



Adaptation of the *Cyanobacterium fischerella* sp. ISC 107 to the combined effects of pH and carbon dioxide concentration

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Abstract

The aim of this study was to investigate the adaptation of the cyanobacterium *Fischerella* sp. ISC 107 to combined effects of carbon dioxide concentration, acidic and alkalinity. Axenic strain was incubated in BG 11 medium. Carbon dioxide treatments were limited and relatively non-limited. Acidic (pH 5), neutral (pH7), and alkaline (pH 9) conditions were employed in each treatment. Survival, growth, chlorophyll, phycocyanin, allophycocyanin, and phycoerythrin contents were evaluated in each treatment along with ammonium liberation, frequency, and biometry of heterocyst. Results showed that like other explored stigonematalean and nostoclean cyanobacteria, this strain cannot grow in acidic condition. Under limited carbon dioxide condition, difference in growth rates were not significant between acidic and alkaline conditions; however, carbon dioxide enrichment caused significant increase in growth rates. Phycobilisome system of this strain lacked phycoerythrin and may complete its structure both at the core and the rod under alkaline condition. Heterocyst frequency and biometry were maximum at alkaline condition showing significant correlation with nitrogenase activity. Heterocyst showed cylindrical and sub-cylindrical morphology on days 4 and 5 after inoculation. Overall, the results showed that this strain may be qualified to be used as a biofertilizer similar to the other cyanobacteria.

Keywords: Acclimation, Cyanobacterium, Golestan, Fischerella, paddy field

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Introduction

Cyanobacteria in paddy fields are under influence of a number of stresses including CO₂ as the main stress. Flooding irrigation changes CO₂ contents of the paddy field which the Cyanobacterium must tolerate along with other

stress conditions such as salinity, light, and darkness in its attempt to survive. Biotechnologically important strains e.g., as biofertilizers in paddy fields must be robust enough to tolerate fluctuations in the CO₂ content (Boussiba, 1988; Dashti et al., 2015).

Paddy field Cyanobacteria are exposed to a range of acidic and alkaline changes which can even vary in the paddy fields daily. Flooding the

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paddy fields causes a balance between CO₂ and bicarbonate. The determining factor for this balance is the environment acidity (Stal, 1995). Active concentration mechanism was shown in *Nostoc* (Amir-latifi, 2007) and *Fischerella* strains (Shokravi et al., 2002). In flooding irrigation where there is limitation in free CO₂, inducing this mechanism is necessary for survival and therefore, the strains with biofertilizing applications must have the flexibility to induce this mechanism and obtain the resources required for obtaining energy (Shokravi, et al., 2008).

Fischerella sp. ISC 107 is among cyanobacteria strains reported in the paddy fields in Golestan province. Because of their potentials at various levels stigonematalean cyanobacteria can be seriously considered in applied agrobiotechnology of microalgae. Their ability to output nitrogen compounds such as ammonium has made them applicable as biofertilizers in Golestan province which is a center of agriculture (Shokravi and Sateyi, 2005). In addition, the special morphology of this group of Cyanobacteria and the form of their filaments result in their expansion in agricultural soils and paddy fields which output a wide range of antimicrobial compounds and protect, maintain, and disinfect the soil (Soltani et al., 2006).

Combined, these features make investigation of stigonematalean cyanobacteria in Golestan province an agrobiotechnologically important endeavor (Anand et al. 1990; Elmekawy et al. 2015). In this case, characterization of this strain in terms of various features including morphology and ecophysiology can pave the way for future applications. Regarding the important position of rice as a staple food in Iran and the necessity of applying biofertilizers in future and since this plant is considered as a strategic crop in Iranian agriculture, the issue of survival and growth of this strain in the rather hard conditions of the paddy fields is of importance.

There are some studies on other stigonematalean cyanobacteria which are also related to the field of ecophysiology. Shokravi et al. (2007) investigated the *Stigonema* specimens morphologically and taxonomically, respectively. Baftechi et al. (2001) studied the growth and pigments of *Fischerella* sp. Under 12 h photoperiod. Shokravi et al. (2003) studied the

growth capability of the specimen under continuous lighting condition. Soltani et al. (2006) investigated nitrogenase activity of an unknown serovars (at the level of a strain) under combined condition of acidity and light intensities. The effects of salinity and acidity on survival and growth of *Fischerella* and *Nostoc* strains were also studied by Safai et al. (2007) and Amir-latifi (2007). Findings of a study on *Chlorella vulgaris* in pH 5-8 suggested the maximum growth under pH 7.5 condition (Sakarika and Kornaros, 2016). Vakili (2006) studied the effects of photoperiods on growth and frequency of heterocyst Cyanobacterium *Fischerella*. There are also reports on the effects of nitrogen resources and salinity on the stigonematalean cyanobacterium *Fischerella* sp. under laboratory conditions (Soltani et al., 2007, 2008).

The aim of the present study was characterization of a *Fischerella* sp. ISC 107 specimen under natural condition. High density of this specimen in agricultural lands and paddy fields of Golestan province necessitates its precise ecophysiological evaluation. As a first step, the survival and growth of the specimen under combined CO₂, acidic, and alkaline condition was studied. It is assumed that combinatory analysis under natural conditions results in a more realistic result compared with the laboratory conditions (Poza-carion et al., 2001).

Materials and Methods

Soil samples were collected from Golestan Province. The soil samples were cultured following the methods of terricolous cyanobacteria culture (Kaushik, 1987). After formation of colonies, isolation, and subsequent culturing, pure *Fischerella* sp. cyanobacterium was prepared (Kaushik, 1987). Preliminary identification at the strain level was done based on Geitler (1932), Desikachary (1959), Prescott (1962), Anagnostidis and Komarek (1990), and John et al. (2003). The *Fischerella* sp. ISC 107 sample was cultured in a liquid BG-11 medium under 2 μM Quanta/ms-1 lighting condition (supplied by fluorescent lamps), 28° C, and pH 8 (Soltani et al., 2006). For acidity balance 25 μM HEPES was used (Soltani et al., 2006). Physiological assays were carried out in 500 ml Erlenmeyer flasks containing 300 ml

suspension. The cultures were stirred for 1 h before being transferred to the light condition. The sample was introduced into the liquid medium for adaptation for 24 h prior to the inoculation. The preliminary analysis of the acidity treatments were done under acidic, neutral, and alkaline conditions, pH 9, 7, and 5, respectively. The buffers used included Hepes, Mes, Tris, and Phosphate. CO₂ treatments and analysis of concentration mechanism were carried out under CO₂ limited (no aeration) and relative limited (no aeration) conditions (Poza Carion et al. 2001). Growth was measured on the basis of the change in dry weight (Soltani et al., 2006). Chlorophyll assay was done after derivation with methanol based on the method explained by Soltani et al. (2005). Phycobilin proteins were also assayed based on the method of Soltani et al. (2006). Ammonium liberation was assayed using the method of Solorzano (1969). Morphological analyses were carried out using living samples as well as the samples stabilized in mounted glycerin. Fluorescent microscope was used to observe and identify the heterocyst dimensions. Heterocyst frequency was calculated using Kaushik (1987) and the statistical analyses were carried out using SPSS Ver. 1 and Sigmaplot.

Table 2

The effect of combined treatment of CO₂ and pH on the protein, total sugar, and carotenoid contents of the cyanobacterium *Fischerella* sp. ISC 107

Culture Conditions		Chla	PBP	APC	PC
pH	DIC	$\mu\text{g mg dw}^{-1}$			
5	NA	6.84±1.2	8.89±1.38	ND	8.89±1.4
	AE	6.15±0.3	6.15±2.24	ND	6.35±2.34
7	NA	25.40±09	56.19±9.52	12.32±1.44	8.156±0.76
	AE	18.48±1.2	58.44±8.95	10.43±2.55	47.28±6.42
9	NA	12.89±2.7	61.45±20.82	7.83±9.43	55.68±11.42
	AE	8.34±0.72	96.58±5.92	6.54±1.52	92.02±4.42

Culture Conditions		TP	TC	CAR
pH	DIC	$\mu\text{g mg dw}^{-1}$		
7	NA	236.9±12.4	166.9±23.45	34.5±9.12
	AE	344.9±33.6	188.8±10.4	37.56±6.22
9	NA	446.875±20.2	297.3±38.99	3.82±8.22
	AE	657.5±19.43	388.3±11.46	32.98±4.36

AE: aeration; NA: no aeration; ND: not determined

Table 1

The combined effect of CO₂ and pH on nitrogenase activity, heterocyst, and maximum ammonium liberation in cyanobacterium *Fischerella* sp. ISC 107

Culture Conditions	nMI ethylene	PSII/PSI	MAL ⁵	
pH	DIC	mg dry wt ⁻¹ h ⁻¹	(mg/L)	
5	NA	324 ± 33 ^a	1.64	0.022 ± 0.016
	AE	324 ± 33 ^a	1.64	0.083 ± 0.06
7	NA	324 ± 33 ^a	1.64	0.23 ± 0.06
	AE	421 ± 48 ^a	1.64	0.18 ± 0.08
9	NA	371 ± 87 ^b	1.64	0.29 ± 0.11
	AE	589 ± 122 ^b	1.64	0.44 ± 0.13

NA: non-aeration; AE: aeration; MDW: maximum dry weight; SGR: relative growth rate; MAL: maximum ammonium liberation (the 5th day is marked with 5)

Results

The serovar under study faces problem under acidic conditions and the environment turns into white (Fig. 1). The growth of the specimen under acidity significantly decreases compared with under alkaline conditions ($P \leq 0.05$). Increase in CO₂ concentration did not have any effect on the serovar's inclination to acidic conditions (Table 1). Under neutral conditions and limited CO₂ concentration, growth is significantly increased

compared with the condition when there is no limitation in CO₂. This is also the case with alkaline conditions with limited CO₂ (P≤0.05). The cyanobacterium *Fischerella* sp. ISC 107 seemed to have the highest growth rate in alkaline conditions and under no limitation in CO₂ (Fig. 1). Comparison of mean growth rates (Table 1) confirms this suggesting that under neutral condition, the change in CO₂ concentration has no effects on the growth of the specimen. In fact, inactivity or relative activity of the CO₂ concentration mechanism does not induce growth under neutral pH conditions in this serovar (Leganés et al., 1991). Liberation of nitrogen compounds including ammonium follows the growth pattern (Table 1) whereas the liberation in *Fischerella* sp. ISC 107 is higher than the other stigonematalean cyanobacteria such as *Fischerella* sp. FS33. This increase is particularly noticeable where there is no CO₂ limitation (Soltani et al., 2006). The liberation in nostocal specimen such as *Nostoc* sp. (Shokravi et al., 1999) under limited and unlimited CO₂ condition was not significantly different from that in *Fischerella* sp. ISC 107 (P≤0.05).

The cyanobacterium *Fischerella* sp. ISC 107 does not contain phycoerythrin. Therefore, it is similar to the other investigated stigonematalean cyanobacteria such as *Fischerella* sp. FS33 (Soltani et al., 2006). The efficiency of phycobilisome system reduces under acidic condition and the allophycocyanin region is removed. The change in the CO₂ content has no effect on allophycocyanin recovery (Table 2). On the other hand, under neutral and alkaline conditions, the central part of phycobilisome recovers itself. In neutral condition this part is lower in quantity. The pigments in the stem are phycocyanin and under alkaline conditions, particularly when the CO₂ content is increased, they show a significant increase (P≤0.05). Chlorophyll content shows the maximum quantity when the environment is neutral and there is limited CO₂ (Table 2).

In acidic condition, as the growth of the specimen is significantly reduced, so does the capability of Heterocyst production (results are not presented here since the specimen did not grow under acidic condition). Microscopic analyses showed that under the aforementioned condition Heterocysts mature later and there are few

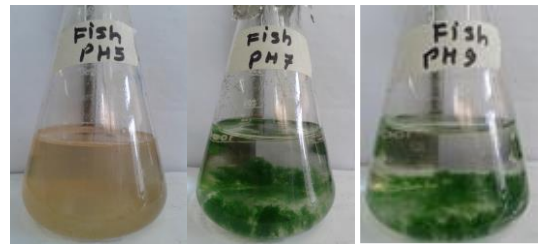


Fig. 1. Macroscopic images of the Cyanobacterium *Fischerella* sp. ISC 107 under combined CO₂ and pH treatment

Table 3

The effects of combined treatment of CO₂ and pH on biometric features (diameter and length) of the vegetative cells and spores and morphology of the branches in the cyanobacterium *Fischerella* sp. ISC 107

pH	DIC	HF (%)	VGD (μ)	VGM
7	NA	3.66±0.2 ^a	34.63±1.785 ^a	CY
	AE	7.89±0.78 ^a	365±1.34 ^a	CY
	NA	4.44±1.15 ^b	42.44±1.78 ^b	SU-CY
	AE	7.22±2.68 ^b	40.45±1.78 ^b	SU-CY
9	NA	9.65±2.56 ^a	9.26±2.76 ^a	CY
	AE	6.24±2.44 ^a	30.18±24 ^a	CY
	NA	8.14±0.86 ^b	33.53±25 ^b	SU-CY
	AE	8.23±2.22 ^b	37.66±16 ^b	SU-CY

HF: Heterocyst frequency; VGD: Vegetative growth duration; VGM: Vegetative growth measurements; CY: cylindrical; SU-SY: sub-cylindrical; the days after inoculation are shown by the 4 and 5.

mature Heterocysts. In addition, the dimension of the Heterocysts do not follow the same pattern. The results of analysis of the Heterocyst under alkaline and neutral conditions showed that they achieve maximum size as well as frequency in alkaline conditions (Table 3). Normally, the frequency of the Heterocysts can indicate their nitrogenase activity (Shokravi et al., 2007). Direct measurements showed that in this specimen, the nitrogenase activity reaches the maximum level under alkaline condition and no CO₂ limitation which is significantly different from the other treatments (results are not shown). Regarding the ammonium liberation condition, it seems that the liberation of nitrogen compounds reaches the maximum level under alkaline conditions and introduction of CO₂ which is a result of high nitrogenase activity. In neutral condition, just as with the frequency of Heterocysts, their dimensions do not follow the same and predictable pattern (Table 3).

The change in the morphology of the Heterocysts is interesting in this specimen. Their shape changed from cylindrical to sub-cylindrical from the day 4 to the day 5. This change in the shape of Heterocysts is observed both in neutral and alkaline conditions. Increase in the CO₂ level seems to have no role here. Similarly, the growth condition and particularly high growth of the specimen and no limitation in CO₂ is not the reason for this change in the morphology (Tables 1 and 3). Therefore, it is highly likely that the change is related to the time and the stages of development in the specimen.

Discussion

Cyanobacterium *Fischerella* sp. ISC 107 survives under acidic condition but is not able to grow. The acidic condition seems to be accompanied with some shock which is manifested in the form of negative growth. Overall, this strain more tends to the neutral and alkaline conditions. As such, it is similar to the other stigonematalean cyanobacteria studied in Golestan province (Soltani et al., 2006). Negative growth was also observed during the first two days under the relative limitation and non-limitation of CO₂. Active concentration mechanism. This suggests that the cyanobacterium does not possess a two way concentration mechanism activated under acidic conditions (Safaei et al., 2007). In their study on the stigonematalean cyanobacteria *Fischerella* sp. isolated from the paddy fields in Golestan province Safaei et al. (2007) that they do not possess CO₂ concentration mechanism under acidic conditions and the change in acidic and alkaline condition could not develop this capability. Survival under acidity does not correspond with the results of studies on the other studied stigonematalean serovars such as *Fischerella* sp.FS18 (Soltani et al., 2006). Weak ammonium liberation under acidic condition is consistent with the survival of the specimen but practically it does not support the cyanobacterium under acidic condition (Anand et al., 1990). Under low or high pH condition, cells may spend their energy on maintaining their internal pH level for the normal activities; therefore, this may lead to a reduction in their growth. The optimum pH range for 6 strains of cyanobacteria is reported between

7-4 and 8 (Rai and Rajashekhar, 2014). *Oscillatoria tenuis*, *Plectonema boryanum*, and *Lyngbya aestuarii* have the maximum biomass production under pH 6.5-7.5. The pH in the culture media plays an important role in dissolving CO₂ which directly influences the metabolism in the strains (Shruthi and Rajashekhar, 2014). Significant increase in the growth of the specimen under alkaline conditions when the CO₂ limitation is removed along with the ammonium liberation is justified by what was explained about the inductive nature of the concentration mechanism of cyanobacteria and some other microalgae (Poza-Carion et al., 2001). The relative reduction in the concentration mechanism causes the energy to tend to the other processes including growth and this explains the significant growth observed in the study (Stal, 1995). Another reason for this is the lack of significant difference in the biomass on different days under the alkaline and neutral conditions and inoculation and relative non-inoculation of CO₂. This was also reported for the cyanobacterium *Synechococcus* PCC7942 (Yu et al., 1994). The cyanobacterium *Lyngbya* sp. FS33 Agardh showed optimum growth not under excessive alkaline condition (pH 9) but rather under neutral conditions. It seems that the one-way concentration mechanism (Poza-Carion et al., 2001) is at work in this serovar. The survival of the specimen under excessive acidic or alkaline condition makes it practically capable (Anand et al., 1990). The presence of such mechanism in the cyanobacterium *Oscillatoria* was seriously discussed (Stal, 1995). However, these mechanisms are mainly two-way and overlap under alkaline conditions resulting in a remarkable growth under this condition. The fluctuations observed in the growth when the cyanobacteria are not under the optimum condition seem to be a strain characteristic at least not observed in the nostocalean and stigonematalean cyanobacteria (Safai et al., 2007).

The capability of producing chlorophyll as an index of photosynthesis under acidic and alkaline conditions was not in agreement with the growth of the specimen and it seems to follow a different trend (Verling and Alberts, 1980). Careful observation of the arrangement and formation of the colonies showed that e.g., under alkaline and no CO₂ limitation condition, the specimens lose

their tendency to cling to the borders of the container and take a light green color which suggests weakened chlorophyll biosynthesis. Under alkaline and neutral condition with both limited and non-limited CO₂ condition, the specimen tends to cling to the container and its color turns to dark green showing increased chlorophyll activity (Reuter and Muller, 1993). Studies by Amir-latifi (2011) and Badeli et al. (2013) showed that *Nostoc* sp. FS 76 and *Anabaena* sp. FS 77 strains survive not only under excessively limited lighting condition, but also enjoy a high photosynthesis and nitrogenase activity.

The efficiency of phycobilisome system observed under neutral condition (optimum for growth) is an evidence for the capability of providing energy for applying CO₂ concentration mechanism (Poza-Carion et al, 2001). Under acidic condition, since there is no central region in the phycobilisome system, the ability to entrap light and send it to the reaction center reduces naturally (Soltani et al., 2006). The inability of the specimen to collect enough CO₂ in the paddy fields particularly under flooding irrigation condition can be attributed to the same reason (Mimuro et al., 1986). When the pH moves to the neutral condition, the phycobilisome system is strengthened and the central region comprising allophycocyanin and also the periphery region comprising phycocyanin remarkably increase in quality. These findings are in line with those reported by Burns et al. (2005) on the cyanobacterium *Synechococcus Elongatus*. The capability of the specimen to grow under neutral condition is because of introduction of the two-way concentration mechanism which itself can result from the phycobilisome system (Shokravi et al., 2008). The integrity of the phycobilisome system is preserved under alkaline condition but its strength is reduced (Soltani et al., 2006). This confirms the studies reported by Vakili (2006). Functionally, considering the indexes of Boussiba (1988), phycobilisome system seems to improve significantly both in structure and performance with an increase in alkalinity and also CO₂ content (Yamamaka and Glazer, 1981). Increase in the central region and also significant increase in the stem pigments improves the photosynthesis performance which is manifested in the significant

increase in growth. This is also reported on nostoclean cyanobacteria *Nostoc* sp.UAM 205 (Valiente and Leganes, 1998) and stigonematalean cyanobacteria *Fischerella* sp.FS33 (Soltani et al., 2006).

The frequency and biometric characteristics of the Heterocysts can be related with diazotrophy (Shokravi et al., 1999). In the Heterocystous cyanobacteria including the samples from the paddy field, it is possible to separate the location of photosynthesis and diazotrophy through the emergence of the Heterocysts. In the general analysis, increase in the frequency of the Heterocysts can be an index of the nitrogenase activity (Shokravi et al., 2007). The relationship between the growth and frequency of the Heterocysts in stigonematalean and nostoclean cyanobacteria has also been investigated. Soltani et al. (2007) showed that the acidic and alkaline conditions under non-limited CO₂ condition changes the dimensions of Heterocysts in the cyanobacterium *Fischerella* sp.FS33. Alkaline condition in this cyanobacterium seems to increase the Heterocyst dimensions even though the light intensity has the main influence on this (Soltani et al., 2006). Studies by Baftechi et al. (2001) showed that in stigonematalean cyanobacteria, the Heterocysts can be influenced by the environmental factors but this variability is more in branches than in the main axis. Under nitrogen deficit condition, synthesis of nitrogen compounds is reduced while the lipid compounds are increased; therefore, C/N ratio is a highly determining factor (Venkata Subhash et al., 2017; Baojun et al., 2017).

Unlike the stigonematalean and nostoclean cyanobacteria studied in Golestan province (Vakili, 2006; Safai et al., 2007; Shokravi et al., 2007; Soltani et al., 2006) there is a considerable morphological flexibility in *Fischerella* sp. FS33. The rationale behind geometric arrangement of the Heterocysts in the cyanobacteria is not fully understood yet (Shokravi et al., 2008). Analysis of nitrogenase activity revealed that there exists a significant relationship between the size of Heterocysts and the nitrogenase activity ($r^2=0.86$). This is yet another reason for the effect of alkaline condition and non-limited CO₂ on the growth of the specimens; however, it is not possible to study the effects of

morphological condition of the Heterocysts on the nitrogenase activity. The correlation between the frequency of the Heterocysts and the nitrogenase activity is positive but weak (results are not shown). This low correlation is more obvious under neutral condition. On the other hand, under alkaline condition, whether with limited or non-limited CO₂, the correlation increases.

Conclusion

Cyanobacterium *Fischerella* sp. ISC 107 possesses some characteristics which qualify it for functional purposes including application as biofertilizers in paddy fields. Firstly, this strain exists at high concentrations in the paddy fields in Golestan province growth is high under alkaline conditions and particularly the CO₂ is as much as that in the air. Under the neutral and alkaline conditions, the specimen can adapt to acidity, alkaline, and CO₂ stresses. CO₂ concentration mechanism is active in this strain and especially under neutral and alkaline condition and limited CO₂, it results in the survival and growth of the specimen. Ammonium liberation under neutral and alkaline conditions is similar to the other serovars of stigonematalean and nostoclean cyanobacteria studied in Golestan province. Therefore, they can be considered as a rich source of nitrogen in farms and paddy fields. Just like other stigonematalean strains studied, this strain does not possess phycoerythrin; however, under alkaline condition and particularly when there is no relative limitation in CO₂, phycobilisome structure completes. The specific feature of this specimen is the flexibility in the dimensions and morphology of the Heterocysts which has relationship with nitrogenase activity under alkaline conditions. Taking all considerations into account, it is logical to employ this specimen not separately, but as combined starter along with Heterosystous cyanobacteria.

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