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Ecophysiological study on soil cyanobacterium in combination of pH and salinity conditions at limited irradiance

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Sınırlı Işınımda Ph ve Tuzluluk Koşullarının Kombinasyonu ile Toprak Siyanobakteri Üzerinde Ekofizyolojik Bir Araştırma

ÖZ

Bu araştırmanın amacı İran'ın kuzeyinde Golestan eyaletinin çeltik tarlalarından toplanan ekofizyolojik siyanobakteri Hapalosiphon sp. FS56 nın araştırılmasıdır. Bu denemede saflaştırılmış örnekleri, BG011 kültür ortamı pH 7.5 ve 25 ° C sıcaklık koşullarında aydınlatma altında (2 umol kuant.m-2.S-1) inkübe edildi. Fotosentez ve büyüme hızı, pigment ve fikobilizom içeriği gibi morfolojik parametreleri değerlendirmek için sınırlı ışınlamada koşullarında (2 µmol quanta.m-2.S-1); pH (5,7,9) ve farklı tuzluluk durumu (% 0,025,% 0,5 ve% 1) kombinasyonları uygulanarak etkileri incelenmiştir. Sonuçlar, Hapalosiphon sp. Fs56 nın % 0.5 tuzluluk (g / l-1) altında en yüksek büyüme oranına sahip olduğunu ve doygun ışık fotosentez oranında bu tuzluluk konsantrasyonunda en yüksek noktaya ulaştığını gösterdi. PH 9'da elde edilen bu optimal büyüme oranı, bu toprak siyanobakterisinin alkalin koşullarına daha duyarlı olduğunu göstermiştir. Bu siyanobakterinin, çeşitli çevresel pH ve tuzluluk koşulları altında görünümünde ve morfolojisinde değişiklikler gösterdiği fark edilmelidir.

Anahtar Kelimeler: Siyanobakteri, hapalasifon, tuzluluk, ışık, asidite ve baziklik, morfoloji, İran

ABSTRACT:
The aim of this research was to study ecophysiological cyanobacterium *Hapalosiphon sp. FS56* collected from paddy fields of Golestan province in north of Iran. In this attempt, the purified strain was incubated in the BG011 culture medium under illumination (2 µmol quanta.m^{-2}.S^{-1}) with pH 7.5 and 25±2 °C temperature. In order to assess photosynthesis and morphological parameters such as growth rate, pigment and phycobilisome content, some treatments under the combination effects of pH (5,7,9), salinity condition (0.025%, 0.5% and 1%) at the limited irradiance (2 µmol quanta.m^{-2}.S^{-1}) intensities were implemented. The Results showed that *Hapalosiphon sp. Fs56* had the highest growth rate under 0.5% salinity (g/l^{-1}), and light-saturated photosynthetic rate peaked at this salinity concentration as well. This optimal growth rate obtained at pH 9 indicated that this soil cyanobacterium is more susceptible to alkaline conditions. It should be noted that this cyanobacterium showed changes for appearance and morphology under various environmental conditions pH and salinity.

Keywords: Cyanobacteria, Hapalosiphon, Salinity, Light, Acidity and alkalinity, Morphology, Iran

INTRODUCTION

Cyanobacteria in agricultural lands, especially paddy fields, are affected by the combined influences of environmental stresses. One of them is the alkalinity-acidity of the soils, which cyanobacteria typically grows in the pH range of 7.7-10. However, there are reports showing growth at pH levels below 3.5 (Aiyer, 1965). Generally, in ecological and ecophysiological discussions, soil acidity is an elective factor for algal population, and, overall, there is a significant positive correlation between cyanobacteria and soil acidity (Roger et al., 1980). Experiment on Hapalosiphon sp. FS 44 (Shokravi et al., 2010) is an example of a study showingv a negligible difference between acidic and alkaline conditions while there was a substantial increase in the growth rate due to the presence of the highest carbon dioxide. Although, phycobilisome system in *Hapalosiphon* sp., FS44 lack phycoerythrin, it could be fulfilled with the core and rode at alkaline conditions. The light is another important factor for all photosynthetic microorganisms, and it plays an essential role in survival and growth (Velu Vijaya, 2009). In floodwater soils, the light intensity available to cyanobacteria depends on the season and geographical location. In paddy fields, the effectiveness of vegetation and vegetative canopy height on the abrupt decrease in the light reaching the floodwater environment is significant (Shokravi et al., 2007). Some studies have shown that the light under received in a paddy field after rice growth is about one percent of obtained before rice growth (Valiente and Leganes, 1989). Cyanobacteria has specific strategies for using limited light. One of these strategies is the existence of a phycobilisome system which could led to cyanobacteria struggle with stresses under the low light conditions, such as paddy fields or inside soils (Soltani et al., 2005). In addition, salinity stress is observed in tidal areas, rocky shores, stromatolites, paddy fields, and especially paddy fields near marine environments. In addition to physiological behaviors, the morphology of cells appears to change in response to salinity. Small cells are solitary or placental in low salinity and large vacuoles and round cells are high in salinity (Shokravi et al., 2007). Generally, echophysiology has not been investigated so far regarding the combined conditions of pH, limited light and salinity in this strain. The genus of *Hapalosiphon*, only studied on characterization in Shokravi et al. (2005), there are some examples of soil cyanobacteria in Golestan province (Shokravi et al., 2008). *Hapalosiphon* sp. was studied e only morphologically and taxonomically by Jorjani et al. (2012), Shokravi et al. (2011) and also another species of this genus, *Hapalosiphon* sp. FS44, in terms of ecophysiological with emphasis on acidity-alkalinity and carbon dioxide by Shokravi et al. (2010). There are some articles on stigonematal cyanobacteria in northern Iran but these articles were mainly focused on species which salinity and acidity on the survival and growth of *Fischerella* and *Nostoc* species has been studied by Safai et al. (2007), Amir Latifi et al. (2007), Neda Soltani (2005). Practical application in the field of agriculture requires enough information on the strains collected. So, these investigations can be considered as an essential step towards using stigonematal algae that can be used as bio-fertilizer. Recently, many companies have been attracted to microorganisms due to high-level metabolites such as lipids, proteins, carotenoids, and carbohydrates and more importantly, easy cultivation. Therefore, they are interested to produce different products from them as human and animal feed supplements, biofuels and pharmaceuticals (Etesami et al., 2020). According to using of this strain or specie in the practical application of the agriculture field, It needs the characterization of the collected samples. At result, the aim of this research could be considered the first fundamental step in the use of stigonematales as candidates for future biological fertilizers.

MATERIALS AND METHODS

Soil cyanobacterium were collected and identified from paddy fields of Golestan province. To cultivate the soil, amount of sample soils were passed through a 2 mm mesh and also 5 grams were removed from the smoothed soils and transferred to pre-sterilized Petri dishes. Then the BG0-11 culture medium was added to them. However, the pH of this culture medium was adjusted base on soil pH. These Petri dishes were placed on the shelves of the culture room, and the fluorescent light with an intensity of 20 micromoles of quanta per square meter per second (depending on treatment conditions) was illuminated. (Kaushik, 1987). After colonization, isolation and subsequent cultures, cyanobacteria *Haplosiphon* sp. It was prepared purely (Kaushik, 1987). Preliminary identification at the strain level was done based on John et al. (2003), Anagnostidis & Komarek (1990), Prescott (1962), Desikachary (1959), Geitler (1932). Specimens after identification as *Haplosiphon* sp. FS56 was coded and recorded in the algae museum of the Institute of Applied Sciences of Shahid Beheshti University in Tehran-Iran. Culture was carried out in BG0-11 liquid medium, actually Stock culture was grown in N-free medium at 2 Mm Quanta/ms^{-1} (provided by fluorescent lamp) at 28 °C and pH 8 (Soltani et al., 2006). Physiological examinations were performed in 500 ml volumes containing 200 ml suspension. The cultures were stirred for 1 hour and then transferred to the light chamber. Prior to inoculation, the sample was incubated for 48 hours in the liquid medium for adaptation. Acidity treatments were done in triple acidic, neutral and alkaline conditions (pH 5,7,9). The buffers used were phosphate, Hepes, Mes and Tris. Salinity treatments were carried out based on the results of the first step in the range of 0, 0.25, 0.5 and 1% sodium chloride. Growth was analyzed by spectrophotometer (OD750). In addition, the pigment composition and PBs system were determined according to Vincent and Williams (2001). Nitrogenase activity was determined by acetylene reduction test (Soltani et al., 2005) and photosynthesis was performed by Clark model oxygen electrode. Moreover, morphological analysis was considered using living sample in terms of cell shape, colony aggreation, thallus and filament structure by binocular and light microscopy along phasecontrast and fluoresscence microscopy. Statistical analysis was performed using SPSS Ver 11 and Sigmaplot software and comparing means with one-way analysis of variance (ANOVA).

RESULTS

These cyanobacteria tend to grow mostly in alkaline condition (Fig. 1). Although it could grow in acidic and neutral conditions, the specific growth rate delinates the tendency alkaline in this strain of cyanobacteria. It seems that the relative decline in growth at day 6 after inoculation in neutral and alkaline conditions was due to the natural behavior of the sample instead of potential changes.

In the environment, even under acidic conditions at the same time an increase in the growth was seen in the curve (Fig. 1).

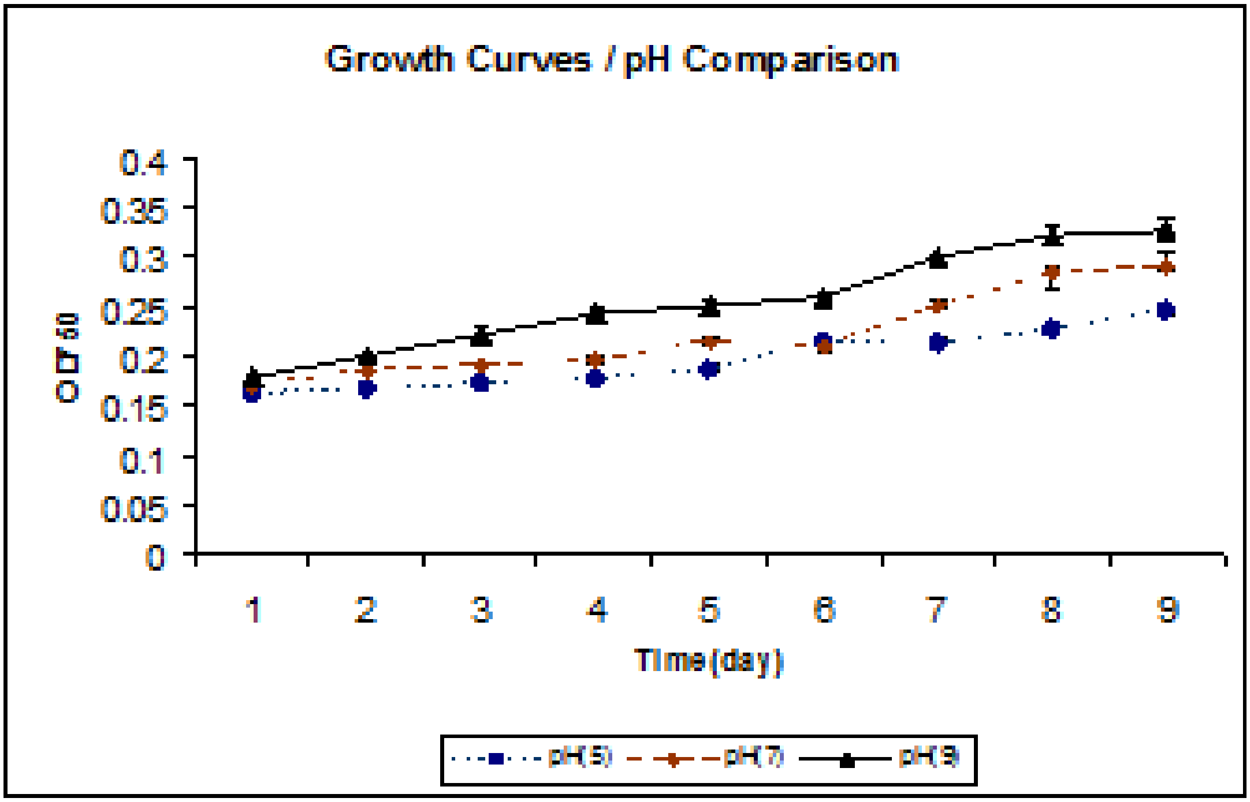

Figure 1- Comparison of the growth curve of *Hapalosiphon* sp. sample under pH conditions (7,5,9)

The amount of salinity above 2% caused disturbance in the photosynthetic system in these cyanobacteria (Table 1). The optimum salinity for this sample is in the range of medium without increasing salinity to medium containing 1% salinity. The table shows that salinity treatment was repeated (0-10%) at 1% intervals. Although there was not growth and survive in 2% salinity, among 3% and 4% salinity even up to 10% salinity treatments were not observed any growth in this strain. The optimum salinity treatment was belonged to 0.5%.

Table 1- Investigation of photosynthetic and photosynthetic pigments (µg/mg dry weight) in different salinities in cyanobacterium *Hapalosiphon* sp. FS56

Chlorophyll	Allophycocyanin	Phycocyanin	Carotenoid	Salinity (%)
064.0	047.0	098.0	197.0	Witness
084.0	118.0	190.0	031.0	0.25
147.0	120.0	104.0	045.0	0.5
018.0	020.0	110.0	110.0	1
011.0	02.0	011.0	012.0	2
012.0	020.0	09.0	01.0	3
012.0	020.0	09.0	01.0	4
012.0	-	08.0	01.0	5
011.0	-	08.0	01.0	6
011.0	-	02.0	01.0	7

The comparative content of phycobilisome pigments (Fig. 2) shows that the phycocyanin content reached 0.25% at salinity level and decreased significantly at 1% salinity. Also, in salt-free medium, allophycocyanin content is not eliminated, only a noticeable decrease.

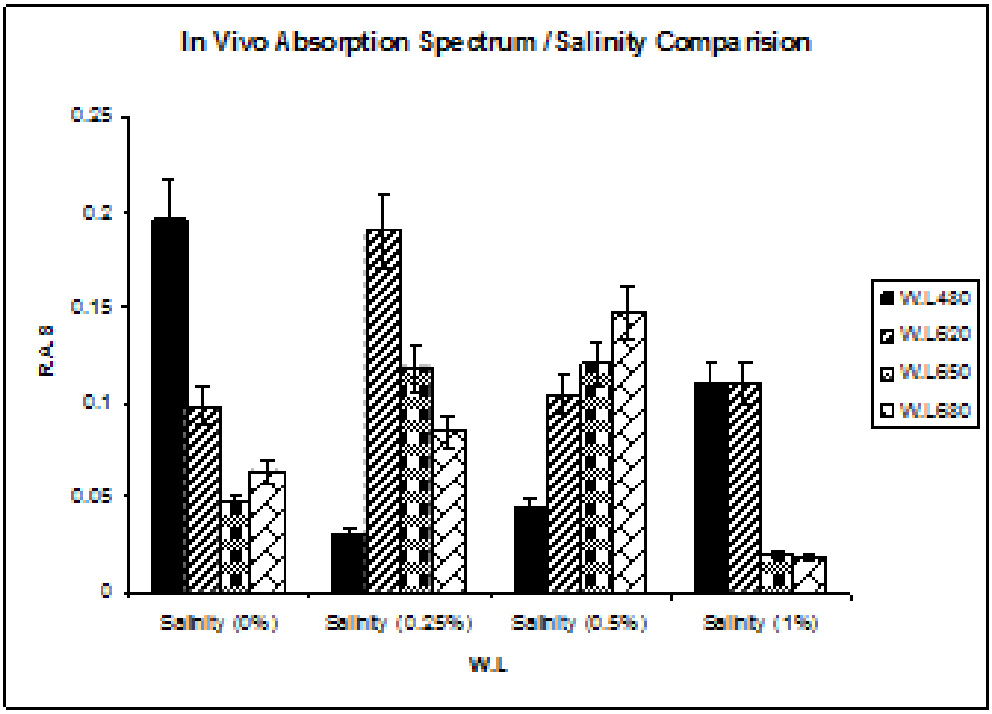

Figure 2- Histogram of pigmentation comparison in cyanobacterium *Hapalosiphon* sp. at different salinity conditions (0%, 25.0%, 5.0% and 1%) on day 6

In Table 2, traits related to sample morphology in alkaline conditions are presented. According to the growth curve (Fig. 1) and relative entry into the stationary phase from day eight, traits were examined daily from first day to eighth day.

Table 2- Specificatio table based on morphological changes of cyanobacterium *Hapalosiphon* sp. at optimum pH

Treatment	**Color of aggregation**	**Form of aggregation**	**Spore cell situation**	**Dimension of Spore cell**
pH9,1th	slightly brownish green	margins slightly in the middle	Spherical, Spherical -elliptical, elliptical with Two multi-Layers	Diameter 17.059-44.604 Length 25.85-33.018
pH9,4th	Dark brown	scattered masses	Spherical, Spherical- elliptical with Two multi-Layers	Diameter 14.311-14.809 Length 14.676-16.5
pH9,6th	Dark brown	margin and slight scattering	Spherical-Oval with two multi-Layers	Diameter 14.468-39.054 Length 15.488-19.092

In optimum pH conditions, the color of the communities appears to be brown. On the first day after inoculation, green color was briefly observed, but gradually in the following days, this color turned brown (Fig. 3). We observed two types of behavior in terms of community behavior.

Communities in the fluid environment sometimes find themselves centrally behaved and sometimes disseminated throughout the container. The presence of layered spores in alkaline conditions in the early days, at least as far as can be detected by conventional microscopy, first multilayered and gradually fixed on two layers (Figs. 4 and 5). Spores are spherical or elliptical-elliptical, more commonly spherical in the early days after inoculation. Spores change from spherical to spherical-elliptical through time (Table 2).

Figure 3- Morphological study of cultured cyanobacterium *Hapalosiphon* sp. At pH 9, 6th day after inoculation

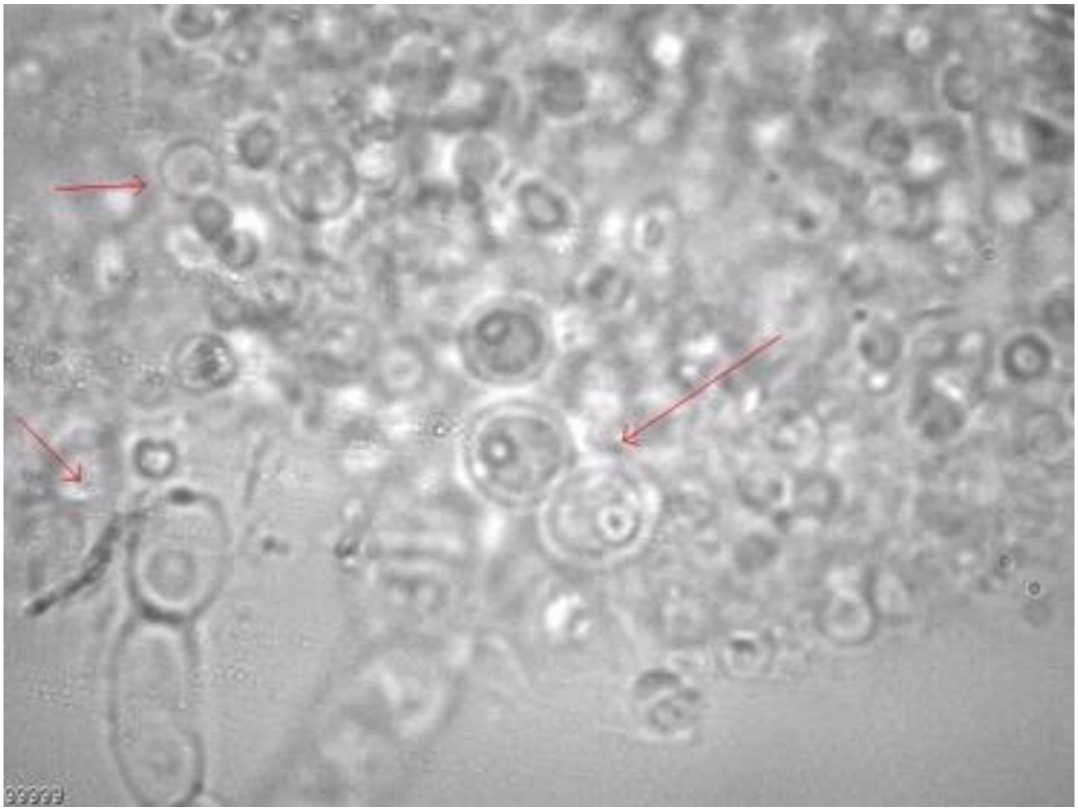

Figure 4- Microscopic image of cyanobacterium *Hapalosiphon* sp. at pH 9, the fourth day after inoculation

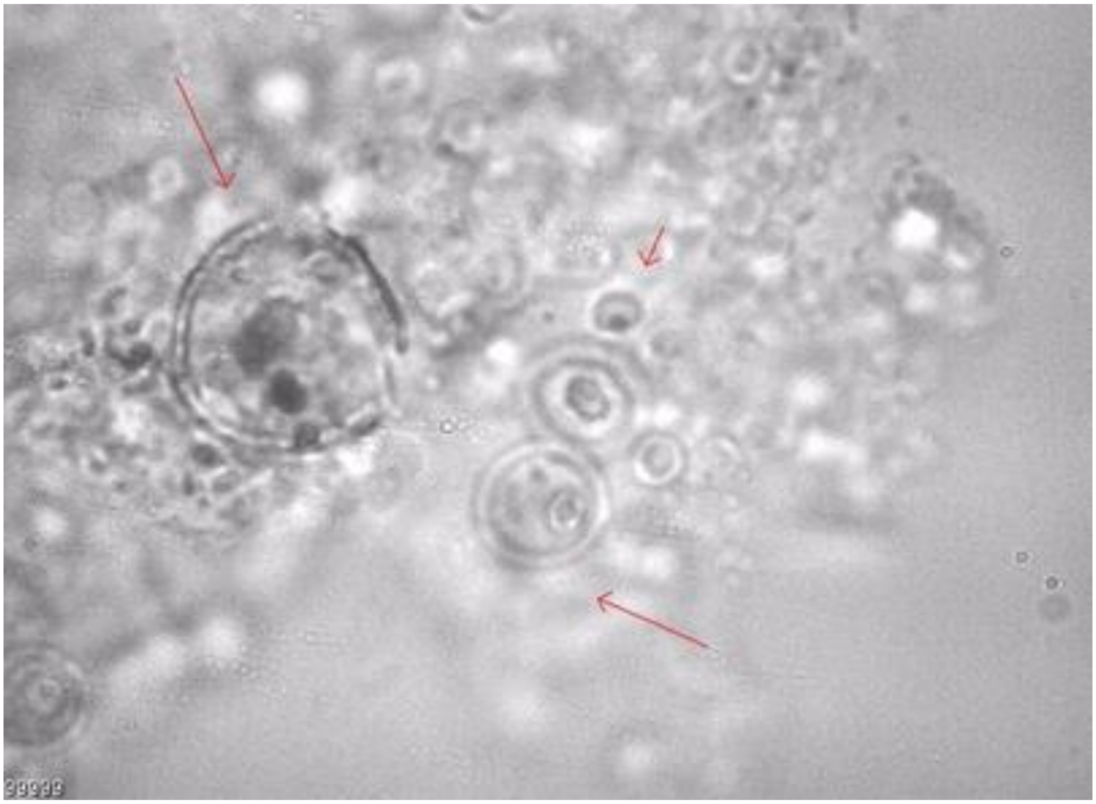

Figure 5- Microscopic image of cyanobacterium *Hapalosiphon* sp. at pH 7, 6th day after inoculation

DISCUSSION

In this study, *Hapalosiphon* sp., FS56 seems to be alkaline-friendly strain. The late phase in alkaline conditions, as well as the logarithmic phase from day one (Fig. 1), indicate that the sample is inherently adaptable to alkaline conditions (Shokravi et al., 2008). This finding complies with most studies which are performed in other countries and Iran. For example, studies on species of *Nostoc* (*Nostoc* UAM205, *Nostoc* UAM 206) in Spanish paddy fields have confirmed the alkalinity of the sample (Leganes and Fenandez Valiente, 1991). *Nostoc* sp. UAM206, while emphasizing the alkalinity of the sample, the effect of carbon dioxide and light content on strain behaviors (Poza-Carion et al., 2001).

It is also consistent with the studies by Amirlatifi et al. (2007), Shokravi et al. (2002), Shokravi et al. (2005) and Soltani et al. (2005) on stigonatomal and nostocal cyanobacteria. In the case of Iranian paddy fields, the reports of Safai et al. (2007), Amir Latifi et al. (2007), Shokravi et al. (2008) show that nostocal and stigonatural cyanobacteria of northern paddy fields from the general pattern of cyanobacterial mycobacteria. The problem of alkalinity is an active concentration mechanism for carbon dioxide. In Sassani et al. (2009) and Shakeri et al. (2009), the issue of the active concentration mechanism of soil oscillatorial cyanobacteria in Golestan province has been emphasized. The same has been the case in the studies of nostocalean cyanobacteria in the studies of Amirlatifi et al. (2007). In the reviews of Shokravi et al. (2009) on Hapalosiphon sp. FS 44, the target species, was able to survive in alkaline conditions, but its survival in extreme alkaline conditions was difficult.

According the effect of salinity, this sample appears to be susceptible to salt tolerance, which has been claimed in relation to soil and salinity cyanobacteria (Shokravi et al., 2008).

The amount of salinity above 2% cause impaired photosynthetic system, higher percent of salt causes serious impairment of growth. It is clear that the optimum salinity for this sample is in the range of medium without increasing salinity (shown in the table as 0%) up to 1% salinity. This seems a bit unusual for soil cyanobacteria (Shokravi et al., 2008). However, it seems this cyanobacterium shows own character in vitro and studying of combination of limitation light but this characteristic in the natural environment is also somewhat ambiguous. In Shokravi et al. (2002) the differences between two environments in terms of species diversity are discussed. Concerning salinity, there is no mention of researches differences between the natural environment and the culture medium which can be due to the lack of focus on the subject.

With the exception of the 1% salinity that a minority reduces growth, especially on the second to fourth days after inoculation while others continue to grow exponentially until the ninth day. The maximum growth was observed in the 0.5% salinity treatment. The treatment of 0.5% salinity and the increase in its growth comply with most of the studies in Golestan province. In Shokravi et al., (1378) salinity of 0.5% in culture medium lead to stimulate the significant growth in *Nostoc* sp. In Shakrovi et al., (2002), the same amount of salinity stimulated the growth in nostocalean and stigonemalean cyanobacterium (*Fischerella* sp.); and also we saw in oscillatorial cyanobacterium *Lyngbya* sp., *Oscillatoria* sp. in Shokravi et al. (2003), Safai et al. (2007) and in Soltani et al. (2009).

Comparative content pigment with emphasis on pigments is given in (Fig. 2). Figure shows that the phycocyanin content reaches its maximum at 0.25% salinity and decreases considerably at 1% salinity. Another significant point is the high content of allophycocyanin in 0.5% salinity due to the complete structure of the basal phycobilisome. At 1% salinity and also in salt-free medium culture, the allophycocyanin content is not eliminated, only a noticeable decrease. Considering the increase of phycocyanin content in 0.5% salinity and high amount of allophycocyanin in the same condition compared between without salt and 1% salinity medium, it is reasonable to assume higher growth in 0.5% salinity. In salinity 0.25% difference between phycocyanin and salinity 0.5% was significant ($p < 0.05$), but the difference between allophycocyanin values was not considerable. However, there is a difference in carotenoid and chlorophyll content, which may justify higher growth in salinity of 0.5%. Despite the significant higher concentration of phycocyanin at 0.25%, it seems that photosynthetic shows intrinsic compatibility with 0.5% salinity. Under these conditions, maximum photosynthesis was significantly ($p < 0.05$) higher than other salinity treatments. It is also more efficient in absorbing light. It seems that changes in the engineering of the phycobilisom system or morphological variation may be some of the factors that explain this efficiency. This issue has been emphasized in the studies of Schwarz et al. (2005) and Nisha et al. (2007).

According to absorbance curves for acidity and alkalinity treatments can be useful for investigating the compatibility conditions at pH 9 in these conditions. It seems that the absorption range of phycobilisome pigments is maximum and in addition there is a relative stability in the amount of comparative pigments. At optimum pH conditions, the exception of first day after inoculation, the brown color tendency in these communities is a constant trait. By changing content pigments, the brown color tends to light brown. It seems that on the fourth day after inoculation, the brown color tends to change into dark brown. Concerning the behavior of aggregation in the liquid culture medium, it is sometimes centered and sometimes disseminated throughout the container. At result, this strain does not comply with the *Fischerella* sp. (Vakili et al., 2006).

The presence of layered spores in alkaline conditions seems to be an almost constant trait. having layered spores maintain under all conditions. These layers can be more than two, but the two layers of spore gradually will be stable, and it seems that changing time may not cause or increase the spore layers. In the reviews of Perona et al. (2003) this is confirmed. Unlike the layers, which appear to be a steady character, spores change their state over time. It can be concluded that the spores tend to be much more spherical-elliptical in alkaline conditions which is common among this strain.

Having unilayer cells at the main axis may be regarded as a relatively constant trait. The form of branches may be considered as another constant trait that is similar to *Fischerella*/*Stigonema* group. In other studies, on other stigonematal cyanobacteria in Golestan province, it has not been found certainly in the unilayer main axis. In Shokravi et al. (2006); Shokravi et al. (2008), and Vakili et al. (2006), the main axis in genus such as *Fischerella* and *Stigonema* are unilayer and multilayer. In Soltani et al. (2005), cyanobacterium stigonemalean as *Fischerella* sp. FS18 has shown both unilayer and multilayer. In Shokravi et al. (2009), a cyanobacterium similar to *Hapalosiphon* sp. FS44 has been introduced as a strain with a unilayer main axis but has not emphasized the issue of morphological diversity and the status of the main axis. With the evidence currently available, the unilayer cells principal axis for this trait can be identified as constant and consistent.

CONCLUSION

Hapalosiphon sp., FS56 could be represented especially potential species. The presence of carbon-concentrating mechanism in this cyanobacterium lead to both acidic and alkaline conditions maintaining the species's survival under adverse conditions, although optimum growth is belonged to the alkaline conditions. Moreover, the most significant potential in this strain is that studies were performed under limited light conditions, with remarkable photosynthetic activity and maintaining phycobilisome system which tolerate under the limited light while the sample is sensitive to salinity, so it is possible to impact on its activities. The high percentage of allophicocyanin with 0.5% salinity due to the complete structure of the basal phycobilisome structure is in paddy fields of Golestan province and in terms of morphological characteristics with the reported species, *Hapalosiphon* sp. FS44 and other members of the stigonamalean that is considered in this province are different. In addition, the most considerable ecophysiological mechanisms has been described about *Hapalosiphon* sp. FS56. As result, the species with its specific characteristics can be an effective model for practical applications in agriculture.

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