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Full Length Research Paper

A morphological, physiological and molecular investigation of *fischerella sp.* MCCS023 (cyanobacteria) from paddy-fields of Iran

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Fischerella ambigua is an important member of the cyanobacterial community in the rice field. In this study morphological, taxonomical variation in blue-green algae Fischerella ambigua was determined. In later stage they are treated with different nitrogen sources (N-free, 1 & 2 mM NH4+ and 5 and 10 mM NO3−). Results indicated that maximum growth rate belonged to NO3− treatment. Dry weights of Fischerella sp. MCCS023 in N-free medium and ammonium were in decreasing order of preference; and NH4 + 2 mM was drastically toxic for this strain and at last molecularly investigated. For Genetic variation, among rice-field, Fischerella isolates were studied using DNA extracted from single filament-derived cultures. Laboratory studies have shown that it is possible to use PCR to amplify individual Cyanobacteria filaments. As a result of these studies, Shiraz University was isolated for direct PCR and sequence analysis. Our results show that two distinct genotypes of Fischerella are present in the population at all stations and are not present in approximately equal numbers, they are not distributed uniformly.

Keywords: Ammonium, cyanobacteria, fischerella ambigua, Iran, nitrate, molecular investigation, paddy-fields, taxonomy.

INTRODUCTION

Heteterocystous cyanobacteria are the only group of organisms that are able to reduce nitrogen and carbon in aerobic conditions whose effect may be responsible for the evolutionary and ecological success (Prosperi, et al., 1992). Nitrogen-fixing cyanobacteria, the major component of microbial flora in rice paddy fields, are the main contributors to photo dependent N2 fixation (Roger & Kulasooriya, 1980).

It seems that in the north paddy fields of Iran, especially Golestan province, there is no clear report about their morphological characterization, taxonomic and molecular situation (Shokravi, et al., 2002).

Fischerella is one of the most diverse benthic N2 fixing cyanobacteria in paddy fields of Iran. This strain can be used commonly as biological nitrogen fixer. Genera of the stigonematales exhibit the highest degree of morphological complexity and differentiations and ratios seem to be a key factor in regulating cyanobacterial growth. Fischerella, as well as all cyanobacteria, can readily be utilize in organic nitrogen compound such as nitrate, nitrite and ammonium salts. Some species can also assimilate nitrogen (N2) from the atmosphere and certain species can use organic nitrogen compound (Perona, et al. 2003). Ammonium- nitrogen although energetically are the most favorable nitrogen, often supports poorer growth than does nitrate supplied

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at comparable level and cause cell lyses. Nitrate nitrogen is the most preferred nitrogen sources in most culture media (Soltani et al., 2006). Biological N2 fixation is the major replacement mechanism. Urea and in organic N sources other than ammonium are first metabolized to ammonium to allow assimilation of their N atoms.

However, a morphology-based classification may provide in sufficient taxonomic resolution and cyanobacteria with similar or identical morphology may have significantly different physiology.

Molecular markes have been used in previous studies of cyanobacteria phylogeny (Willmotte, 1994) and genetic diversity (Wood & Townsend, 1990). The application of molecular tools has the potential to resolve much of the controversy over cyanobacterial taxonomy evolution and the species concept. In comparison to other bacterial division, the 16s sequences have been used as targets for primer- directed DNA amplification by polymerase chain reaction (PCR) for identification of micro-organisms.

The aim of this study was to assess the taxonomic significance of the observed variation in trichome morphology by comparing it with nucleotide sequence variation to show the incorporation of different sources of nitrogen by heterocystous cyanobacterium Fischerella sp. MCCS023 and the correlation between nitrogen fixation, photosynthesis and growth rate with different contents of combined nitrogen as well as N- free medium to evaluate the adaptation of this strain to extreme conditions.

MATERIAL AND METHODS

Organism and culture conditions

In the present study filamentous, heterocystous cyanobacterium Fischerella MCCS023 was used. It was isolated from soils of paddy-field of Golestan province, Iran. Isolation and purification was performed by plating (Vonshak 1986). Stock cultures were grown in the N- free medium previously described (Soltani et al., 2006). Temperature was maintained at 30°C and cultures were bubbled with air under a constant light intensity of 60 µmol photon m-2s-1 supplied by three fluorescent tubes. Cells in logarithmic phase of growth were collected from stock cultures and used as inoculums for experiments. Ammonium with final concentrations 1 and 2 mM was experimentally added separately for treatments. Nitrate was used with final concentration 5 and 10 mM as other treatments. Culture medium was the control. Colony formation and cell shapes were evaluated by binocular and light microscope (in addition to phase contrast) in 2 weeks periods.

PCR amplification, cloning and sequence analysis of 16s rDNA

The template DNA for PCR amplifications of cultured material was prepared from approximately 10 mL of Fischerella filaments by suspending them in 5 µL of PCR buffer, 1 µL dNTP, 1.5 µL MgCl2, 2 µL primer forward and 2 µL primer reverse, 28.4 µL strile water. Reactions involved an initial denaturation step of 94C for 5 min then centrifuged at 12000 rpm. The supernatant was used as a template for PCR. The applied PCR condition has been described by Nubel et al. PCR products were electrophoresis in a 1% (w/v) agarose gel using TBE buffer containing 1µg/ml ethidium bromide. After that DNA was extracted from the gel using the core Bio Gel Extraction Kit. All PCR products were sequenced on both strands by the Cina Gene Company with the primers. Sequence similarity searches were done with BLAST through the website of the NCBI. The sequence of Fischerella sp strain MCCS 023 was recorded in the NCBI under the accession number FJ 392546.

Analytical Methods

Growth was estimated as the increase in dry matter, as described (Leganés et al., 1987). Chlorophyll content was determined performing over night extractions using 90% aqueous methanol. Centrifuged extracts were measured at 665 nm and calculated using the extinction coefficient of (Marker, 1972). 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 O2 electrode. Two ml aliquots of cell suspensions were placed in water-jacketed, temperature-controlled and cuvette were 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

Morphological variations and biometrical analysis

Morphological observations were made in liquid media. Thallus growth, filament structure, type of branching,

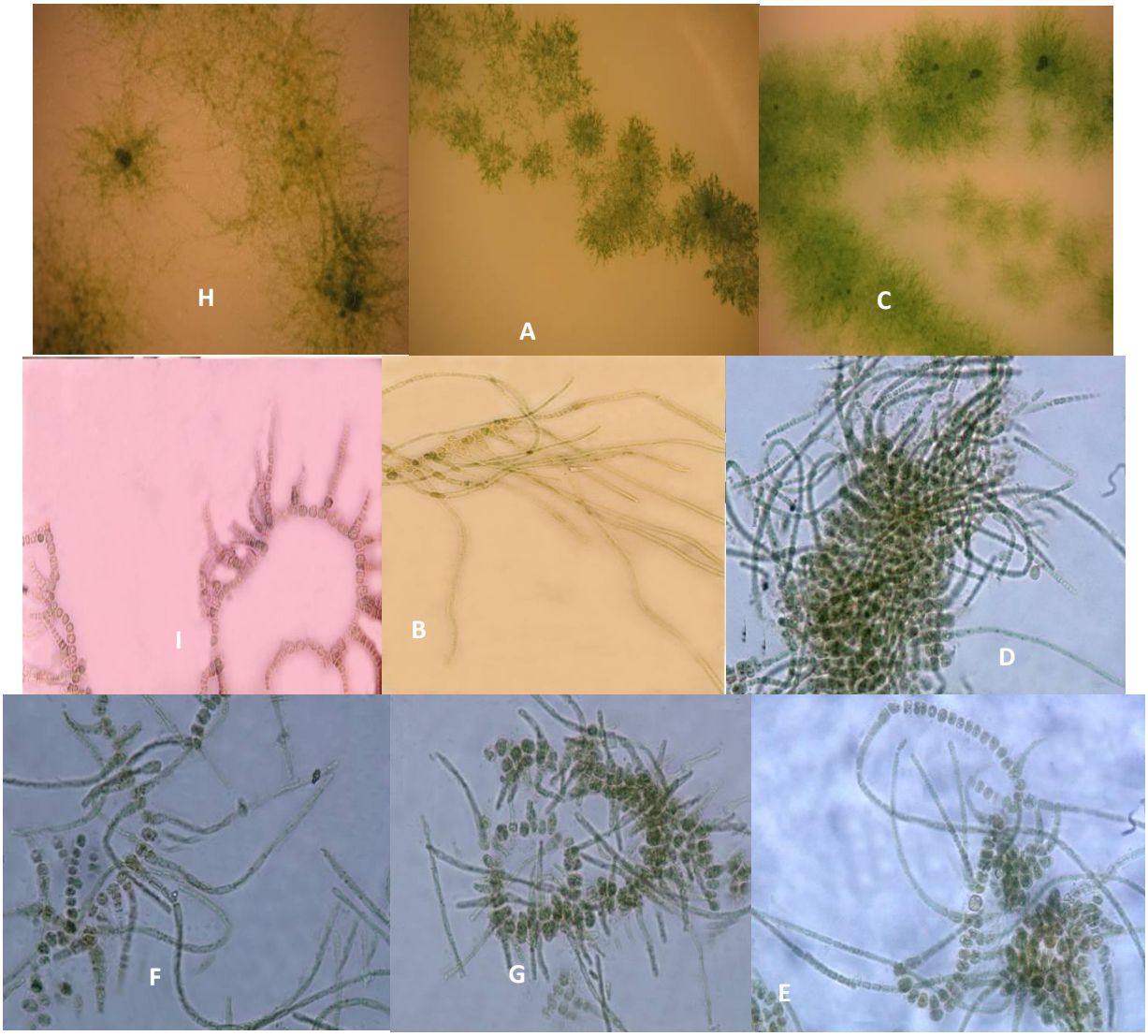

Figure1:A-I: phase contrast photomicrographs of *F.ambigua* , A-B in absence(-N), C- G- in NO3 grown filaments,H-I- in NH4 grown filaments.

position of the heterocysts, in addition to biometrical in formation were recorded (Gugger & Hoffmann, 2004, Shokravi, et al 2007). Colony formation and cells shape were evaluated by binocular and light microscope and phase contrast microscope each day in two week periods.

Microscopic observations in BG11 medium

Morphological observations with traditional approach strongly emphasize the preliminary identification. Filaments showed green color, delicate sheat around main axes and branches, vegetative cells were rectangular, elliptical and square shapes, main axis consist of single row of cell. Branches were short and rare and then long bilateral with distinct walls. Cells were square and narrower than the main axis. There is hormogonium. Branches cell in 16-17 days and heterocyst cell in 7-8-9-15-17 days were significant, that showed a different topological configuration. Figure 1: a, b and Table 1.

Morphological variations of Fischerella sp. MCCS023 can be related with N sources. Variations were seen in 5 mM nitrate. Specimens were green, vegetative cells showed different shape, square, spherical and cylindrical with lateral divisions, main axis in some specimen were uniseriate and other multiseriate, branches were very longer, two side and uniseriate , narrower than in axis with lateral divisions that were gradually attenuated without distinct wall . It seemed that in this condition,

Table1. Biometrical analysis *Fischerella*sp in liquid medium.
DVC, Dimension vegetative cell DHC, Dimension heterocyst cell
LVC, Length vegetative cell LHC, length heterocyst cell
DBC, Dimension branches cell DSC, Dimension spore cell
LBC, Length branches cell LSC, length spore cell

CELL	N- Free medium		Nitrate medium		Amonium medium	
	Min-day	Max-day	Min-day	Max-day	Min-day	Max-day
DVC	2.6 µ - 12	6.7 µ -12	3µ-9	7.13µ-5	2.5µ-8	5.5µ-7
LVC	3.2 µ -14	4.9 µ - 12,13	3.13µ-8	5.5µ-5	2.5µ-11	3.7µ-7
DBC	2.5 µ - 14	4.8 µ -1	2.8µ-9	5.33µ-8	2.7µ-11	4.16µ-7
LBC	2.5 µ - 8,9	6 µ - 6	3µ-8	7.6µ-9	4.5µ-6	5.1µ-8,9
DSC			5.2 µ-5	7.33µ-10	4.53µ-5	6.4µ-11
LSC			5.4µ-8	9µ-10	5.26 µ-9	7 µ-7
DHC	2.2 µ - 14	3.5 µ -11	3.16 µ -10	4.26 µ -6	2 µ -6	3 µ -11
LHC	5.4 µ - 6	8 µ - 8	5.33 µ -6	8.93 µ -11	2.6 µ -11	6.7 µ -10

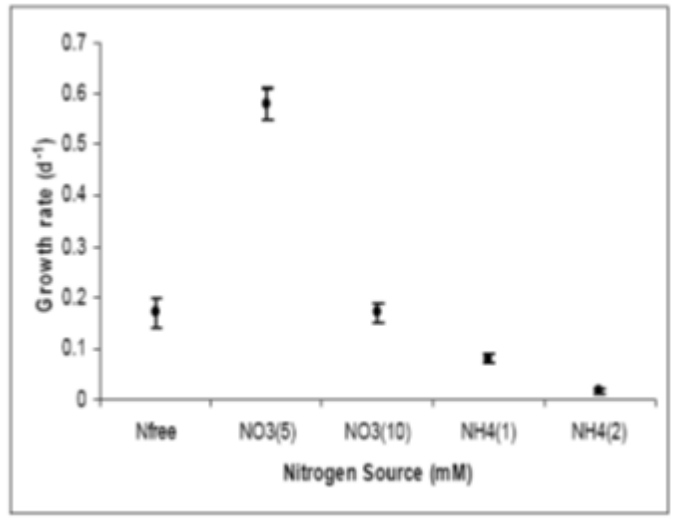

Figure 2. The effect of different nitrogen sources on growth rate of *Fischerella*sp. MCCS023.

organism tends seriously to get a new or at least different topological configuration.

Sporogenesis was high, dark green, spherical, square and bigger than vegetative cell, heterocyst were observed in different , cylindrical and rectangular shapes, in the case of length spore cell in 5-6-8-9-10-11 were significant Figure 1:c-g and Table1. In other conditions, 10 mM nitrate and 1 and 2 mM ammonium, growth was low and completely the same. In ammonium medium, we did not observe markable changes in this specimen aspect in heterocyst, that was low frequency and we did not observe difference significant among cells. Figure 1: h, i.

Physiological changes

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 was almost zero in 2 mM ammonium (Fig. 2). The effect of altered N sources on chlorophyll can be seen in Figure 3. 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 NH4+ (ANOVA, $P<0.05$). Inorganic nitrogen does not seem to change the chlorophyll significantly except mentioned case.

Table 2 describes the phycobiliproteins measured in Fischerella sp. MCCS023. The amount of PBP in the presence of NO3− (5 mM) was higher than in N free and NH4+ (Table 2). Differences were statistically significant (ANOVA, $P<0.05$) only with NH4 +. The photo dependence 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. 3).

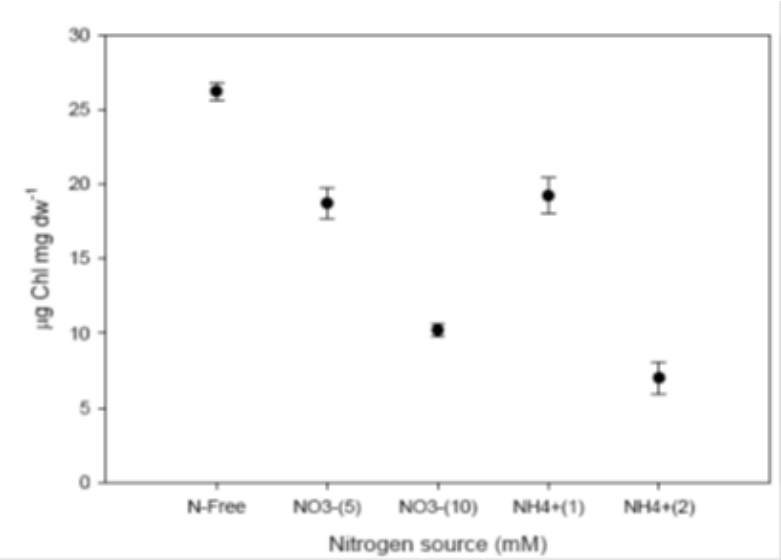

Figure 3. The effect of different nitrogen sources on chl. content of *Fischerella*sp. MCCS023

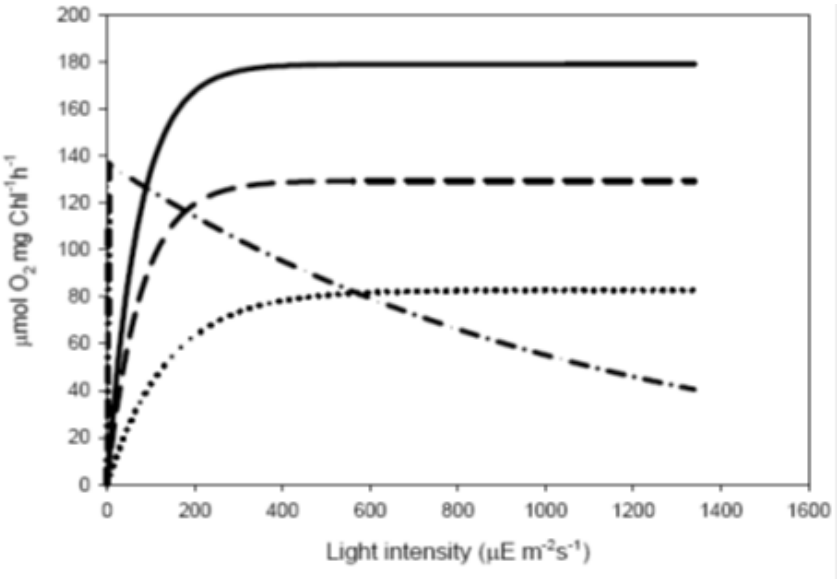

Figure4.photosynthesis versus irradiance curve of in different nitrogen sources.— N-Free;----NH^{4+}(1mM);…..NO^{3-} (5mM); -··· NO^{3-}(10mM).

Figure 4 shows the highest Pmax in N-free medium (179 µmol O2 mg chl−1 h−1). The lower obtained contents for NH4+ (1 mM), NO3− (5mM) and NO3− (10mM) respectively (129, 82 & 137 µmol O2 mg chl−1 h−1). With increasing light intensity, in the case of 10 mM NO3−, photosynthesis rate decreased. The N-free medium also indicated the steepest initial slope (α=2.4 µmol O2 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. 5). 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 higher than those usually found in rice field. Figure 5 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. MCCS023 fixed 856 nmol ethylene mg

Table2. Phycobiliproteins contents in different nitrogen sources (µg ml^{-1}) in *Fischerella* sp. MCCS023.

Parameter	N-Free	$N0_3^-$(5mM)	NH_4^+ (2mM)
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

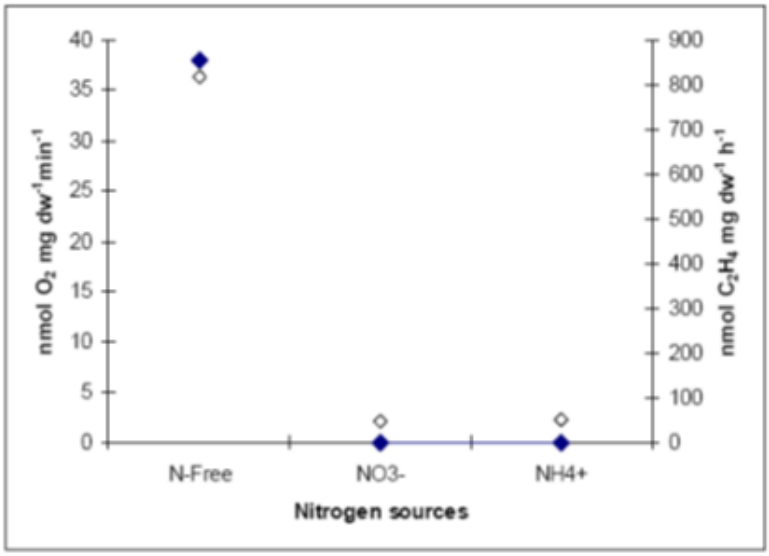

Figure5. The effect of different nitrogen sources on photosynthesis andnitrogenase activity *Fischerella*sp. MCCS023.

dw−1 h−1 in N free medium. This ability disappeared in the presence of combined nitrogen.

16s r RNA gene sequences

The partial sequence of the 16s r RNA sequence of the fischerella sp is as fallows:
´TGGGGAATTTTcCgAATGGGCGAAAGCCTGACGGAG
CAATACCGCGTGAGGGAGGAAGGCTCTTGGGTTGTA
AACCTCTTTTCTCAGGGAATAAGCAAGTGAAGGTACC
TGAGGAATCAGCATCGGCTAACTCCGTGCCAGCAGC
CGCGGTAATACGGAGGATGCAAGCGTTATCCGGAAT
GATTGGGCGTAAAGCGTCCGTAGGTAGCAGTGTGTG
TCTATTGTTAAAGAGTTTGGCTTAACCAAATAAAGGC
GGTAGAAACTACACAGCTAGAGTGCGTTCGGGGCAG
AGGGAATTCCTGGTGTAGCGGTGAAATGCGTAGAGA
TCAGGAAGAACACCGGTGGCGAAAGCGCTCTGCTAG
GCCGCAACTGACACTGAGGGACGaaaGctagGggAGC
GAATGGGATTAgataCCCCAgTAGT
The sequence of Fischerella sp. strain MCCS023 was recorded in the NCBI under the accession number FJ 392546.
Phylogeny
Comparing 16s rRNA sequences (Figure 6) with the gene bank (Htpp://www.ncbi.nlm.nih.gov/BLAST) showed the highest level of similarities with Fischerella sp. partial 16s rRNA gene, strain CR_18M, EF545608/1, center of investigation in cellular and molecular biology, laboratory of environmental microbiology and genetic prospection, University of Costa Rica (2007) Morales et al. and Uncultured cyanobacterium partial 16s rRNA gene, strain MPB14, EF429504/2. J. Ecology and Biodiversity. Environ. Microbiol. 9 (12), 3065-3076 (2007) Lacap and Pointing (99%) And narrow borders with the fallowing species:
Fischerellasp partial 16s - rRNA gene, strain MV9, DQ786169/1, J. Environ.Microbiol. 10(2), 460-473 (2008) Finsinger et al (97%).
Filamentous thermophiliccyanobacterium partial 16s rRNA gene, strain tBTRCCn101, DQ471442.1, Department of Plant and Environmental Sciences/the Hebrew University of Jerusalem, Ionescu et al. (2006).
However, results could be able to draw a relatively primitive molecular morphological and taxonomical situations Fischerella species in paddy fields of Iran.

DISCUSSION

High frequency of Fischerella sp. MCCS023 were seen in paddy fields of Iran in the last researches, (Ghasemi, et

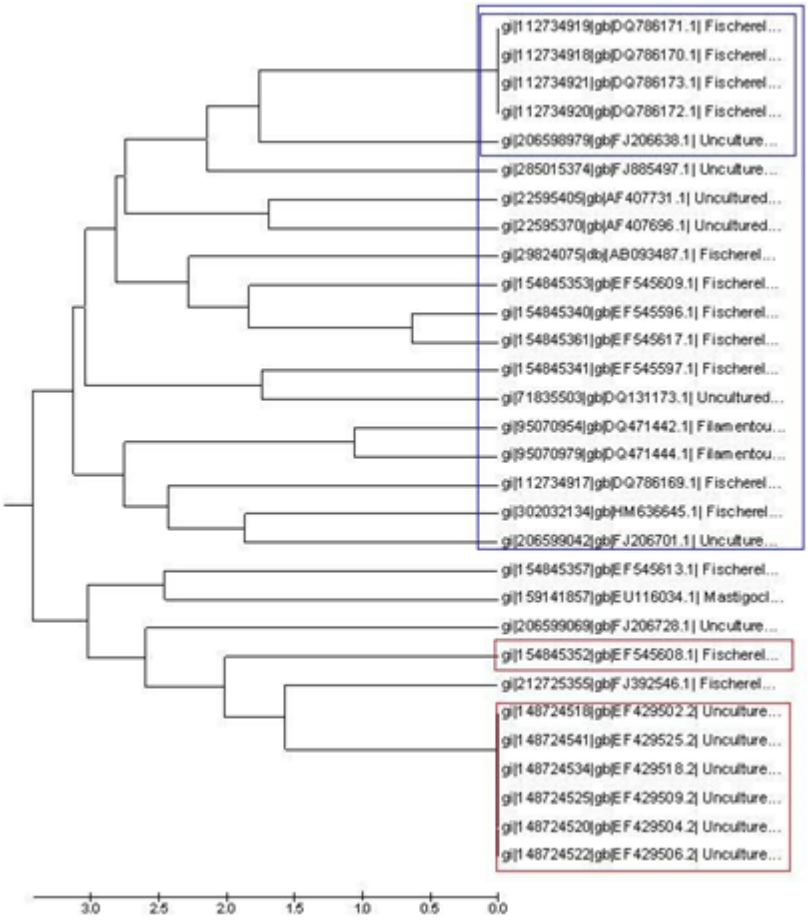

Figure6. Phylogenetic tree based on 16s rDNA sequence of Stigonematalean cyanobacterium including *Fischerella*sp MCCS023.

al. 2004). However, results could be able to draw a relatively primitive picture of the effect of N - sources in morphological analysis of the organism. This organism showed variable characters from morphological point of view and the morphological variation has shown many difficulties for the taxonomist. Nitrate (5mM) showed the points for stating the highest variation. In this condition there was a remarkable effect on the ability of germination and hormogonium production. It seems that the potential branch producing (especially main axes) increased sharply in these conditions. Cells contain granules in all growth cycle, the length of the branches increased; tapering at the end and hair development was reduced. In the case of cell dimensions, length and width of vegetative cells in the main axes increased. By statistical analysis, it is difficult to reach a unique pattern in morphological variation in vegetative cells of this strain, in this condition; Fischerella tends to get a different topological configuration. Growth rate decreased in nitrate 10 mM and 1mM ammonium and there is no growth in 2mM ammonium, indicating its toxicity for this strain. However results showed that this organism can be considered an alkalophilic organism. Optimal growth rates were observed at pH 7(Soltani et al., 2010).

Repression of growth with ammonium is concluded in other researches too (Lehtimaki et al., 1997) and (Rajini& Subramanian, 2007). 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. In Fischerella sp. MCC023, phycocyanin is the major biliprotein and occupies half of PBP content approximately .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., 1996.

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 (Pmax) and the steepest initial slope (α) in N-free grown

cells revealed the highest efficiency of Fischerella sp. MCCS023 to fix carbon autotrophically in the limitation of light. Determination of Pmax was impossible in ammonium (2mM) due to the high depression of growth. 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.

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. 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, 1994. 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 (Quesada, et al 1997). This study indicated the inhibition of nitrogenase activity after 24-48 h by ammonium.

Our results indicate that Fischerella sp. MCC023 is a diazotrophic species with a considerable potential to be used in biofertilizers. Nitrate (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.

CONCLUSION

Description of Fischerella sp. MCCS023

Fischerella sp. MCCS023 {Fischerella ambigua (Bornet & Flahault) Gomont 1895, P.52 }.

Filament with a delicate sheath, Trichomes coiled into a tight, closed helix, curved branches in witch one and two sides, cells rectangular, elliptical and square, usually loosely arranged in one to series in the main axis and in a single series only in the branches, heterocysts cylindrical and globose. Able to grow at N- combined sources (nitrate 5mM, 1mM ammonium). But the growth rate decreased with increasing nitrate (10mM) and ammonium (2mM). this variability was related with both nitrate and ammonium. High ammonium concentration had a remarkable inhibitory effect on the ability of germination. pc is the main component of phycobiliproteins, there was no growth of this species at ammonium , chlorophyll contents were very low at N- combined sources.

Fischerella sp strain is MCCS023, which was isolated from a microbial mat in a paddy –field near Gorgan, Golestan, Iran and has been deposited in the Iranian Microbial Culture. The DNA sequence of strain was recorded in the NCBI under the accession number FJ392546.

Fischerella sp. partial 16S rRNA gene, strain MCCS 023.

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