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Growth and Some Metabolic Features of Cyanobacterium *Fischerella* Sp. FS18 in Different Combined Nitrogen Sources

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Abstract

In the present research the growth and metabolism of cyanobacterium *Fischerella* sp. FS18, isolated from rice fields of Gillan Province (Iran), were evaluated in different nitrogen sources. This strain is a good candidate as biological fertilizer due to high efficiency to N_2 fixation. In this way, cells in logarithmic phase of stock were treated with different nitrogen sources (N-free, 1 & 2 mM NH_4^+ and 5 and 10 mM NO_3^-). Results indicated that maximum growth rate belonged to NO_3^- treatment. Dry weights of *Fischerella* sp. FS18 in N-free medium and ammonium were in decreasing order of preference; and NH_4^+ 2 mM was drastically toxic for this strain. The highest chlorophyll concentration was detected in N-free medium. NO_3^- 5 mM and NH_4^+ 2 mM had the lower contents of this pigment. Photosynthesis rate varied the same as chlorophyll. phycocyanin and phycoerythrin had the highest rate in nitrate. The highest light-saturated photosynthetic rate and also steepest initial slope clearly belonged to nitrogen free-medium. All types of combined nitrogen sources had inhibitory effect on nitrogenase activity, regardless of the concentrations used. Our results indicated that *Fischerella* sp. FS18 is a potent diazotrophic species with increasing growth in the presence of nitrate; and ammonium 2mM seems to be toxic for it.

Keywords: Ammonium; Cyanobacteria; *Fischerella* sp.; Nitrate; Nitrogenase; Photosynthesis; Photosynthetic pigments

Introduction

Nitrogen-fixing cyanobacteria, the major component of microbial flora in rice paddy fields, are the main contributors to photodependent N_2 fixation [21]. The input of nitrogen fertilizers to fields reduces nitrogen

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fixation since the presence of combined nitrogen inhibits nitrogenase activity. *Fischerella* is one of the most diverse benthic N_2 fixing cyanobacteria in paddy fields of Iran. This strain can be used commonly as biological nitrogen fixer.

Fischerella, as well as all cyanobacteria, utilizes nitrate, nitrite and ammonium as the sole nitrogen source for growth. However, in the presence of different nitrogen sources, cells uptake ammonium, nitrate or N_2 (for the nitrogen fixing species), in decreasing order of preference [6]. Urea and inorganic N sources other than ammonium are first metabolized to ammonium to allow assimilation of their N atoms [4]. Biological N_2 fixation is the major replacement mechanism [22].

As cyanobacteria are the early microorganisms on the earth which contribute to altering the atmosphere, they have several adaptative mechanisms to survive in extreme or variable environments [25]. The nutrient medium can affect the growth, metabolism [14], phycobiliproteins [11] or even antimicrobial properties [1].

Studies of Iranian rice fields have revealed not only favorable conditions for biological nitrogen fixation but also development of this cyanobacterium [5, 23 and 24]. This work aims to show the incorporation of different sources of nitrogen by heterocystous cyanobacterium *Fischerella* sp. FS18. Besides the correlation between nitrogen fixation, photosynthesis and growth rate with different contents of combined nitrogen as well as N-free medium were studied to evaluate the adaptation of this strain to extreme conditions.

Materials and Methods

Organism and Culture Conditions

In the present study filamentous, heterocystous cyanobacterium *Fischerella ambigua* FS18 was used. It was isolated from soils of paddy fields of Gillan province, Iran. Isolation and purification was performed by plating [26]. Stock cultures were grown in the N-free medium previously described [24]. Temperature was maintained at 30 °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 inoculum for experiments. Ammonium with final concentrations 1 & 2 mM was experimentally added separately for treatments. Nitrate was used with final concentrations 5 & 10 mM as other treatments. Culture medium BG110 was the control.

Analytical Methods

Growth was estimated as the increase in dry matter, as described by Leganés *et al.* [9]. Chlorophyll content was determined performing overnight extractions using 90% aqueous methanol. Centrifuged extracts were measured at 665 nm and calculated using the extinction coefficient of Marker [12]. Phycobiliproteins were extracted after osmotic shock and measured spectrophotometrically at 652, 615 and 562 nm.

Determination of Nitrogenase Activity

Nitrogenase activity was determined by acetylene reduction using Shimadzu GC-8 gas chromatograph. Prior to incubation, 10 % of the air inside the vial was replaced with the same volume of acetylene. Cells were incubated for 1 hour under the same conditions as they were cultured.

Oxygen Exchange

Oxygen exchange was measured with a Hansatech O_2 electrode. Two ml aliquots of cell suspensions were placed in water-jacketed, temperature-controlled cuvette and placed in dark or illuminated with a quantum flux density of 300 µmol photon m^{-2} s^{-1}(which was supplied with florescent lamps).

Statistical Analysis

Data are presented as the means and standard deviation of at least four replicates. Statistical differences were examined using the ANOVA test.

Results

Our results showed that maximum growth rate belonged to 5mM nitrate. Lower growth rates were demonstrated in N-free, 10 mM nitrate, 1 and 2 mM ammonium respectively. The growth rate of last treatment (2 mM ammonium) was almost zero (Fig. 1).

The effect of altered N sources on Chl can be seen in Figure 2. Maximum rate of chlorophyll belonged to N free medium (26.2±0.6 µg Chl mg dw^{-1}). But the difference was significant only with NH_4^+ (ANOVA, $P<0.05$). Inorganic nitrogen does not seem to change the chlorophyll significantly except mentioned case.

Table 1 describes the phycobiliproteins measured in *Fischerella* sp. FS18. The amount of PBP in the presence of NO_3^- (5 mM) was higher than in N free and NH_4^+ (Table 1). Differences were statistically significant (ANOVA, $P<0.05$) only with NH_4^+. Taking

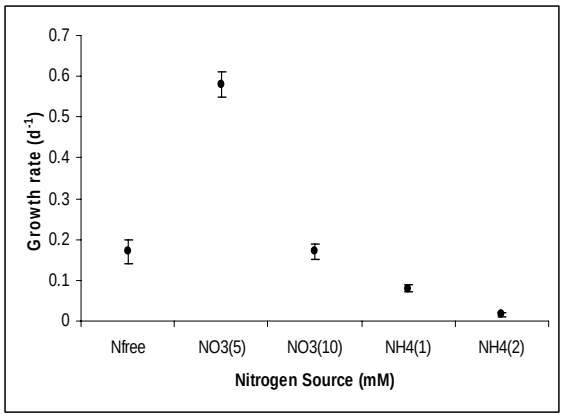

Figure 1. The effect of different nitrogen sources on growth rate of *Fischerella* sp. FS18.

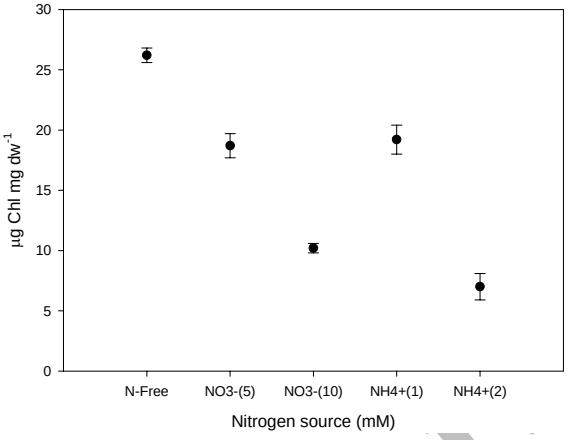

Figure 2. The effect of different nitrogen sources on chl. content of *Fischerella* sp. FS18.

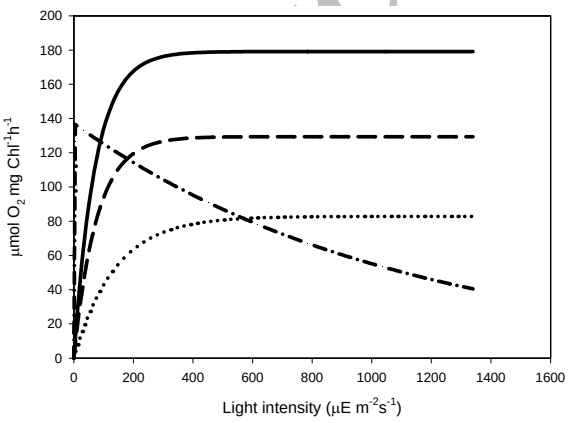

Figure 3. Photosynthesis versus irradiance curve of *Fischerella* sp. FS18 in different nitrogen sources. —— N free; – – – NH_4^+ (1 mM); ····· NO_3^- (5 mM); –·–·– NO_3^- (10 mM).

into account all three sources of N, the PC and PE contents followed the same trend than total PBP, with higher values at NO_3^-. The PC is the major biliprotein in *Fischerella* sp. FS18 (152.24±12.2 µg ml^{-1} in NO_3^- treatment).

The photodependence of C assimilation was investigated by mean of irradiance curve in all three nitrogen sources to analyze the functional significance of the altered pigment pattern referenced to chlorophyll (Fig. 2).

Figure 3 shows the highest P_{max} in N-free medium (179 µmol O_2 mg chl^{-1} h^{-1}). The lower obtained contents for NH_4^+ (1 mM), NO_3^- (5mM) and NO_3^- (10 mM) respectively (129, 82 & 137 µmol O_2 mg chl^{-1} h^{-1}). With increasing light intensity, in the case of 10 mM NO_3^-, photosynthesis rate decreased. The N-free medium also indicated the steepest initial slope (α=2.4 µmol O_2 mg chl^{-1} h^{-1}/µE $m^{-2}s^{-1}$). This medium seems to have the highest efficiency to use light. These data give a linear correlation of photosynthesis with nitrogenase (Fig. 4). The maximum rate of photosynthesis belonged to N-free medium completely matched to nitrogenase activity. This correlation was seen in all other cases.

The amount of combined nitrogen used in the above mentioned experiments was in the range of that normally supplied in most culture media, but it was however higher than those usually found in rice field [16]. Figure 4 shows the inhibitory effect of nitrate (10 mM) and ammonium (2 mM). To check this feature, lower concentrations of N sources were used. Nitrogenase activity was fully repressed in the presence of nitrate 5 mM and ammonium 1 mM (Data not shown). *Fischerella* sp. FS18 fixed 856 nmol ethylene mg dw^{-1} h^{-1} in N free medium. This ability disappeared in the presence of combined nitrogen.

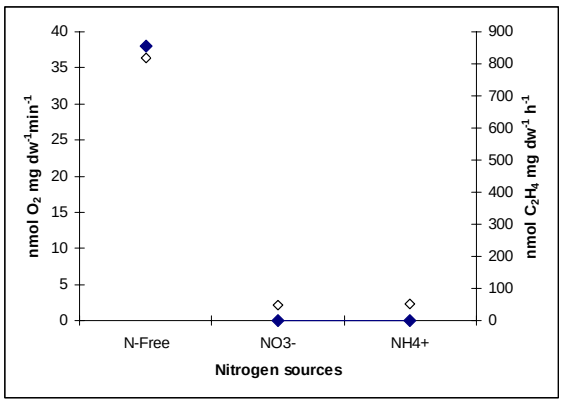

Figure 4. The effect of different nitrogen sources on Photosynthesis (◊) and nitrogenase activity (♦) of *Fischerella* sp. FS18.

Table 1. Phycobiliproteins contents in different nitrogen sources (μg ml^{-1}) in *Fischerella* sp. FS18

Parameter	N-Free	NO_3^- (5 mM)	NH_4^+ (2 mM)
PC	144.36±10.8	152.24±12.2	129.7±14
PE	60.54±2.2	126.5±5.4	74.51±8.7
PBP	247.7±10.8	278.7±17.5	220.9±20.7

Discussion

High frequency of *Fischerella* sp. FS18 were seen in paddy fields of Iran in the last researches [5, 23 and 24]. The adaptation of this unexplored strain to variable culture medium and ability to fix N_2 was the main aim of the present research. As nitrogen constitutes 5-10 % of dry weight of a cyanobacterial cell, physiological responses of this strain in different nitrogen sources were examined. Ammonium can be directly taken up from environment and cyanobacteria are in general able to grow with ammonium as N source, although nitrate is probably the most abundant source of combined N for their nutrition. But preference and selection of each N source is dependent of the strain.

Our results revealed that *Fischerella* sp. FS18 has the highest biomass in nitrate 5 mM. Ammonium seems to be more effective in decreasing the growth rate of this strain. The growth rate was almost zero in ammonium (2 mM), indicating its toxicity for this strain. Repression of growth with ammonium is concluded in other researches too. Lehtimaki *et al.* [10] and Rajini & Subramanian [19], for instance, showed decreasing of biomass of *Nodularia spumigena* and *Nostoc* sp. in the presence of ammonium respectively. Although based on logics of cell economy, ammonium should be the most preferred nitrogen source, but results gave nitrate as the best in *Fischerella* sp. FS18. This study confirms that the good or poor growth of cyanobacteria need not only be due to their efficiency to metabolize nitrogen but is actually the sum of the entire physiology and genetics of these organisms. Moore *et al.* [13] demonstrated the incapability of *Prochlorococcus* to use NO_3^- due to the lack of the genes required for NO_3^- uptake. Thus contrary to our finding, the ability of utilizing of NO_3^- is not a common feature among phytoplanktons.

In *Fischerella* sp. FS18, phycocyanin is the major biliprotein and occupies half of PBP approximately. These three nitrogen sources similarly influenced the phycobiliprotein composition of the phycobilisomes, the major light harvesting antennae. Taking into account all treatments, the amount of phycocyanin was diminished 5.3% & 15.1% in N-free medium and ammonium (2mM) respectively in comparison with NO_3^-. The concentration of phycoerythrin showed similar trend in altered nitrogen sources. The maximum amount of PE was seen in the presence of nitrate (5 mM). Regarding ammonium treatment, our finding of PE is in agreement with Liotenberg *et al.* [11]. In contrast, the observed changes in cell pigmentation are reminiscent of the phenomenon of complementary chromatic adaptation. Soltani *et al.* [24] has demonstrated similar variation in photosynthetic structure in cyanobacterium *Fischerella* sp. FS18, when light quantity was altered. These results demonstrated some common features of acclimation of this strain to spectral light quality and nitrogen sources.

Variation of chlorophyll was the same as photosynthesis. Our results also showed the higher photosynthetic activity in N-free medium. The highest rate of photosynthesis (P_{max}) and the steepest initial slope (α) in N-free grown cells revealed the highest efficiency of *Fischerella* sp. FS18 to fix carbon autotrophically in the limitation of light. Determination of P_{max} was impossible in ammonium (2mM) due to the high depression of growth. Also our other results, in the case of NO_3^- showed photoinhibition of photosynthesis. It seems that Increasing light seemed to decrease the rate of photosynthesis.

With regards to nitrogenase activity the highest rate was seen in N-free medium as well as photosynthesis. This was predictable from the same data seen in the case of nitrogenase activity. Normally carbon skeleton of nitrogen fixation supplies by photosynthesis. Maximum nitrogenase activity in N-free medium is in agreement with Leganes & Valiente [8] in the case of *Nostoc*, confirming the correlation between photosynthesis and nitrogenase. Consideration of high demand of energy for biological N_2 fixation can explain our finding. The highest nitrogen fixation rates were shown at the beginning of the exponential growth phase, and usually the nitrogen fixation rate decreased towards the stationary growth phase, may be due to physiological changes in the cultures.

Fields investigations revealed that lack of nitrogen fertilizer is the main limit to crop production globally. Cyanobacteria incorporate to nitrogen supply in paddy fields due to N_2 fixation. The relation between nitrogen and abundance of cyanobacteria is investigated in laboratory experiments [14]. Besides it has shown that nitrogenase activity decreases linearly as the amount of N fertilizer increases in paddy fields [2].

Our results showed the inhibition of nitrogenase activity in cells grown in the presence of nitrate and ammonium regardless of concentration used. These data are in agreement with Flores and Herrero [4] and Guerrero and Lara [6] who indicated that ammonium and nitrate repressed nitrogenase activity in laboratory

experiments. However, the amount of ammonium used in laboratory experiments is usually higher than that observed in most rice fields [18]. Also recently, Ernst *et al.* [3] published their work on two strains of *Synechococcus* spp. showing that nitrate with higher dose than that found in the natural environment, caused lower growth rate. Field studies indicated that at concentrations usually found in the field, nitrogenase activity is partially and in some cases not significantly affected by ammonium and nitrate [17].

These results are similar to the data concluded of experiments with nitrogen-fixing bacteria, in which nitrogenase activity is inhibited after adding ammonium. Decreases of heterocyst differentiation and percent of vegetative cells produced were also seen in the same experiment [7]. According to Reich *et al.* [20], the important role of pH in the inhibition of nitrogenase activity in *Anabeana variabilis* in the presence of ammonium, may be due to carbohydrate sources. Prosperi *et al.* [14, 15] determined the nitrogenase activity of four species in the presence of ammonium and nitrate. Data indicated that ammonium was more effective in inhibition of nitrogenase activity. The maximum contents of nitrate and ammonium in paddy fields were 1.5 and 0.25 mM respectively [16]. This study indicated the inhibition of nitrogenase activity after 24-48 h by ammonium.

In conclusion, our results indicate that *Fischerella* sp. FS18 is a diazotrophic species with a considerable potential to be used in biofertilizers. Nitrate (5, 10 mM) and ammonium (1, 2 mM) inhibit nitrogenase activity after three days. Nitrate 5 mM promotes growth and survival and ammonium seems to be toxic for this strain.

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