The effect of salinity on morphological and molecular characters and physiological responses of *Nostoc* sp. ISC 101

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Abstract

Taking into account the high potential of cyanobacteria to tolerate salinity stress, researches have evaluated the morphological and physiological behavior of these microorganisms in recent years. This study is conducted to investigate the impact of different concentrations of NaCl on the morphological and physiological traits of *Nostoc* sp. ISC 101. Biometrical and morphological observations are carried out by light and scanning electron microscopy. Results indicated that vegetative cells and heterocysts were wider in control treatment in comparison with samples under different amounts of salinity. Akinete formation began in 3% NaCl and reached to highest level in 5%. The relative degeneration of structure of the cells in 5% salt was demonstrated. According to physiological impresses of salt it was found that growth rate decreased with increasing salinity. Total chlorophyll content stimulated in 1% salinity, but in the higher concentration it decreased vice versa. The rate of APC, PE, PC increased in 1% salinity, although in high level concentration they would be diminished. Photosynthesis rate was also decreased with increasing salinity but it was stimulated slightly in 1% NaCl. All in consequence, despite of acclimation of this strain to marine environment, not much tolerance was seen in the mentioned treatments, and increasing salinity to upper than 1% NaCl had destructive effects, and cyanobacterium maintained its growth rate at slightly saline environments.

Keywords: Growth, Morphology, *Nostoc*, Photosynthesis, Salinity, SEM, 16S rRNA.

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Introduction

Salinity, as one of the most significant ecological factors, affects growth and metabolic activities of different living organisms. Up to 20% of the world's lands, which produce one third of the world's food, are salt affected (Gaber, 2010), and they will grow up to 50% by the year 2050 (Zhu, 2001). Salinity has begun to accumulate in soil and water tables and may soon reach to toxic levels. Various anthropogenic activities have accentuated the existing stress factors (Mahajan and Tuteja, 2005). When a change in salinity of the environment exceeds a certain threshold level, the morphology and physiology of organisms are impressed. Cyanobacteria, the ancient oxygen evolving photoautotrophs, are present in environments with different salinities. It is well documented yet that cyanobacteria have a remarkable flexibility to adapt to a wide range of environmental conditions. Although it is shown that Na^+ is essential for physiological activities such as growth and photosynthesis (García-González *et al*., 1987), increasing salinity induce some adaptive responses. Salinity does not affect all cyanobacteria to the same extent due to their morphological and genomic diversity (Stal, 2007). The responses differ based on cyanobacterial species involved.

Despite the considerable negative impact of salinity on physiology of pure cultured cyanobacteria, little is known regarding its effect on morphological variation of them in laboratory conditions under different salt levels. Morphology (vegetative cells and heterocysts extent and akinete formation) and life cycle of these microorganisms can vary considerably in response to fluctuations in salinity. So the microscopic techniques which are most frequently used to determine the diversity of cyanobacteria in terms of morphology are not only using classical light microscopy, but also scanning electron microscopy (SEM) (Hernández-Mariné *et al*., 2004).

Regarding investigations under salt conditions, cyanobacterial growth (Kumar Rai and Abraham, 1993; Rosales *et al*., 2005), photosynthesis (Allakhverdiev *et al*., 2000; Suleyman *et al*., 2008; Srivastava *et al*., 2011) Chlorophyll *a* (Singh and Kshatriya, 2002; Shanleyraj and Anand, 2006); carotenoids and phycocyanin (Sundaram and Soumya, 2011), have been reported to be adversely affecting.

We wanted to draw the attention into the responses of *Nostoc* sp. ISC 101, a heterocystous cyanobacterium to salinity stress. *Nostoc* is a cyanobacterium from Nostocales order that assume a special significance in these environments (Soltani *et al*., 2007) and has been isolated from a wide range of habitats, largely varying in their salinity (Vonshak and Tomaselli, 2000).

The present study was undertaken (i) to evaluate morphological and molecular taxonomy of the cyanobacterium *Nostoc* sp. ISC 101, and (ii) to elucidate physiological responses of this species under salinity stress.

Materials and methods

Isolation of the strain

The strain of *Nostoc* sp. ISC 101 was isolated from the coasts of north of Iran (N 363444; E 520045). Isolation and purification of *Nostoc* sp. was made by ordinary methods (Andersen, 2005). Following achievement of axenic culture, cyanobacterium was cultivated in $BG11_0$ liquid and solid medium (Soltani *et al*., 2006).

Culture conditions

Stock cultures were grown in the liquid medium $BG11_0$. Temperature was maintained at 30±1°C and cultures were bubbled with air under a constant light intensity of 60 µmol photon m^{-2} s^{-1} supplied by three fluorescent tubes. Cells in logarithmic phase of growth were collected from stock cultures and used as inoculate for experiments. Three sets of experiments were performed depending on desired salinity (1, 2, 3, 4, 5 %). Cells from stock culture were inoculated in 300 ml of $BG11_0$ medium in 500 ml erlenmeyer flasks stoppered with cotton plugs. Cultures were aerated daily and illuminated with 40 W cool white fluorescent tubes. Fresh samples were taken and used for determinations during the logarithmic phase of growth.
For morphological studies solid medium was prepared and maintained in the same condition as liquid, following inoculation. Semi permanent slides were prepared every other day and used in light and florescence microscopes inspections.

Microscopy Techniques

Morphological variation evaluation was carried out by light and scanning electron microscopy. For SEM, samples were fixed in 2.5% glutaraldehyde 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.

PCR amplification, cloning, and sequence analysis of 16S rRNA

For extracting DNA, fresh biomass of *Nostoc* was obtained by centrifuging at 12000 rpm and using Fermentas kit (#k0512). The applied PCR condition is described by Nübel *et al*. (2000). PCR amplification, cloning and sequence analysis of 16S DNA content was first extracted from the cyanobacterium, and then PCR was applied using two set of primers. Sequences were amplified using the primers PA
5´-AGA GTTTGATCCTGGCTCAG-3´ as forward and
5´-TTACCTTGTTACGACTT-3´ as reverse 1492R, which 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 CinaGene Company primers. The sequence data was analyzed using a similarity search by using the BLAST

through the website of NCBI. The nucleotide sequences described in this study were submitted to NCBI under the accession number NCBI: JF290484.

Analytical methods

Growth rates were calculated as Soltani *et al*. (2006). For 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).

Statistical analysis

Data are presented as mean and standard deviation of at least three replicates. Also Statistical differences were examined by ANOVA test using SPSS ver. 18 software.

Results

Morphological observations

Morphological observations showed that *Nostoc* sp. ISC101 is a solitary filamentous cyanobacterium in a dense sheath. Also all the trichomes aggregated in a thick sheath and colonies in solid medium grew as small balls. Colonization in treatments indicated their color change in various salinities even in 1% it varies from green to brown. Also the colonizations grew slowly and weekly with increasing of the salinity (Fig.1)

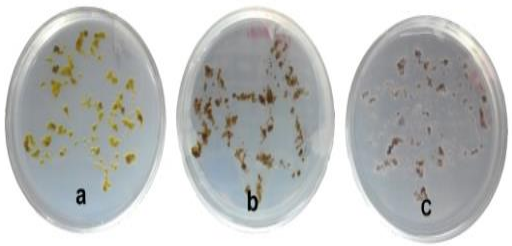

Figure 1: Changes in color and colonization in control (a), 1 (b) and 5% (c) treatments.

As we estimated the sensitivity of this cyanobacterium to salinity, microscopic evaluation of the shape of cells and trichomes in response of salinity was carried out. Results implied that 1% salinity did not affect the shape of trichomes so much. But by increasing the salinity, changes in shape of cells were seen. In 0 and 1% salinity the cells were square or rectangular. But in 2% salinity, cells tended to shape like beads of rosary. This was the beginning of isolation of cells which was observed in 4 and 5% salinity. Also the filaments degenerated in upper than 3% salinities (Fig 2).

The heterocysts of this strain were seen terminally but intercalary in rare cases. The shape of heterocysts did not change (ovate) but their dimensions more or less increased with increasing of salinity. There was no akinete in control and 1% salinity, but in upper NaCl concentration presence of them was seen in coordination with degeneration of filaments. After generation of numerous akinetes, they

were seen as free cells in the medium (Table 1, Fig 2).

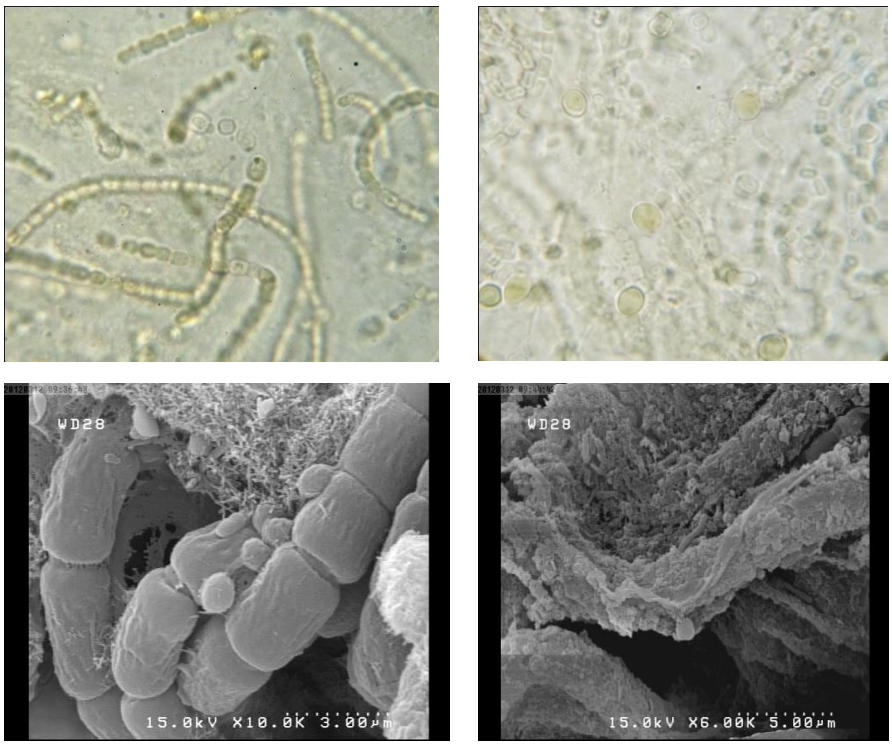

Figure 2: Shape of cells in control (left) and 5% salinity (right), by light microscope (up) and SEM (down).

Tabel 1: Selected morphological characteristics of *Nostoc* sp. ISC 101 under different salinity.

Salinity (%)	Trichome width (µm)	Vegetative dimensions (µm)*	Heterocyst		Akinete	
			dimensions (µm)*	Shape	dimensions (µm)*	Shape
Control	7.8- 11.7	3 x 3-4.5(7.5)	4-4.5 x 5.8	Ovate	NS†	
1%	7.8- 11.7	4 x 4-5.8	4-5.8 x 5.8-7.8	Ovate	NS	
2%	7.8- 11.7	4 x 5.8	5.8 x 5.8-7	Ovate	4.3 x 5.8	rare
3%	7.8- 11.7	3.1-4 x 4.6-5.8	4-4.3 x 5.8-6.6	Ovate	4 x 5.9-6.6	moderate attached
4%	7.8- 11.7	3.5-4 x 5.8-6.5	4-4.3 x 4.3-7	Ovate	4.3-5.1 x 6.6-7.8	frequent separate
5%	7.8- 11.7	3 x 3-4.5	4.5-6 x 6-7.5	Ovate	5.8 x 7.5-9.7	frequent separate

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

Sequence analysis

The sequence of the 16S rRNA gene was determined for *Nostoc* sp. ISC101. The sequences were compared with those of representative heterocystous *(Nostoc)* cyanobacteria available in GenBank (http://www.ncbi.nlm.nih.gov/BlAST).

The 16S rRNA sequences were combined with other *Nostoc* species available in the database (Casamatta *et al*., 2003; Ezhilarasi and Anand, 2009). 16S rRNA gene sequence similarities of 97% within *Nostoc* sp. were observed. The nucleotide sequences described in this study were submitted to NCBI under the accession number NCBI: GU138682.

Physiological results

Our result showed that maximum growth rate belonged to control. Growth rate decreased with increasing salinity whereas in 5% salinity cells were shrunk. Growth rate altered from 0.02 d^{-1} in control to -0.07 d^{-1} in 5% salinity.

The effect of salinity on pigments content is shown in Table 2. Results revealed that chlorophyll content increased in 1% salinity, however in higher concentration it would be decreased vice versa, but the difference is only significant within 3-5%.

According to phycobiliproteins, the same results were seen but all of the differences were insignificant (Table 2).

Table 2: Effect of salinity on pigment contents of *Nostoc* sp. ISC101 grown in four days. Data are mean values of the four experiments ± SD.

Salinity (%)	APC	PC	PE	Chl
	µg mg dw^{-1}			
0	0.979±0.834$^{a\,b}$	0.396±0.300^{a}	0.374±0.381$^{a\,b}$	1.860±0.233^{b}
1	1.562±0.097^{b}	0.399±0.042^{a}	0.631±0.039^{b}	1.924±0.074^{b}
2	0.953±0.518$^{a\,b}$	0.273±0.225^{a}	0.381±0.201$^{a\,b}$	1.661±0.538^{b}
3	0.668±0.07$^{a\,b}$	0.097±0.047^{a}	0.275±0.026$^{a\,b}$	1.145±0.136^{a}
4	0.233±0.143^{a}	0	0	0.968±0.030^{a}
5	0	0	0	0.878±0.010^{a}

Different letters in superscript following values indicate statistical significance.

These results were more or less in relation to photosynthesis. In the latter case, the highest rate was 51.11 $nmolO_2$ mg dw^{-1} min^{-1} in 1% and decreased afterwards, although the difference was not significant.

Discussion

Salinity stress induces various types of morphological and physiological changes in cyanobacterial cells. These structural changes provide useful information as to the underlying mechanism of salinity stress. It is well documented that sodium is an essential nutrient for cyanobacteria (García-González *et al*. 1987). This assessment has, however, been criticized on the grounds that morphology can vary considerably in response to fluctuations in environmental conditions, that has not received much attention yet.

Our biometrical observations suggested a reduction in length and width of the

vegetative cells in comparison with the control. Regarding akinete formation, results showed enhanced formation of this phenomenon in higher salinity. Cells tend to have akinetes and a greater production and accumulation of metabolites, though they were unable to grow as fast. Data obtained for inclusion indicated that increasing salinity has destructive effects on morphology, although it is not in agreement with other rare researches (Allakhverdiev and Murata, 2008). It seems that such impresses varies from one species to another. In addition, SEM techniques confirmed the changes in structure. Presence of these inclusions might be considered to indicate toxicity in different salinities. As biological material, cyanobacteria tend to be susceptible to dehydration. Sheaths and mucilaginous outer layers maybe condensed or blurry the surface of the specimen (Hernández- Mariné *et al*., 2004).

Further, we focused on the effects of salt (NaCl) stress on physiology of *Nostoc* sp. ISC101. Our results revealed that *Nostoc* sp. ISC101 had the lowest biomass in 5% salinity. The growth rate was more or less decreased by increasing salinity. Salinity has negative effect in the growth indicating the toxicity of this salinity treatment for evaluated strain. Repression of growth is in agreement with Shanleyraj and Anand (2006); Soltani *et al*. (2007) confirmed that salinity does not cause any significant negative effect on growth up to 1% NaCl depending on the cyanobacterial species. In *Anabaenopsis* it increases from 0 to 1.5% but in 2% growth declines. *Cylindrospermopsis* is more sensitive and its growth is negative in salinities upper than 0.6% (Moisander *et al*., 2002). Tolerance of growth in lower salinities, maybe related to adaptation to the marine environment in which it is isolated (Rosales *et al*., 2005).

The present study confirms the adverse effect of salinity stress on pigments like chlorophyll a. The observed changes in cell pigmentation and the reminiscence of the phenomenon on complementary chromatic adaptation of chlorophyll content is increased in 1% salinity, however in the higher concentrations it would be decreased vice versa; reduced chlorophyll contents at higher salinities are because of salt osmotic and toxic ionic stress. Maybe the toxicity causes inhibition of chlorophyll biosynthesis by inhibition of a-aminolevulinic acid dehydrogenase and protochlorophyllide reductase (Ouzounidou, 1995). As evident from the results on NaCl-dependent increase in the chlorophyll a, much lower concentration (5-10 mM) of NaCl are found to be favorable for the growth and photosynthesis. But higher concentration-dependent cause a decline in the level of photosynthetic pigments. Earlier workers have also demonstrated this (Singh and Kshatriya, 2002; Shanleyraj and Anand, 2006; Soltani *et al*., 2007). Phycobiliproteins (PBPs) that are attached to the stromal surface of thylakoid membranes serve

as the primary light-harvesting antennae for PSII. The composition and function of PBPs in cyanobacteria was changed in response to stress conditions. In *Nostoc* sp. ISC101 phycoerythrin is the major biliprotein. Salinity has influenced the phycobiliprotein composition of the phycobilisome, the major light harvesting antennae; PE was increased in 1% salinity, however in the higher concentration it would be decreased vice versa. APC and PC has similar trends in attended salinity (Lu and Vonshak, 2002; Sudhir *et al*., 2005).

Maximum photosynthesis rate was seen in 1% salinity, however it was decreased in higher concentrations, otherwise the difference with the control was not significant. Taking the results into account, it is concluded that salinity has a significant impress on photosynthesis and affects the usage of minimum light for photosynthesis, these results are in agreement of other researches (Allakhverdiev *et al*., 2000; Lu and Vonshak, 2002; Sudhir *et al*., 2005; Soltani *et al*., 2007). Shaila and Mathad (2010) and Rosales *et al*. (2005) also explained that salinity repressed photosynthesis activity. These results draw attentions to the decline in the pigments. Phycobiliproteins and chlorophyll proteins act as light harvesting antenna and transfer the absorbed energy to PSII reaction center which is essential to initiate the electron transport process. Under salt stress, there is also a loss in the chlorophyll protein and a core membrane link-protein that can attach phycobilisomes to thylakoids (Garnier *et al.*, 1994). Present results showed that the process of O_2 evolution is relatively more sensitive to NaCl stress.

The increased photosynthesis under salt stress could be explained as increased demand for carbon by the salinity acclimation mechanisms. Higher p_{max} in the medium with 1% NaCl compared to that in the medium with no added NaCl, could indicate that the organism is preferentially a "salt species". For this species this could be true since its growth rate was slightly slower at 0% than at 1%.

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