Molecular Identification and Morphological Characterization of Indigenous Soil cyanobacterium *Anabaena* sp. ISC 55

Shaghayegh Iranshahi[1]*, Neda Soltani[2], Taher Nejadsattari[1], Shadman Shokravi[3], Mahrouz Dezfulian[4]

Received: 2016-05-21 Revised and accepted: 2017-03-12

Abstract

Cyanobacteria have a high morphological flexibility, so their identification is problematic. In this research, identification and morphological variability of indigenous cyanobacterium *Anabaena* sp. ISC 55 Bory ex Bornetet Flahault (Nostocaceae) is investigated disciplines-wide. For this purpose, soil samples were cultured in BG110 medium and *Anabaena* sp. ISC 55, after isolation and treatment with different pH (5, 7 and 9) and salinities (0, 1, 2, 3, 4 and 5%), which then were followed biometrically and morphologically in solid and liquid medium via light and florescent microscope. Also, wall and cell variation was evaluated by scanning electron microscope (SEM). Morphological and molecular identification was carried out by valuable keys and 16s rDNA partial sequencing respectively. The results indicated that morphological variation of *Anabaena* sp. ISC 55 is dependent on the effect of pH and salinity. Biometric analysis implied that heterocyst and akinet shape is one of the most stable features and can be considered as a distinguishable character. The effect of pH is more highlighted in comparison with to salinity. In salinities less than 1% and alkaline condition, optimum condition for colonization is prepared and clump shape of colonies can be considered as a stable character. Molecular identification confirmed the morphological one.

Keywords: *Anabaena*, Morphological variation, pH, Salinity, SEM, 16s rDNA.

Introduction

Cyanobacteria, the ancient form of life, form massive diversity in morphology and genetic during their long and slow evolution from simple unicellular organism to complex filamentous organism (Whitton 1992). Genera of the Stigonematales and Nostocales exhibit the highest degree of morphological complexity and differentiation within the cyanobacteria (Anagnostidis and Komarek, 1985; Castenholz, 2001). The complex variety of forms or developmental stages exhibited by terrestrial and marine cyanobacteria may include versatility of aggregations, shape of colony, cell form and dimensions, granule position and unicellular reproductive agents including heterocysts and akinetes, make them the most difficult and discouraging organism from taxonomical point of view. However, a polyphasic study includ-

1- Department of Biology, Science and Research Branch, Islamic Azad University, Tehran, Iran
2- Department of Petroleum Microbiology, Research Institute of Applied Science, ACECR, Tehran, Iran
3- Associate prof., Department of Biology, Gorgan Branch, Islamic Azad University, Gorgan, Iran
4- Department of Biology, Karaj Branch, Islamic Azad University, Karaj, Iran
*email: sh.iranshahi@gmail.com

ing traditional and modern taxonomy, physiology, and biochemistry is needed to determine the real place of a special organism as a whole; although it has been emphasized that taxonomy had based on morphological characters yet (Anagnostidis and Komarek, 1985).

Cyanobacteria are exposed to many variable factors in the habitats and their survival depends on the ability to their acclimization. Salinity, one of the most important environmental factors,determines the natural distribution of cyanobacteria (Valiente-Fernandez and Leganes, 1989). Furthermore, pH clearly impacts it. A wide range of adaptation to pH has been observed not only among different genera but also between different isolates of the same species, although most cyanobacteria are alkaliphile and their best growth is observed in cultures with pH range between 7 to 10 in laboratory, (Soltani et al., 2006; Poza-carrión et al., 2001).

Genus *Anabaena*, belonging to the order Nostocales, family Nostocaceae, is one of the famous and ubiquitous cyanobacteria with unbranchedheterocystous filaments (Komarek, 2010). Previous studies show that epidaphic and endaphic forms of this genus may have been affected by a network of environment fluctuations, including salinity and pH. As field observation cannot provide complete information on morphological plasticity in response to the chaotic and hard variety of environmental conditions, so laboratory studies seem to be essential at least at the first step of characterization. Despite the considerable studies on cyanobacteria in the world including growth (Rosales et al., 2005), photosynthesis (Suleyman et al., 2008; Srivastava et al., 2011; Allakhverdiev et al., 2000); carotenoids and phycocyanin (Sundaram and Soumya, 2011), little is known about morphological variation against fluctuation of environmental factors. Accordingly, the microscopical techniques, most frequently used to determine the diversity of cyanobacteria in terms of morphology, not only using classical light microscopy, but also by scanning electron microscopy (SEM) (Hernández-Mariné et al., 2004). With respect to the molecular taxonomy and floristic researches, a few reports are presented from Iran (Shariatmadari et al., 2011; Shokravi et al., 2012; Soltani et al., 2011).This research was conducted to evaluate the morphological characterization of cyanobacterium *Anabaena* sp. ISC 55 exposed to elevated pH and salinity.

Materials and Methods

Collection

Strain selection and Incubation Soil samples were collected from south of Iran (Khuzestan Province). The collected soils were cultured by usual methods and isolation was carried out by plate agar (Andersen, 2005). Stock cultures were grown in BG110 medium. The cultivation was done under constant illumination 60μE $m^{-2}s^{-1}$, supplied with three 40W cool white fluorescent tubes; different pH (5, 7 and 9) and salinities (1,2,3,4 and 5%) both in liquid and solid cultures with same medium. The temperature was adjusted on 30 ± 1ºC.

Morphological studies

Morphological identification was done according to John et al. (2003) and Desikachary (1959). Biometrical information and morphological observations were made in liquid as well as solid media. Thallus growth, form of aggregations, filament structure, presence or

absence of sheath, form and position of the heterocysts and akinetes, multiplication, were recorded (Gugger and Hoffmann, 2004). Colony formation and cell shapes were evaluated by light, epifluorescence, and electron microscope every other day during two weeks. The growth curves were attained via measurement of chlorophyll daily.

PCR amplification, cloning, and sequence analysis of 16S rRNA to extract DNA from the *Anabaena*, 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 primers106F: (5'-CGGACGGGTGAGTAACGCGTGA-3'and Rb: (5'- GACTACAGGGGTATCTAATCCCTTT-3'). 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 GeneFanAvaran Company with the primers. The sequence data was analyzed using a similarity search by using the BLAST and through the website of the NCBI.

Electron Microscopy Techniques

Morphological variation was carried out by light and scanning electron microscope. For scanning electron microscopy (SEM), samples were fixed in 2.5% glutaraledehyde and washed in buffer phosphate. They were then centrifuged and dehydrated in successively increasing concentrations of methanol (10%, 30%, 50%, 70% and 100%). Finally, all samples were mounted on metal stubs and coated with a layer of gold.

Statistical analysis

Data are the means and standard deviation of at least three replicates. Statistical differences were examined by ANOVA test using software SPSS ver. 18.

Results

Sequence analysis

(The sequence of the 16S rRNA) gene was determined for *Anabaena* sp. ISC 55. The sequences were compared with those of representative heterocystous *(Anabaena)* cyanobacteria available in GenBank (http://www.ncbi.nlm.nih.gov/BLAST). The 16S rDNA sequences were combined with other *Anabaena* species available in the database. 16S rDNA gene sequence similarities of 97% within *Anabaena* sp. were observed. The nucleotide sequences described in this study have been submitted to the NCBI under the accession number NCBI: GU290484.

The effects of different salinities (1, 2, 3, 4 and 5%) and pHs (5, 7 and 9) on solid cultures of *Anabeana*sp. ISC 55, are shown in Figure 1. It seems obvious that the saltless media with alkaline pH may provide better condition for survival of the strain (Fig. 1). At this provision, aggregations tended to make a clump and separated green and brown community. Attention to the aggregations revealed the mucilage layer around whole communities that may be seen at the outer sides as in Figure 2B. Presence of individual sheath in each trichome may be seen in salt free culture medium, especially in pH9 (Fig. 2, 2A). Our observation indicated that heterocyst had both apical and intercalary positions. There

were numerous heterocysts obviously in control (Figs 3 and 4). It seems that frequency and dimensions of heterocysts were more affected by salinity and pH fluctuations than their form (Table 1 and 2).

By increasing salinity, colonization grew slowly in both solid and liquid conditions in comparison with those of control. Akinetes were formed at chains in about 5% salinity. They appeared conspicuously larger than the other cells. Different salinities,especially at the second week (8 days after inoculation), caused conspicuous morphological changes which were seen in all treatments.

Extreme high salinity (more than 1%) caused separation and degeneration of the aggregations and cells (Figure 4 B). As it is shown, the length and width of remainder vegetative and heterocyst cells were reduced by elevated salinity (Table 1 and 2). In fact, the results implied that salinity stimulated degeneration of filaments; moreover, cells began to drift apart from each other.

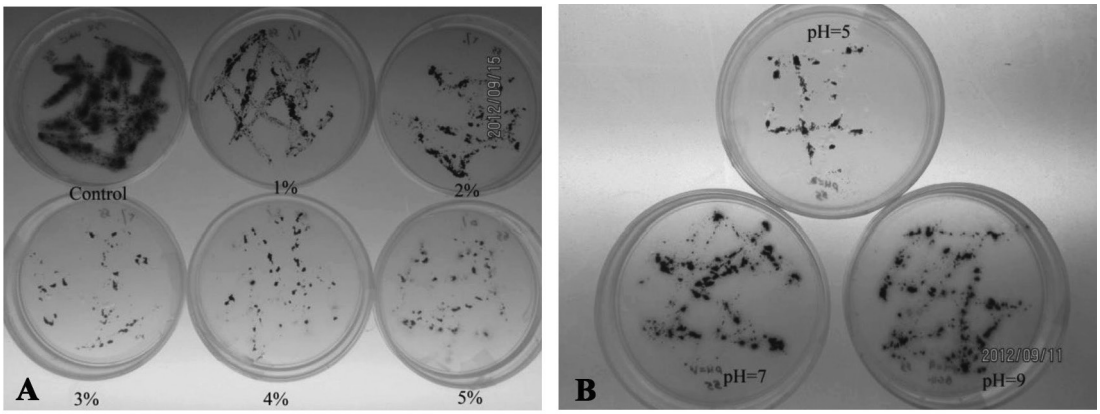

Fig.1. Samples in various concentrations of salinity (A) and pH (B) in solid culture.

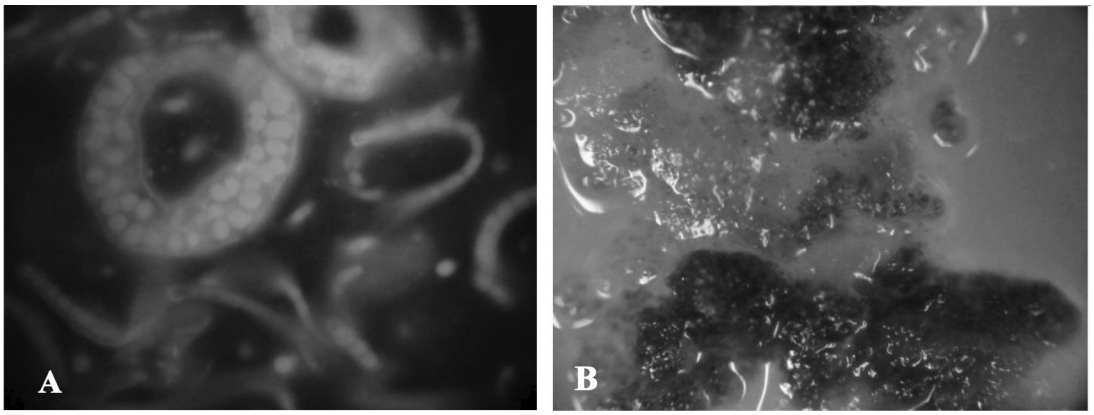

Fig. 2. Sheath is shown in green color in solid culture of colony (A) in close shot in solid culture (B).

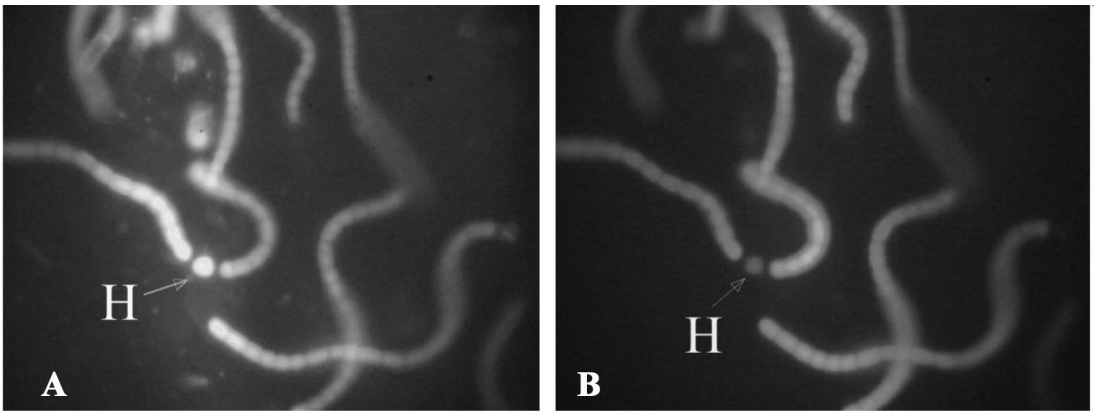

Fig. 3. Heterocysts cells by epifluorescence (A), fluorescence microscope (B).

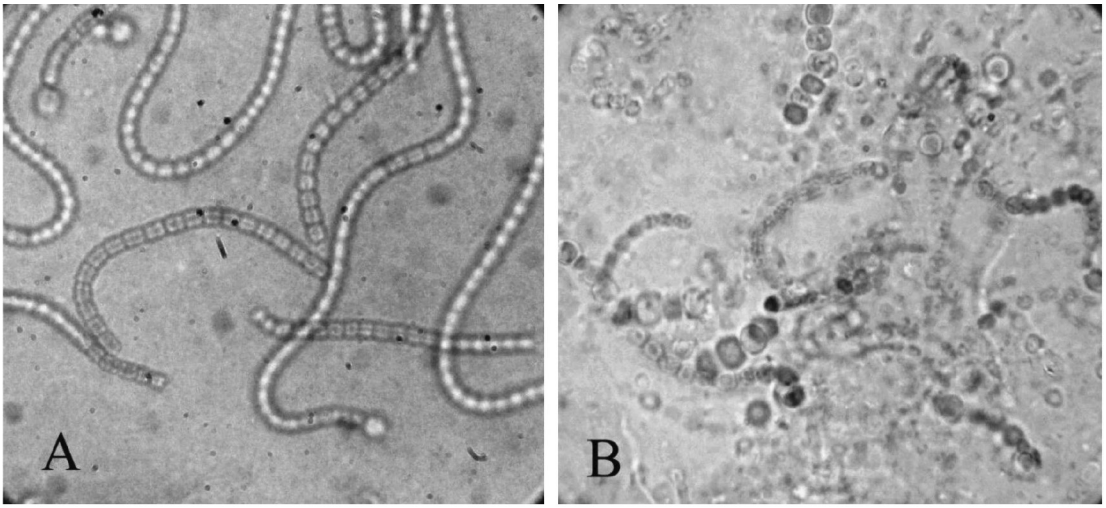

Fig. 4. The degeneration of cells is shown in 5% salinity (B) in comparison to control (A).

Table 1. Selected morphological characteristics of *Anabaena* sp. ISC 55 under different salinities.

Salinity (%)	Vegetative cells		Heterocyst			Akinet		
	Dimensions (µm)*	Frequency	Dimensions (µm)*	Shape	Frequency	Dimensions (µm)*	Shape	Frequency
Control	29.3x26	+++++	52.3 x 38	Rectangle-cylindrical	+++++	NS†	NS†	NS†
1%	33.5x3.8	+++	77.3x77.6	Rectangle-cylindrical	+++	54.6x38.6	Circle	NS†
2%	24.1x27.6	++	32.6x22.3	Rectangle-cylindrical	++	45.3x47	Circle	++
3%	27.5x19.1	++	45.6x26.3	Rectangle-cylindrical	++	65.3x48.6	Circle	++
4%	27.6x24.8	+	33.3x20.3	Rectangle-cylindrical	+	48.6x41.6	Circle	+++
5%	26.6x24.3	+	30.3x31.6	Rectangle-cylindrical	+	59x56.5	Circle	++++

* Numbers are (minimum and maximum) of width x length
†NS= Not seen

In high acidic condition (pH 5), cell shapes were changed, filaments were degenerated and their colors turned to light brown. At neutral or alkaline conditions (pH 7 and 9), organism would continue natural growth but as was expected at pH9 the best growth rate was achieved (Fig. 5) Photomicrographs of SEM showed that in 5% salinity cells were degenerated completely and permanently (Fig. 6B). Despite this, at the other salinities, injuries may be repaired and the frequency of the contact cells may be recovered by time. However, it showed that though high salinity may be toxic for the strain, defense systems can compensate damages but the degree of recovery may be dependent on salt content and time of treatment. Morphological behaviors at these conditions seemed interesting; circular shapes in 1,2 and 3% salinities which were not seen in 4 and 5% (Fig. 6).

Discussion

Molecular identification According to taxonomic classification, it should be mentioned that one cyanobacterial genotype can be represented by more than one morphotype (Palińska et al., 1996; Bittencourt-Oliveira et al., 2001). Under altering environmental conditions, both in nature and in cultures, some morphological features, such as colony formation, cell size and trichome aggregation, can be changed or lost (Otsukaet al., 2000).

Since classification of *Anabaena* based solely on morphology has turned out to be unreliable, genetic methods have to be applied in taxonomic studies (Mazur-Marzec et al., 2010). Comparison with the same sequence of *Anabaena* strains from GenBank revealed a high level of genetic identity, strongly indicating that studied strain belongs to *Anabaena* genus.

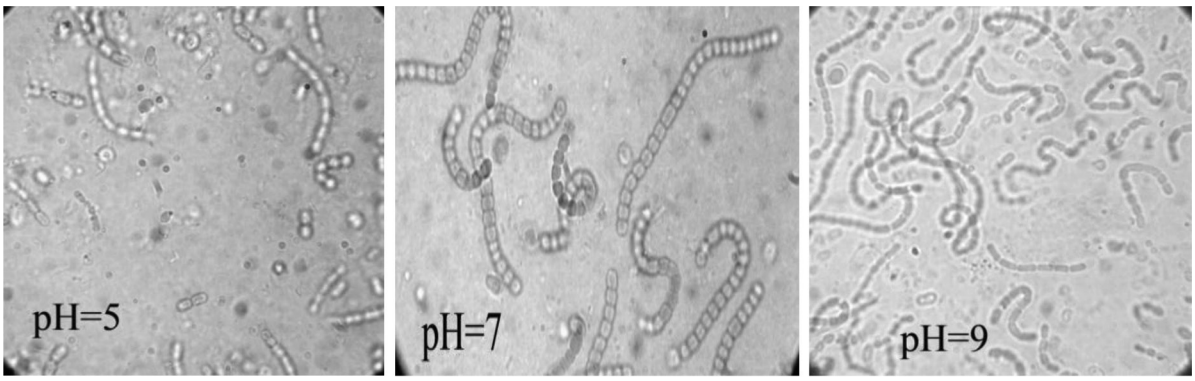

Fig. 5. The shape of cells in different pHs (5, 7and 9)

Table 2. Selected morphological characteristics of *Anabaena* sp. ISC 55 under various pHs.

pH	Vegetative cells		Heterocyst			Akinet		
	Dimensions (µm)*	Frequency	Dimensions (µm)*	Shape	Frequency	Dimensions (µm)*	Shape	Frequency
5	28.1x30	++	30.6x29	Rectangle-cylindrical	+++++	45.3x47	Circle	+++
7	28x24.8	+++	32.6x20.6	Rectangle-cylindrical	+++	NS†	NS†	NS†
9	34.6x25.8	++++	40x31	Rectangle-cylindrical	++	NS†	NS†	NS†

* Numbers are (minimum and maximum) of width x length
†NS= Not seen

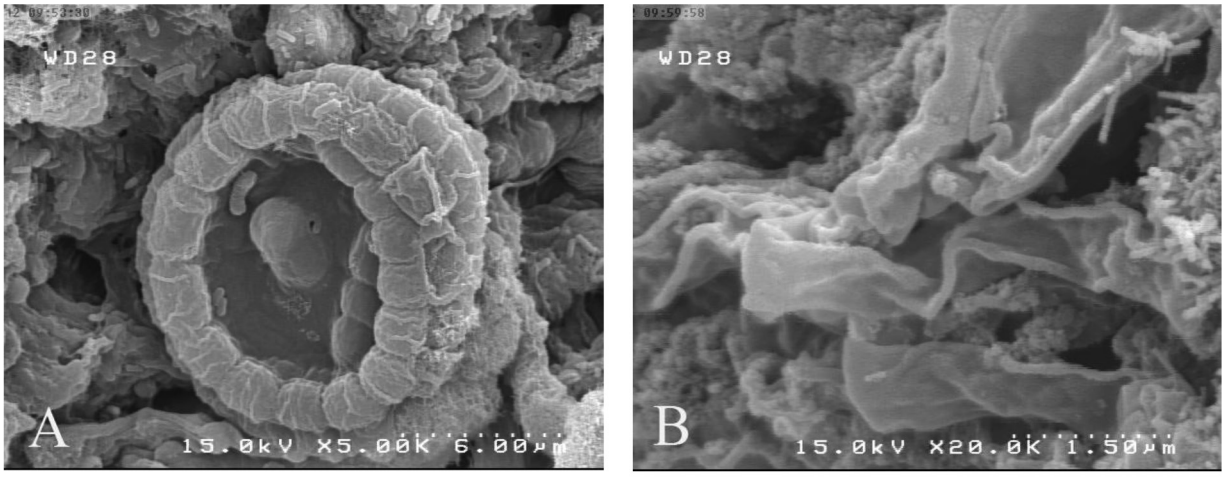

Fig. 6. The shape of cells in control (Left) and 5% salinity (Right) by SEM.

Morphology

Only a few Nostocalean morphotypes have been cultured, and therefore the high variability of morphotypes found in nature is under-represented in culture (Gugger and Hoffmann, 2004). However, results could be able to draw a relatively primitive picture of the combination effect of salinity and pH in morphological analysis of the organism. These organisms showed variable characters from morphological pointof view, and this variability was related with both acidity and salinity. Previous researches have mentioned that environmental variation such as salinity could impact their morphology. These results were confirmed by microscopic manuals as SEM (Diestra et al. 2007). As shown, cells went to death phase and caused cell reduction density due to salt tension which has affected microorganism growth (Whitton and potts 2000). Salt influences cells not only by ionic and osmotic stress but also oxidative one (Zhu 2001).

Up to now, the researches on morphological characterization of native cyanobacteria have mostly been performed at salinity less than 1% (Shokravi et al., 2007; Soltani et al., 2010) and it is the first report about the effect of extreme high salinity on morphological variations of soil cyanobacteria of north of Iran. Also, different pHs were evaluated rarely by indigenous cyanobacteria (Soltani et al., 2006).

High saline condition had a remarkable inhibitory effect on the hormogonium production. It was applied to both the first and second weeks. It has been emphasized that when mature cultures are agar-solidified medium motile hormogonia formed and easily isolated after migration on agar (Castenholz, 2001). Besides, the heterocysts formation was diminished. It could be presumed that the potential of nitrogenase activity and photosynthesis decreased sharply in these conditions. This fact showed that the morphology of heterocyst and nitrogenase activity is correlated. Also, biometrical analysis showed that both salinity and acidity may affect cell dimensions significantly (ANOVA, $p< 0.05$).

Regarding akinet formation, the results showed enhanced formation of this phenomenon in higher salinity. Cells tend to have akinetes and more production and accumulation of metabolites, though they are unable to grow as fast. It is in agreement with Myers et al., (2011). In fact akinetes are a resting stage in the life cycle

of all cyanobacteria that belong to the orders of Nostocales and Stigonematales and may help these organisms avoid unfavorable growth conditions, particularly those which would otherwise cause their death, and provide a potential inoculum for future growth (Baker and Bellifemine, 2000; Karlesson-Elfgren and Brunberg, 2004). Besides, it should be mentioned that the fluctuation in pH and salt could not affect the topology of the spores. There were significant differences between length and breadth of different salinities and acidities (ANOVA, $p< 0.05$). It is in agreement with our last studies (Shokravi et al., 2012).

Form and topology of the vegetative cells seemed versatile especially at extreme acidity and salinity. The quadrate or cylindrical (rarely sub-cylindrical or even oval-cylindrical) form might be shown in extreme conditions. Epifluoresence analysis showed granule accumulation both on cross walls and all around the vegetative cell especially at the first week of growth. This feature could be related to storage vesicles that appeared at the end of log phase, reaching stationary and death phase of growth. We could not draw a realistic pattern for granule accumulation and so far we are doubtful about their situation in a taxonomical plan. With respect to the sheaths, the results showed that the effect of pH is more outstanding compared to that of salinity. As biological material, cyanobacteria tend to be susceptible to dehydration. Sheaths and mucilaginous outer layers maybe condensed or blur the surface of the specimen (Hernández-Mariné et al., 2004). It is difficult to explain this physiologically but we can suggest the presence of sheath for this strain which is nearly thin and obscure in light microscopy.

Form and color of aggregations seems not to be a stable character. In vivo absorption spectrum evidence (not shown) revealed that photosynthetic pigments ratios may fluctuate even at a short time. It reflects as short time chromatic adaptations which turn to change color. However, green, light green, light brown, and dark brown aggregations may be seen in different periods of life in both liquid and solid media. Naturally, stability of colors seems more existent in solid medium but not as constant as to be regarded in the domain of constitutive traits. According to the form, confluent trichomes may be gathered as clump-shape communities and this shape seems constant in liquid and solid cultures. At extremely high salinities (4 and 5%), aggregations tend to make new unclear and versatile communities (expand, mat form, hairy and gelatinous cushion like) but at lower salinities clump-shape may be regarded as the prominent and stable form of aggregations.

In conclusion, although the Na^+ ion is essential for growth and metabolism of cyanobacteria, salt increase, depending on strain, has destructive effects on cells. Furthermore, data obtained for pH effects confirmed the alkaliphilic behavior of cyanobacteria.

Acknowledgments

This research was supported by grant from Department of Petroleum Microbiology, Research Institute of Applied Science, ACECR, Tehran, Iran.

References

Allakhverdiev SI, Sakamoto A, Nishiyama Y, Inaba, M, Murata N. (2000). Ionic and osmotic effects of

NaCl induced inactivation of photosystem I and II in *Synechococcus* sp. Plant Physiology.

Anagnostidis K and Komarek J. (1985). Moden approach to the classification system of cyanophytes. Introduction. Algological Studies. 38-39:291-302.

Andersen RA. (2005). Algal culturing techniques. Academic Press, San Diego CA. 596 pp.

Baker PD and Bellifemine D. (2000). Environmental influences on akinet germination of *Anabaena* circinalis and implications for management of cyanobacterial blooms. Hydrobiologia. 427: 65-73.

Bittencourt-Oliveira MC, Oliveira MC, Bolch JS. (2001). Genetic variability of Brazilian strains of the *Microcystis aeruginosa* complex (Cyanobacteria/ Cyanophyceae) using the phycocyanin intergenic spacer and flanking regions (cpcBA). Journal of Phycology. 37 (5): 810-818.

Castenholz RW. (2001). Cyanobacteria. In: Castenholz WR, Boone D, editors. Bergey's manual of systematic bacteriology. Oxygenic photosynthetic bacteria. Phylum BX. Baltimore: Williams and Wilkins.

Desikachary TV. (1959). Cyanophyta. New Delhi, In dian council of agricultural research.

Diestra E, Esteve I, Castell O, Solé A. (2007). Ultrstructureal changes in *Microcoleus chthonoplastes* growing in the presence of crude oil. Applications for ecological studies. In: Modern Research and Educational Topics in Microscopy.(Méndez-Vila and Diaz, eds). Formatex. pp: 453-460.

Gugger M and Hoffmann L. (2004). Polyphyly of true branching cyanobacteria (Stigonematales). International Journal of Systematic and Evolutionary Micro biology. 54:349-357.

Hernández-Mariné M, Clavero E, Roldán M. (2004). Microscopy methods applied to research on Cyanobacteria. Limnetica. 23 (1-2): 179-186.

John DM, Whitton BA, Brook AJ. (2003). The freshwater algal flora of the British Isles, an identification guide to freshwater and terrestrial algae. Cambridge University Press.

Karlesson-Elfgren I and Brunberg AK. (2004). The importance of shallow sediments in the recruitment of *Anabaena* and *Aphanizomenon* (cyanophyceae). Journal of Phycology. 40: 831-836.

Komárek J. (2010). Modern taxonomic revision of plank tic Nostocaceae cyanobacteria: a short review. Hydrobiologia. 639 (1): 231-243.

Mazur-Marzec HG, Browarcyzyk-Matusiak K, Forycka J, Kobos Plinski, M. (2010). Morphological, genetic, chemical and ecophysiological characterization of two *Microcystis aeruginosa* isolated from Vistula Lagoon, southern Baltic. Oceanologia. 52 (1): 127-146.

Myers JH, Beardall J, Allinso G, Sazman S, Robertson, S, Gunthorpe L. (2011). Potential triggers of akinete differentiation in *Nodularia spumigena* (cyanobacteriaceae) isolated from Australia. Hydrobiologia. 671: 165-180.

Nübel U, Garcia-Pichel F, Clavero E, Muyzer G. (2000). Matching molecular diversity and ecophysiology of benthic cyanobacteria and diatoms in communities along a salinity gradient. Environmental Microbiology. 2: 217-226.

Otsuka S, Suda S, Li R, Matsumoto S, Watanabe MM. (2000). Morphological variability of colonies of *Microcystis* morphospecies in culture. Journal of General and Applied Microbiology. 46 (1):39-50.

Palińska KA, Liesack W, Rhiel E, Krumbein WE. (1996). Phenotypic variability of identical genotypes: the need for a combined approach in cyanobacterial taxonomy demonstrated on *merismopedia*-like isolates. Archives of Microbiology. 166 (4): 224-233.

Poza-Carrión C, Fernández-Valiente E, Piñas FF, Leganés F. (2001). Acclimation to photosynthetic pigments and photosynthesis of the cyanobacterium *Nostoc* sp. strain UAM206 to combined fluctu-

ations of irradiance, pH, and inorganic carbon avail ability. Journal of Plant Physiology. 158: 1455-1461.

Rosales N, Ortega J, Mora R, Morales E. (2005). Influence of salinity on the growth and biochemical composition of the cyanobacterium *Synechococcus* sp. Ciencias Marinas. 31 (2): 349-355.

Shokravi Sh and Soltani N. (2012). The Effect of ammonium on viability, growth, pigment composition of soil cyanobacterium *Fischerella* sp. International journal on Algae. 14 (1): 62-70.

Shokravi Sh, Soltani N, Fernandez-Valiente E. (2007). Morphological variation of paddy field cyanobacterium *Fischerella* sp. from Iran under combined influence of pH and irradiance. Journal of Plant Science Researches. 1 (8): 1-6.

Soltani N, Baftechi L, Dezfulian M, Shokravi Sh, Alnajar N. (2012). Molecular and morphological characterization of oil polluted microalgae. International journal of environmental research. 6 (2): 481-492.

Soltani N, Khavari-Nejad R, Tabatabaei M, Shokravi Sh, Fernandez-Valiente E. (2006). Variation of nitrogenase activity, photosynthesis and pigmentation of cyanobacterium *Fischerella ambigua* strain FSI8 under different irradiance and pH. World. Journal of Microbiology and Biotechnology. 22 (6): 571-576.

Soltani N, Siahbalaei R, Shokravi Sh. (2010). A new description of *Fischerella ambigua* (Nag.) Gom. A multidisciplinary approach. International Journal on Algae. 12 (1): 19-36.

Srivastava K, Alexova R, Jeon YJ, Kohli GS, Neilan BA. (2011). Assessment of salinity-induced photo respiratory glycolate metabolism in *Anabaena* sp. PCC 7120. Microbiology. 157: 911–917.

Suleyman I, Allakhverdiev SI, Murata N. (2008). Salt stress inhibits photosystems II and I in cyanobacteria. Photosynthesis Research. 98: 529-539.

Sundaram Sh and Soumya KK. (2011). Study of physiological and biochemical alterations in cyanobacterium under organic stress. American Journal of Plant Physiology. 6 (1): 1-16.

Valiente-Fernández E and Leganés F. (1989). Regulatory effect of pH and incident irradiance on the levels of nitrogenase activity in the cyanobacterium *Nostoc* UAM 205. Journal of Plant Physiology. 135: 623-627.

Whitton AB and Potts M. (2000). The ecology of cyanophyta, their diversity in time and space. Kluwer Academic Publishers, Dordrecht. 233-255.

Whitton BA. (1992). Diversity, ecology and taxonomy of the cyanobacteria. In: Photosynthetic prokaryotes (Mann NH and Carr. NG. eds). Plenum Press. New-York. pp. 1-51.

Shariatmadari Z, Riahi H, Shokravi Sh. (2011). A Taxo nomic Study on Soil Taxa of *Anabaena* Bory ex Bornet et Flahault (Nostocaceae) in Iran. Journal of Botany. 17 (1): 105-118.

Zhu JK. (2001). Plant salt tolerance. Trends in Plant Science. 6: 66-71.