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Crude Oil-induced Morphological and Physiological Responses in Cyanobacterium *Microchaete tenera* ISC13

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ABSTRACT: In this research the effects of crude oil on morphological and physiological characterization of the cyanobacterium *Microchaete tenera* ISC13 were investigated. Isolated cyanobacterium treated with different oil concentrations (control, 1, 2.5, 5 and 7%) in carbonless $BG11_0$ medium. Morphological characteristics such as morphology of filament, cellular shapes and sizes, relative position of heterocytes and akinetes were described for these treatments. Biometrical and morphological observations carried out by light and scanning electron microscopy. Dimensions of cells did not significantly impress, although a slightly increase in length of vegetative cells was observed in 2.5 and 5% crude oil in comparison to control. With respect to the physiological responses, cyanobacterium growth increased with elevated oil concentration but no changes was observed in chlorophyll content. Phycobiliproteins (PBP), phycocyanin (PC) and allophycocyanin (APC) had the highest rate in control. Increasing crude oil decreased all PBP. This study demonstrated that crude oil doesn't have destructive effect on *Microchaete tenera* ISC13 and suggest probable potential of this microorganism to use oil hydrocarbons as carbon source.

Key words: *Microchaete*, Morphology, Oil pollution, SEM, 16S rRNA

INTRODUCTION

Crude oil is a highly toxic mixture of more than 10000 different hydrocarbons. Accidental spills of crude oil in environment cause severe contamination of marine and continental ecosystems. Contamination due to spill of processed petroleum derivates (especially diesel and fuel) is an important problem in waters (Lopez-Rodas *et al.*, 2009), generated from both natural and anthropogenic processes (Haghighat *et al.*, 2008; Atlas and Bragg, 2009; Hughes *et al.*, 1997). These pollutants can potentially be degraded by a great variety of soil and aquatic microorganisms. Bacteria, filamentous fungi, yeasts, and cyanobacteria are known to be important hydrocarbon degraders (Raghukumar *et al.*, 2001; Das and Chandran, 2011). Study of these key organisms is important for understanding and evaluating bioremediation strategies (Harayama *et al.*, 2004). In most studies, degradation of crude oil by various species of cyanobacteria has been reported (Kumar *et al.*, 2009; Agbozu and Opuene, 2009; Atlas and Bragg, 2009; Dubey *et al.*, 2011). Despite of this high potential of microalgae there is not much attention to the variation of these microorganisms under petroleum contamination. The flexibility of these microorganisms in morphology (vegetative cells, heterocyst and akinet formation and location) and life cycle give them the ability to survive in such extreme environments (Stal, 1995). The microscopy techniques most frequently used to determine the diversity of cyanobacteria in terms of morphology, not only by the classical light microscopy, but also by scanning electron microscopy (SEM) (Hernández-Mariné *et al.*, 2004).

Also these environmental stresses influence the physiological activities of living organisms. When a change in the environment exceeds a certain threshold

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level, some metabolic pathways are inhibited or abolished and some of others are enhanced or induced. There are a number of studies on responses of cyanobacteria to oil pollution (Rajaniemi *et al.*, 2005; Willame *et al.*, 2006; Soltani *et al.*, 2012), but information is almost accumulating on the structure of cyanobacteria under fluctuations in oil concentration. With regards to documents, cyanobacterial morphology (Rajaniemi *et al.*, 2005; Soltani *et al.*, 2012; Diestra *et al.*, 2007); its molecular characters (Willame *et al.*, 2006); growth and photosynthesis (Gaur and Singh, 1990; Kabli, 1998) and pigments (Yamamaka and Glazer, 1981; Bermejo *et al.*, 2002; Pimda and Bunnag, 2012) adversely affected, but further studies are needed in relation to characterization of endemic oil polluted microalgae. We wanted to investigate the responses of *Microchaete tenera* ISC13, a heterocystous cyanobacterium to petroleum stress. The present investigation is an attempt to study the morphological and physiological responses of the cyanobacterium *Microchaete tenera* ISC13 isolated from oil polluted regions of Iran at elevated concentrations of petroleum.

MATERIALS & METHODS

The strain *Microchaete tenera* ISC13 was isolated from soils polluted of Iran. Cultivation phases done were as following: Isolation and purification by solid agar (Andersen, 2005), liquid cultures by routine procedures with carbonless $BG11_0$ as media culture. Temperature was maintained in 30±2 ºC and cultures were bubbled with air under a constant light intensity of 60 µmol photon m^{-2} s^{-1}. Treatments were including 0 (control), 1, 2.5, 5 and 7% crude oil. Growth rate was calculated as dry weight (Soltani *et al.*, 2006). In order to chlorophyll determination, cells were extracted with pure methanol for 24 hours at 4 °C, and the chlorophyll content was determined spectrophotometrically at 665 nm according to Marker (1972). Phycobiliproteins were extracted after osmotic shock and measured spectrophotometrically at 652, 615 and 562 nm. O_2 evolution was measured with a Clark-type O_2 electrode (Shokravi and Soltani, 2012).

With respect of morphological studies solid medium was prepared and maintained in the same condition as liquid, following inoculation. Semipermanent slides prepared every other day and seen by light and florescence microscopes. The morphology of cells and filaments was studied by Olympus CX 40 light microscope. The following parameters were selected to describe the morphology of the studied strain: Biometrical characteristics; dimensions of vegetative cells, heterocyts and akinetes, presence or absence of terminal heterocyts and shape of filament and its aggregation in colonies (Gugger and Hoffmann, 2004). Regarding to study of ultrastructure by scanning electron microscopy (SEM) samples was fixed in 2.5% glutaraldehyde for 4 hours and washed in buffer phosphate (PBS). Then samples were centrifuged and dehydrated in successively increasing concentrations of alcohol (10%, 30%, 50%, 70%, 90% and 100%). Finally, all samples were mounted on metal stubs and coated with a layer of gold (Diestra *et al.*, 2007).

To extract DNA from the *Microchaete* a fresh biomass was obtained by centrifuging at 12000 rpm and using Fermentas kit (#k0512). The applied PCR condition has been described by Nübel *et al.* (2000). PCR amplification, cloning and sequence analysis of 16S DNA content was first extracted from the cyanobactrium, and then PCR was applied using two set of primers. Sequences were amplified using the primers 979F (CGATGCAACGCGAAGAAC) as forward and 1092R (GCGCTCGTTGCGGGACTT), amplify a ~2000 –bp region of the 16Sr RNA gene. PCR products were obtained by electrophoresis in a 1% (w/v) agarose gel using TBE buffer containing DNA set stain. The sequence was determined by the Genfanavaran Company with the primers. The sequence data was analyzed using a similarity search by using the BLAST through the website of the NCBI. The nucleotide sequences described in this study have been submitted to the NCBI under the accession number NCBI: JF290484. Data were statistically analyzed using a one-way ANOVA followed by Duncan's new multiple range test using SPSS version 16.0. Three independent variables were considered for each experiment.

RESULTS & DISCUSSION

Many cyanobacteria have isolated from oil polluted regions (Al-Hasan *et al.*, 1998). Taking this point into account, present study focused on both morphological and physiological behavior fluctuations of *Microchaete tenera* ISC13 in elevated crude oil concentrations. This information is interesting, not only for basic science but also for the application point of view. According to the morphological variation of cyanobacteria under oil treatment due to theirs flexibility, cell morphology and colonization in liquid cultures were examined by light microscope.

The effects of crude oil on the morphological characters showed that filaments of *Microchaete tenera* form mats on the oil globules and *Microchaete* cells were seen in the close vicinity of oil droplets (fig.1). The morphological characteristics of the strain *Microchaete tenera* are summarized in Table1. This strain had straight or curved trichomes; solitary or

bundled (Table1, Fig. 2). Vegetative cells were more squared or cylindrical. Heterocysts were present apically. The terminal cells of strain were slightly tapered, and clearly distinguishable by the absence of rounded terminal cells. In the treatments the cells didn't tend to enlarge. As seen in the Table 1, the length of vegetative cells increased with enhancing the oil concentration. But these differences are significant only in 2.5 and 5% oil. The width of vegetative cells did not change significantly. Same results were seen in the case of heterocysts. Also there was no indication of akinetes in any treatments. These results covered the physiological observations. Electron microscopy techniques (SEM) were also used to determine morphological variation. The images obtained by SEM indicated that in 7% oil a slightly increase of length of the cells and also the relative degeneration of walls was observed. Also the hyaline mucilaginous sheath was observed clearly in control and treatments(fig.3).

Cell dimensions are regularly used in determination of cyanobacterial species. Dimensions of cyanobacterial cells in optimally growing natural populations appear to be fairly stable with little intraclonal variability, although interclonal variability is higher within the same basic morphotype (Campbell and Golubic, 1985; Abed *et al.*, 2003). Detailed biometrical observations of this strain showed no remarkable change in vegetative and apical heterocyst cells dimensions. Furthermore the cells didn't tend to show akinetes in any treatments. As we know, the ability to develop akinetes (spore-like cells) is a survival trait of the Nostocales that provides these species with a competitive advantage over extreme conditions. These dormant cells survive in the harsh conditions in different extreme environments. As conditions improve,

Fig. 1. Colonization of *Microchaete tenera* growing on agar plates (Left) and filaments close to oil droplets (shown with arrow key) in liquid culture (Right)

Fig. 2. Filaments of *Microchaete tenera* in control (Left) and polluted cultures (Right)

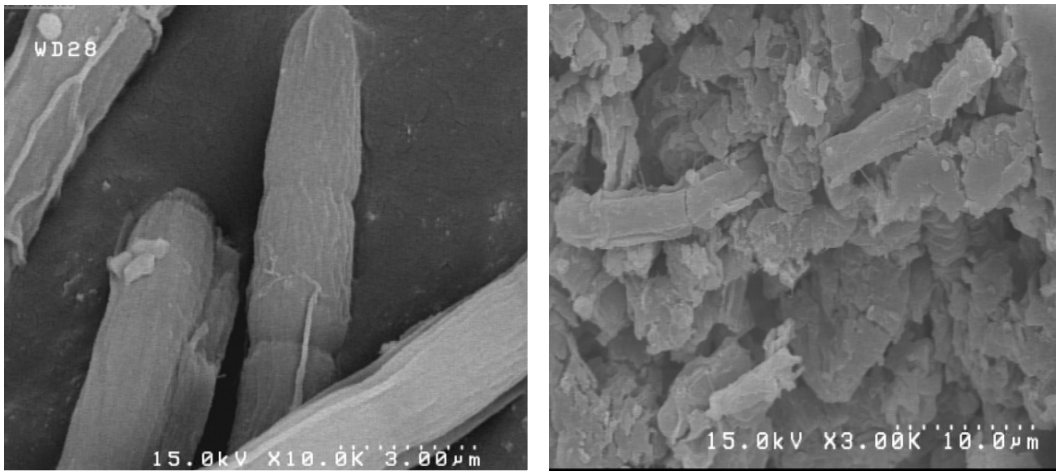

Fig. 3. Scanning-electron microscopy of control *Microchaete tenera* filaments (Left) and treated (7% oil) one (Right). Arrows show the sheath of trichomes

Table 1. The morphological characteristics of *Microchaete tenera* ISC13 grown under the above conditions. Data shows X±SE

morphological characteristics	Crude oil (%)				
	Control (0)	1	2.5	5	7
Morphology of filaments	Solitary or bundled, curved	Solitary or bundled, slightly curved	Solitary or bundled, curved or straight	Solitary or bundled, curved or straight	Solitary or bundled, slightly curved or straight
Mucilage sheath	+	+	+	+	+
Terminal cell	Semi-rounded	Rounded-tapered	Semi-rounded	Semi-rounded or tapered	Rounded or Semi-rounded
Vegetative cells shape	Squared-cylindrical	cylindrical	Squared-cylindrical	Squared-cylindrical	Squared
Vegetative Width (µm)	$5.42\pm0.77^{a,b}$	5.17 ± 0.63^{a}	4.98 ± 0.73^{a}	6.24 ± 0.83^{b}	4.76 ± 0.63^{a}
Vegetative Length (µm)	$6.34\pm1.28^{a,b}$	$6.49\pm1.29^{a,b,c}$	7.96 ± 1.27^{c}	$7.43\pm1.58^{b,c}$	5.03 ± 1.07^{a}
Heterocyst Width (µm)	7.01 ± 0.13^{a}	6.82 ± 0.51^{a}	7.50 ± 0.39^{a}	7.06 ± 0.30^{a}	7.06 ± 0.70^{a}
Heterocyst Length (µm)	6.52 ± 1.14^{a}	6.07 ± 0.97^{a}	6.57 ± 0.81^{a}	7.09 ± 1.94^{a}	6.16 ± 1.57^{a}

akinetes germinate and the resulting vegetative cells disperse (Hense and Beckmann, 2010). These results confirm the appropriate conditions of treatments for the *Microchaete tenera* ISC13. In addition, electron microscopy techniques (SEM) supported these structures. This finding is in agreement with results of our previous study with Anabaena sp. ISC55 (in press).

Molecular techniques were applied to identifying strain *Microchaete tenera* ISC13. Phylogenetic analysis was carried out based on the 16S rRNA gene sequence for strain *Microchaete tenera* ISC13. The sequences were compared with those of representative heterocystous cyanobacteria available in GenBank (http://www.ncbi.nlm.nih.gov/BlAST). The 16S rRNA

sequences were combined with other *Microchaete* species available in the database (Casamatta *et al.*, 2003). The nucleotide sequences described in this study have been submitted to the NCBI under the accession number NCBI: JF290484. According to physiological results the presence of the crude oil didn't abolish the growth (Fig.4). The elevated treatment with crude oil enhanced the growth. But the difference did not significant (ANOVA, >0.05) except in comparison with control. The control culture attained a maximum standing biomass of about 3.88 mg/mL at the 12 day, while 1% oil concentration exhibited algal biomass 4.35 mg/mL and 7% oil concentration 4.60 mg/mL at the 12 day. As can be seen in fig. 4, the optimal growth occurred in the presence of light and crude oil, and under aerobic conditions.

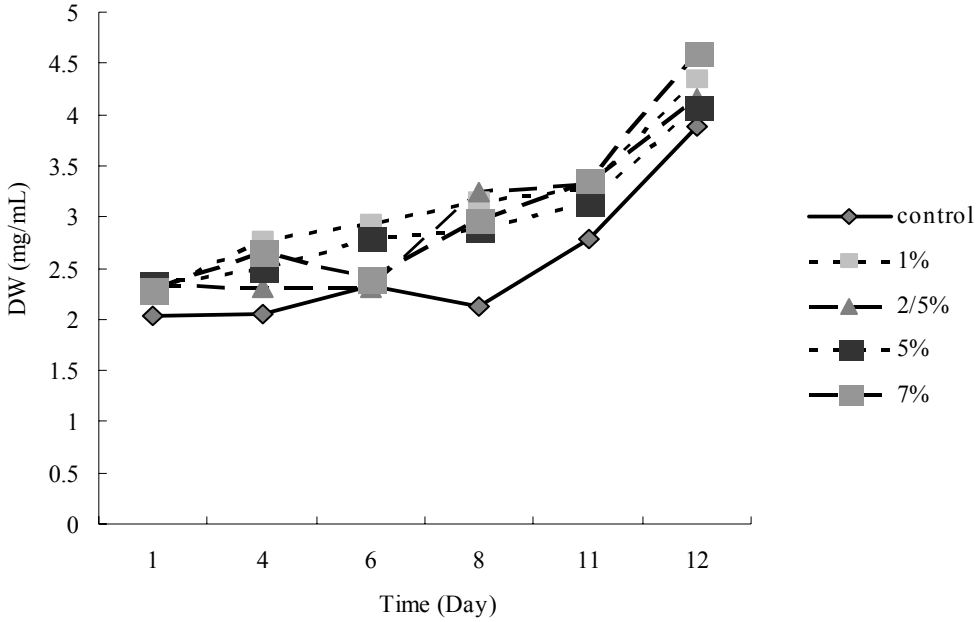

Fig. 4. Dry weigh amounts in different crude oil concentrations (Control, 1, 2.5, 5 and 7%)

Table 2. Photosynthetic pigments amount in different crude oil concentrations in *Microchaete tenera* ISC13 grown under the above conditions. Data shows X±SE

Crude oil	APC	PC	PE	Chl	PBP
(%)			(µg/gDw)		
0	0.693±0.06 ^{b}	1.702±0.05 ^{c}	1.942±0.06 ^{c}	0.253±0.06 ^{a}	16.836±0.6 ^{c}
1	0.538±0.11 ^{b}	1.206±0.22 $^{b, c}$	1.055±0.19 ^{b}	0.241±0.04 ^{a}	12.172±2.24 $^{b, c}$
2.5	0.398±0.09 ^{b}	0.783±0.08 ^{b}	0.750±0.02 ^{b}	0.201± 0.03 ^{a}	8.013±0.72^{b}
5	0±0 ^{a}	0±0 ^{a}	0.025±0 ^{a}	0.191±0.02 ^{a}	0.104±0^{a}
7	0.584± 0.24 ^{b}	0.910±0.39 ^{b}	0.814±0.36 ^{b}	0.179±0.06 ^{a}	10.620±4.53^{b}

The photosynthetic pigments concentrations are shown in different crude oil treatments (Table 2). The crude oil did not affect on some photosynthetic pigments. In the case of chlorophyll content, no remarkable reduction after adding crude oil was seen, although the most chlorophyll amount, 0.253 µg/gdw belonged to control. Regards to other pigments, different results were seen. The highest concentration of phycobiliprotein was distinguished in control and it decreased with increasing the oil concentration up to 5% significantly. But it increased in 7% crude oil in the case of PC and PE.

The size of phycobilisomes can be usually represented by the ratio (PE+PC)/APC (Wyman and Fay, 1986). At 5% oil concentration it was not possible to calculate since no APC was detectable. The ratio was higher at control and significant differences between the treatments and control were seen (Table 3).

Table 3. Effect of oil concentration on (PE+PC)/APC, PBP/Chlorophyll ratios, of *Microchaete tenera* ISC13 grown under the above conditions. Data shows X±SE

Oil concentrations (%)	(PC+PE)/APC	PBP/Chl *a*
Control (0)	5.284 ± 0.47^{d}	17.16 ± 0.61^{b}
1	4.217 ± 0.18^{c}	11.628 ± 2.14^{b}
2.5	$3.951\pm0.7^{b,c}$	11.880 ± 1.07^{b}
5	ND†	0.133 ± 0^{a}
7	2.947 ± 0.10^{b}	12.897 ± 5.51^{b}

†Nd= Not determined

The ratios of PBP/Chlorophyll are used to show the relationship between photosystem II and photosystem I (Yamaka and Glazer, 1981; Poza- Carrión *et al.*, 2001). No remarkable reduction was shown except in comparison with 5% oil. It could be refer to the absence of APC.

Extra information was achieved by physiological experiments. Numerous reports of effects of oil hydrocarbons on cyanobacteria have been published (Diestra *et al.*, 2007). As mentioned above carbonless medium was used for this set of experiments. The results imply physiological flexibility of this strain. The treatment of algal culture with crude oil led to prolongation the growth phase as well as high algal biomass production. There was no evidence of reduction in chlorophyll content, photosynthesis rate and also most of phycobiliproteins. So it is concluded that inhibition of chlorophyll biosynthesis (like inhibition of α-aminolevulinic acid dehydrogenase and protochlorophyllide reductase) was not occurred (Sundaram and Soumya, 2011). The amount of accessory photosynthetic pigments was depending on the crude oil concentration. Essein and Anti (2005) explained the reason of phycobilliprotein reduction in algae under stressful condition. They mentioned that responses to stress in algae are often indicated at the level of proteins while stressful environmental condition can induce synthesis of specific proteins, As well as can affect protein stability and turnover by increasing the rate of proteolysis of specific proteins. Also the growth rate in different concentrations of crude oil demonstrated the ability of consumption of both CO_2 and hydrocarbons by *Microchaete tenera* ISC13. Of course different types of crude oil have distinct effects on cyanobacteria (Llirós *et al.*, 2008). Our results are in agreement with Ibrahim and Gamila (2004) who studied the effect of 0.1% crude oil on algal growth. They concluded that the treatment of algal culture with crude oil resulted in a characterized prolonged growth phase accompanied by high algal biomass production. Gaur and Singh (1990) carried out the growth experiment under crude oil treatments. They concluded that oil inhibited the growth rate of the studied strain. Also Gaur and Kumar (1981) published similar results from the effect of oil on four unicellular algae. As the cyanobacterial cultures were grown photoautotrophically, the degradation of the crude oil is a means of detoxification of the environment (Narro *et al.*, 1992).

Considering the results of the pigments, the rate of photosynthesis is more or less related and explainable. No significant difference was detected in photosynthesis and the highest rate was 4.749±0.09 $nmolO_2$ μgdw/min (Data not shown). Results of photosynthesis rate are compatible with the Sundaram and Soumya (2011) and Altamarino *et al.* (2000). We concluded that adding crude oil can not affected on photosynthesis rate significantly. This result is in conflict with Cohen (2002). These additional pigments can exhibit a high sensitivity to variation of light quality/intensity (Reuter and Müller, 1993). In this respect the variability of phycobilisomes size and structure was examined. In *Microchaete tenera* ISC13, PC is the main component of phycobiliproteins, so the changes on total PBP mostly reflect the changes in PC. Total PBP and PC were affected by oil concentrations. This strategy is similar to other extreme conditions and confirm the results of Soltani *et al.* (2006). Likewise, light intensity has no significant effect on the PC/APC ratio. According to different strategies of adaptation of photosynthetic apparatus by irradiance (Reuter and Müller, 1993) it seems that *Microchaete tenera* ISC13 modulate slightly the number of phycobilisomes, not the size of them, when transferred to higher concentrations of crude oil.

CONCLUSION

The marine environment is highly susceptible to pollution by petroleum, and so it is important to understand how microorganisms degrade hydrocarbons. In this research the effects of crude oil on morphological and physiological characterization of the cyanobacterium *Microchaete tenera* ISC13 were investigated. This study showed that *Microchaete tenera* ISC13 has the potential of survive under extreme environmental condition such as oil pollution. The critical growth factors have not affected by mentioned range of petroleum, cyanobacterium growth increased with elevated oil concentration but No changes was observed in chlorophyll content. The phycobiliproteins are significantly reduced, increasing crude oil decreased all PBP. We are currently attempting to determine whether the total protein is affected by crude oil or not. The toxicity of petroleum has not shown in this strain. Also no significant difference in the size of the vegetative cells and heterocysts has detected. Overall, the results indicated resistance of the studied cyanobacterium under crude oil treatments and also its potential as candidate of use for biodegradation of petroleum pollution.

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