment content in *Leptolyngbya* sp. ISP 25

Treatment with naphthalene (%)	Chl. *a*	PC	APC	PBP	PBP/Chl. *a*
			μg · mg dw^{-1}		
Control	2.685 ± 0.108^{a}	35.72 ± 0.329^{c}	2.645 ± 0.181^{a}	37.72 ± 0.382^{b}	14.09 ± 0.543^{c}
0.05	1.593 ± 0.057^{b}	40.93 ± 1.228^{b}	2.712 ± 0.263^{a}	42.48 ± 0.928^{a}	26.78 ± 1.584^{c}
0.20	1.235 ± 0.062^{c}	44.12 ± 1.798^{a}	0.765 ± 0.043^{b}	42.93 ± 2.096^{a}	34.78 ± 0.127^{c}
0.40	0.495 ± 0.101^{d}	13.24 ± 0.399^{d}	0.393 ± 0.007^{c}	13.55 ± 0.098^{c}	32.36 ± 6.663^{c}
0.60	0.129 ± 0.015^{e}	8.533 ± 0.434^{e}	0.249 ± 0.022^{bc}	8.048 ± 0.45^{d}	63.34 ± 8.567^{b}
1	0.075 ± 0.008^{e}	7.942 ± 0.056^{e}	0.217 ± 0.019^{c}	7.905 ± 0.139^{d}	112.2 ± 17.28^{a}

D e s i g n a t i o n . APC – allophycocyanin; Chl. *a* – chlorophyll *a*; PBP – phycobiliproteins; PC – phycocyanin.

Phycobiliproteins content after treatment with 0.05 and 0.2 % of naphthalenes showed no significant difference from that of the control. In the presence of naphthalene at more than 0.2 %, phycobiliprotein decreased severely as the lowest amount was observed in 1 % of naphthalene. In cyanobacterium *Leptolyngbya* sp. ISC 25, phycocyanin (PC) is the main

component of phycobiliproteins, so in the cyanobacterium the changes on total Phycobiliproteins (PBP) mostly reflect the changes on PC. The allophycocyanin (APC) concentration decreased with increasing naphthalene concentration. The PBP/Chl. *a* ratio is usually used to quantify the relationship between photosystem II (PSII) and photosystem I (PSI) (Yamanaka and Glazer, 1981). This ratio increased significantly with increasing naphthalene concentration as the highest values was noted in the presence of 1 % of naphthalene. Generally, there was an inverse correlation between naphthalene and the content of photosynthetic pigment in cyanobacterium *Leptolyngbya* sp. ISC 25.

In cyanobacteria, phycobiliproteins that are in stroma surface of thylakoid membrane act as primary light harvesting antenna for PS II. Transfer of energy within these additional pigments follows the path from phycoerythrin (when present) to phycocyanin, then to allophycocyanin (APC), and finally to long-wavelength pigment (Mimuro et al., 1986). The structure and activity of phycobiliproteins change under stress conditions (Sundaram and Soumya, 2011).

These results implied that phycobiliproteins were affected by naphthalene. Phycoerythrin doesn't exist in cyanobacterium *Leptolyngbya* sp. ISC 25 and the principal part of phycobiliprotein's structure is phycocyanin. Phycocyanin content increased from control to 0.2 % of naphthalene. Probably, it is because of rising in amount of phycobilisomes and phycobiliprotein's content in response to stress. However, with increasing naphthalene concentration up to 0.2 %, the strain couldn't tolerate the toxicity of naphthalene and decreased significantly. As APC is a component of phycobilisome's core, and the core remains constant, a change in APC content reflects a change in quantity of phycobilisomes. In the cyanobacterium, APC content decreased gradually with increasing naphthalene concentration. The PBP/Chl. *a* ratio is usually used to quantify the relationship between PS II and PS I (Yamanaka and Glazer, 1981). The PBP/Chl. *a* ratio significantly increased in higher naphthalene concentration. These results confirmed the studies of Hasan et al. (1994), wherein demonstrated that PBP content was affected more than chlorophyll content, under stress condition.

The effect of naphthalene on photosynthetic activity of the cells was also examined to analyze functional significance of altered pigment pattern. These results showed that photosynthetic activity decreased with increasing naphthalene concentrations, but not significantly in 0.05 and 0.2 % (Fig. 1) and became 37.602 $\mu g \cdot mg\ dw^{-1}$ in the presence of 1 % naphthalene. Oxygen release decreased with increasing naphthalene concentration. Although in this cyanobacterium there was no significant difference between control, 0.05 and 0.2 % naphthalene, but generally the oxygen release decreased with increasing of naphthalene. According to previous studies, photosynthetic activity decreased slightly because of toxicity of naphthalene. Kabli (1998) showed the oxygen evaluation of three algae was greatly inhibited by crude oil and naphthalene. Also Soto et al. (1975) found that the addition of 100 %

naphthalene to *Chlamydomonas angulosa* Dill cultures caused immediate and almost complete loss of photosynthetic activity.

In general, the amount of ammonium excretion decreased with increasing naphthalene concentration (Fig. 2). Although, it showed little increase in 0.4 % naphthalene. Ammonium release can be important in economic and scientific aspects in investigation on growth and adaption in cyanobacteria (Boussiba, 1988). The results showed that ammonium release generally reduced with increasing naphthalene concentration. Accordingly, ammonium release is due to active nitrogen assimilation and its accumulation inside of cell, therefore a reduction in ammonium excretion with increasing naphthalene concentration, was expected.

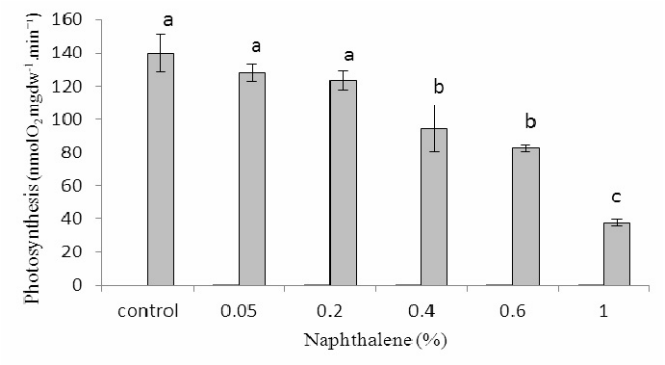

Fig. **1**. The effect of naphthalene on photosynthetic activity of *Leptolyngbya* sp. ISC 25

According to Fig. 2, the ammonium excretion was increased in 0.4 % of naphthalene. Maybe it is because of existence of nitrogen component due to entrance of cell into death phase (Borowitzka and Borowitzka, 1988).

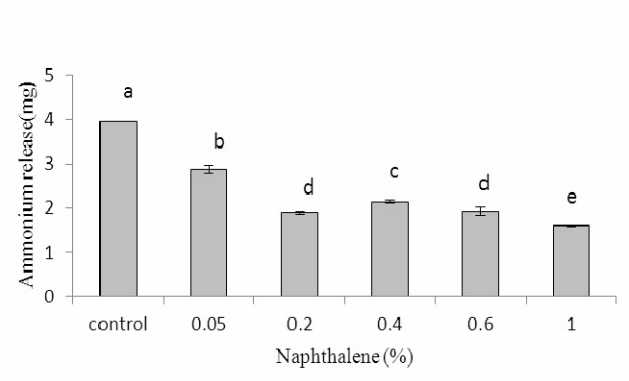

Fig. **2.** The effect of naphthalene on ammonium excretion of *Leptolyngbya* sp. ISC 25

Degradation of naphthalene by the cyanobacterium *Leptolyngbya* sp. ISC 25 was assayed also in 0.05 % naphthalene. Results of these analyses indicated

significant reduction in amount of naphthalene within 10 days incubation (Fig. 3) in comparison with control sample (Fig. 4).

GC/MS analysis was assayed after 10 days treatment of *Leptolyngbya* sp. ISC 25 with 0.05 % naphthalene detected the degradation products (Table 3).

These results showed that naphthalene as a toxic component in the environment, can affect physiological responses of cyanobacterium *Leptolyngbya* sp. ISC 25. Also we found that this cyanobacterium could tolerate 0.05 and 0.2 % of naphthalene.

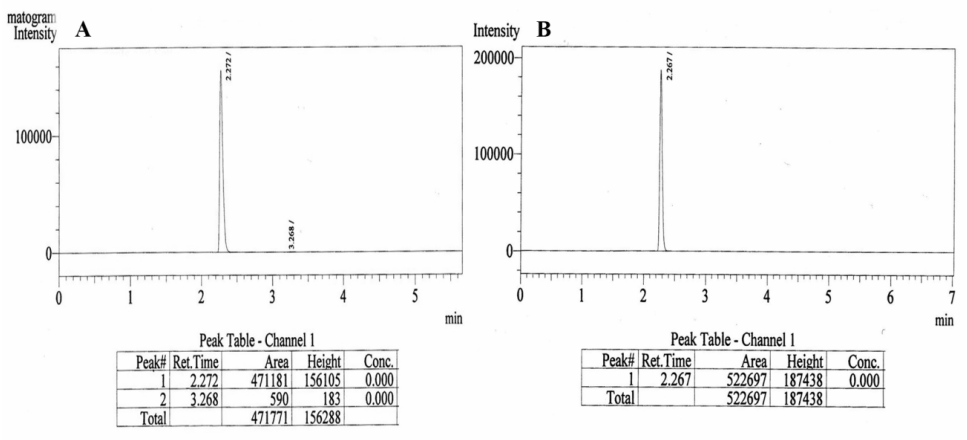

Fig. **3**. Diagram of GC analysis after treatment with 0.05 % naphthalene during 1 (*A*) and 10 (*B*) day

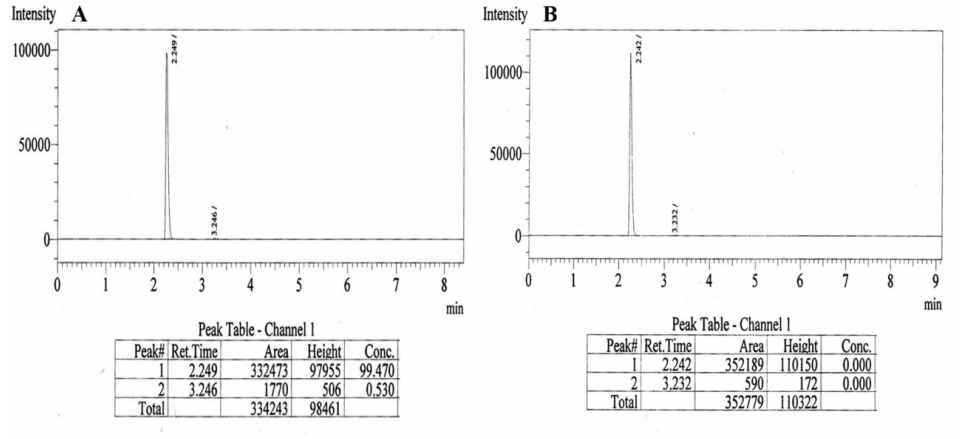

Fig. **4**. Diagram of GC analysis of control after 1 (*A*) and 10 (*B*) day

The more important part of this research was in relation of biodegradation ability of naphthalene by cyanobacterium *Leptolyngbya* sp. ISC 25. GC analysis of naphthalene degradation indicated that it has been removed by cyanobacterium after incubation for 10 days comparing with control

(cyanobacterium free) and GC/MS analysis indicated the formation of component that presumably arising by an inducible enzyme system. So, the cyanobacterium could oxidize naphthalene under photoautotrophic condition. According to Table 3, the most important component produced in oxidation of naphthalene, is 2(4H)-benzofuranone, -tetrahydro-trimethyl. Other component identified by GC/MS is intermediate of naphthalene oxidation (Zhang et al., 2004).

Oxidation of aromatic ring carbon in an energy providing process often leads to complete degradation of the substrate. These results confirmed previous studies about photooxidation of naphthalene by cyanobacteria. The first investigation on biodegradation ability of *Oscillatoria* sp. and *Agmenellum quadruplicatum* was accomplished by Cerniglia et al. (1980a,b). They identified *cis*-1,2-dihydroxy-1,2-dihydronaphthalene, 4-hydroxy-1-tetralone and 1-naphthol, as initial compounds from naphthalene oxidation. Narro et al. (1992) showed that *Oscillatoria* sp. could oxidize naphthalene to naphthalene-1,2-oxide. Kumar et al. (2009) demonstrated that *Phormidium tenue* had ability to oxidize naphthalene to 1,2-naphthoquinone and naphthalene-1,2-diol.

Table 3

Identified components of GC/MS analysis

Component	RT (min)
Naphthalene	15.95
Benzothiazole	17.266
Isoquinoline	18.39
Dimethylnaphthalene	22.08
Naphthalene, 1,4,6-trimethyl	23.54
2(4H)-benzofuranone, -tetrahydro-trimethyl	24.04

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А. Панах[1,2], Ф. Наджафи[1], Н. Солтани[2], Р.А.Х. Неджад[1, 3], С. Бабаи[1, 2]

[1]Биологический факультет, Университет Харазми, Тегеран, Иран

[2]Отдел микробиологии нефти, ACECR, Исследовательский ин-т прикладных наук, Тегеран, Иран

[3]Отдел биологии, Научно-исследовательское отделение, Исламский Университет Азад, Тегеран, Иран

СПОСОБНОСТЬ К БИОДЕСТРУКЦИИ НАФТАЛИНА И ВОЗДЕЙСТВИЕ НА НЕГО ФИЗИОЛОГИЧЕСКИХ РЕАКЦИЙ ЦИАНОБАКТЕРИИ *LEPTOLYNGBYA* SP. ISC 25

Исследование посвящено изучению физиологических реакций цианобактерии *Leptolyngbya* sp. ISC 25 при воздействии нафталина. Показано, что данный штамм устойчив к нафталину и способен эффективно разлагать его токсичные соединения в окружающей среде. Воздействие различных концентраций нафталина на способность

цианобактерии к выживанию, содержание хлорофилла *a*, скорость фотосинтеза и экскреции аммония были исследованы в логарифмической фазе роста культуры. Способность цианобактерии к биодеградации нафталина измеряли методами газовой хроматографии и масс-спектрометрии (ГХ/МС). Показано, что концентрация хл. *a* снижалась с увеличением концентрации нафталина в среде, достигая нуля в присутствии 1 % нафталина. Содержание фикобилипротеинов в присутствии нафталина сперва уваличивалось (до 0,2 % нафталина), но при более высоких его концентрациях существенно снижалось. Скорость фотосинтеза и экскреции аммония снижалась в присутствии нафталина в любой концентрации. ГХ подтвердила разложение нафталина в присутствии штамма *Leptolyngbya* sp. ISC 25 по сравнению с контролем (без цианобактерии). Продукты деградации нафталина культурой *Leptolyngbya* sp. ISC 25 определены методами ГХ/МС. Установлено, что низкие концентрации нафталина не являются смертельными для цианобактерии и этот штамм способен к биодеструкции нафталина до 2(4H)-бензофуранозон-тетрагидро-триметила.

К л ю ч е в ы е с л о в а : цианобактерия, *Leptolyngbya* sp., нафталин, хлорофилл *a*, скорость фотосинтеза, экскреция аммония, логарифмическая фаза роста.