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Physiological variability in cyanobacterium *Phormidium* sp. Kützing ISC31 (Oscillatoriales) as response to varied microwave intensities

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The influence of microwave radiation on physiological behaviors of *Phormidium* sp. Kützing ISC31 (Oscillatoriales) was investigated. The organism grown in BG-11 medium was microwave-treated at a frequency of 2450 MHz using a microwave oven. Fifteen (15) microwave pretreatments were established, combining five intensities (180, 360, 540, 720 and 900 W/cm^2) and three periods of pretreatment [10, 20 and 30 second(s)]. Results revealed that samples exposed to microwave various intensities showed significantly higher growth rates and biomass than that of non-irradiated controls. The content of chlorophyll a, which exists in the thylakoid membrane, decreased with increase in field strength and duration of exposure. Synthesis of the phycobiliproteins (PBP), phycocyanin (PC), phycoerythrin (PE) and allophycocyanin (APC), except in 720 & 900 W/cm^2(30s), increased in all exposures as compared to that of control Photosynthetic activity rate compared to nitrogenase activity increased in all microwave exposures except in 180W (10s) and 720W (10s). Identification was carried out by molecular method. The result of PCR blasted with sequenced cyanobacteria in NCBI showed 97% homology to the 16S rRNA of *Phormidium* sp. This study revealed that various microwave intensities induce different physiological effects, depending on field strength and duration of exposure.

Key words: 16S rRNA gene, PCR, *Phormidium*, Microwave treatment, Physiological characteristic, Paddy field

Introduction

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Life on earth has evolved in a sea of natural electromagnetic fields (EMFs). Over the past century, this natural environment has sharply changed with introduction of a vast and growing spectrum of man-made electromagnetic fields. From models based on equilibrium thermodynamics and thermal effects, these fields were initially considered too weak to interact with biomolecular systems, and thus incapable of influencing physiological functions. Since the 18th century, scientists have been intrigued by the interaction of EMFs and various life processes. Attention has been focused on EMFs in various intensities and frequency ranges, of which microwave intensity and frequency range forms an important part. Several biological effects of the direct exposure of biosystems to microwaves have been reported (Rai *et al*., 1994a,b; Rai *et al*., 1999a,b). Microwaves are part of the electromagnetic spectrum and are considered to be that radiation ranging in frequency from 300 million cycles per second (300 MHz) to 300 billion per second (300 GHz), which correspond to a wavelength range of 1 m to 1 mm. This non-ionizing electromagnetic radiation is absorbed at molecular level and manifests as changes in vibration energy of the molecules or heat (Banik *et al*., 2003). Identifying and evaluating the biological effects of microwaves have been complex and controversial. Microwaves have been reported to cause thermogenic and athermal bioeffects, which were found to vary depending on far-field versus near-field location, power density, duration, frequency, polarization, modulations, pules etc. There is, however, little information on bioeffects of microwaves on microorganisms. The present study is an attempt to identify the effects of microwave irradiation on biologic systems, especially cyanobacteria. Cyanobacteria are a widely distributed group of photosynthetic prokaryotes and can be found in aquatic as well as in terrestrial ecosystems (Whitton, 2000; Potts, 2000; Qiu *et al*., 2002). Due to cyanobacteria ability to fix atmospheric nitrogen into ammonium with the help of enzyme nitrogenase, some of these organisms play a vital role in nature to enrich soil fertility, particularly in rice paddy fields as a natural biofertilizer (Sinha *et al*., 1996; Zulpa *et al*., 2008; Pereira *et al*., 2009). Although cyanobacteria have been shown to be resistant to a variety of environmental stress factors such as heat, drought, salinity etc (Pócs, 2009), it has been shown that electromagnetic radiation affects cyanobacteria in many different ways including pigmentation, motility, photosynthesis and nitrogen fixation (Singh *et al*., 1994; Rai *et al*., 1999a,b). During their long evolutionary history cyanobacteria have developed many strategies to protect themselves from excessive electromagnetic radiation including active avoidance of brightly light habitats or the production of electromagnetic waves-absorbing compounds, for example, mycosporine-like amino acids (MAAs) and/or scytonemin (Sinha *et al*., 2002). The present study examines the changes in

growth, pigmentation, photosynthesis activity and nitrogenase activity of cyanobacterum *Phormidium* sp. Kützing ISC31 (Oscillatoriales) isolated from the paddy fields in Iran, while they are exposed to different intensities and duration of microwave radiation. Since cyanobacteria are strongly influenced by environmental stimuli morphologically, it has been difficult to classify cyanobacteria in appropriate taxonomic groups. For example, many species of the genera *Oscillatoria, Lyngbya, Phormidium, Schizothrix, Plectonema* were included in *Schizothrix calcicola* (Turner *et al*., 2001), which was originally classified on the basis of sheath characterization and the presence or absence of false branching. Accordingly, this strain was identified by 16S rRNA gene partial sequencing. Oxygenic photosynthetic prokaryotes, cyanobacteria and prochlorophytes are genetically related on the basis of 16S rRNA sequences (Urbach *et al*., 1998; Casamatta *et al*., 2003; Ezhilarasi and Anand, 2009). Cyanobacterial genera namely *Anabaena, Nostoc, Phormidium, Microcystis, Synechococcus* and *Synechocystis* have been analyzed using molecular techniques such as DNA sequencing (Neilan *et al*., 1995; Ezhilarasi and Anand, 2009). 16S rRNA gene sequences have now become the most widely used methods for identification, classification and phylogeny of cyanobacteria (Nübel *et al*., 1997; Crosbie *et al*., 2003; Salomon *et al*., 2003; Premanandh, 2006).

Materials and methods

Organism and culture conditions

The soil cyanobacterium, *Phormidium*, used in this research was obtained from microalgal culture collection of ACECR, RIAS, for screening their physiological and molecular identification. This species was isolated from paddy fields in Iran. Soil samples were cultured by usual methods (Andersen, 2005). Cyanobacterium was grown in 250 mL conical flasks containing 100 ml of BG-11 medium adjusted to pH 7.4. The cultures were illuminated continuously (50 $\mu Em^{-2}s^{-1}$) supplied by six fluorescent lamps and following incubation at 30 ± 1ºC. Preliminary identification of cyanobacterium was done according to Desikachary (1959) and John *et al*. (2003). Exponentially growing cyanobacteria (14-day old cultures) were used for experiments.

DNA extraction, PCR amplification and sequence analysis of 16S rRNA

The genomic DNA was extracted using the method described by Sambrook *et al*., (2001). The PCR reaction was performed with universal primers (CYA106F: 5`-CGG ACG GGT GAG TAA CGC GTG A-3` and CYA781R (b): 5`-GAC TAC AGG GGT ATC TAA TCC CTT T-3`) specific for the 16S rRNA gene (Nübel *et al*., 1997). Amplification was performed on a

Programmable Thermal Controller (CR CorBett Research, USA) as it has been described by Nübel *et al*. (1997). PCR amplified products were subjected to 1.5% (w/v) agarose gel using TBE buffer stained with 6 µg/ml DNA safe stains. For photo documentation, an Uvi-DOC BTX-20-M, EEC system with MITSUBISHI P91E software was used. PCR products were purified with the Real clean Spin Kit. Automated sequencing was determined using the TAG-Copenhagen Company with primers. The sequence data was analyzed using a similarity search by using the BLAST through the website of the NCBI. The nucleotide sequences described in this study have been submitted to the NCBI under the accession number NCBI: GU138682.

Microwave treatment

For organisms grown in BG-11 medium, sixteen flasks were marked. One flask was marked for control, and the remaining 15 were assigned for microwave treatment. The organisms were microwave-treated at a frequency of 2450 MHz, combining two variables: five intensity levels (180, 360, 540, 720 and 900 W/cm^2) and three times of exposure [10, 20 and 30 second(s)], using a microwave oven (LG, model CC-4284TCR). The distance between magnetron lamp and medium was 23 cm in each case. The experimental samples were shaken by hand after treatment to ensure a uniform distribution of temperature. After treatment, they were kept in culture room. The physiological parameters were recorded after 2 days of incubation. Each one of the fifteen treatments was carried out in triplicate.

Analytical methods

Growth rate was estimated as biomass yield and was determined by the cell dry weight as described by Leganés *et al*. (1987). Culture density was determined turbidometrically at 750 nm (OD_{750}) with a spectrophotometer (Lightwave (WPA) S2000 UV/Vis Spectrophotometer version 2.6). For chlorophyll a determination, 1 mL of sample was extracted for 24 hour in total darkness using 90% aqueous methanol and centrifuged at 10000 rpm for 5 minutes. Chlorophyll a content was spectrophotometrically measured at 665 nm and concentration was calculated using the extinction coefficient of Marker (1972). Phycobiliproteins were extracted in 1 mL of cell suspension by the method of osmotic shock, modified by Soltani *et al*. (2006) after Wyman and Fay (1986) and measured spectrophotometrically at 652, 615 and 562 nm.

Nitrogenase activity measurements

Nitrogenase activity was determined by acetylene reduction technique. 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. After incubation 0.5 mL of gas samples were taken and ethylene concentration was determined in a Shimadzu GC-15A gas chromatograph as nmol ethylene/mg dry weight hour.

Photosynthetic activity measurements

Oxygen evolution was measured with a Clark-type O_2 electrode in a Chlorolab oxymeter (Hansatech Instruments, Norfolk, UK). Two mL aliquots of cyanobacterial cell suspensions, with a cell density of 0.3 mg/mL, were placed in an airtight cylinder-shaped cuvette (DW2/2, Hansatech Instruments, Norfolk, UK) with magnetic stirring and dark adapted for 30 min before the light source was switched on at growth temperature. Photosynthetic activity of treated and untreated cyanobacterial cells was estimated by measuring O_2 evolution for 2 minutes and expressed as nmol O_2 evolved/ μg chlorophyll hour.

Statistical analysis

All experiments were repeated three times. Data are means of triplicate tests ± SE. Statistical differences were examined using SPSS software.

Results

When *Phormidium* sp. ISC31 was treated with various microwave intensities and duration of exposure, distinct effects were seen on growth and metabolic characteristics. Our results revealed that there is a significant difference in the group means of growth rate and dry weight at 0.05 levels (Figs. 1, 2). The maximal increase of biomass yield measured in treated cells was higher than 3.936 mg/mL, which was similar for all exposures (Fig. 1). Exposure of *Phormidium* sp. ISC31 cells with various microwave intensities showed that specific growth rate was higher at low intensities. Also, Marked inhibitory effect was observed in 900W (20s) and 900W (30s) treatments (Fig. 2).

Effect of five microwave intensities and three duration of exposure on pigment contents of *Phormidium* sp. ISC31 grown under the above conditions are indicated in Fig. 3. Chlorophyll a content of cells treated with various microwave intensities showed a but significant (ANOVA, $P<0.05$) decrease in comparison to control (from 0.362 to 1.267 μg/mg dw relative to 1.318 in control).

Total phycobiliproteins (PBP) were affected by various microwave intensities (Fig 3). The effect of all tested exposures on total PBP were significantly (ANOVA, $P<0.05$) higher than from controls values (from 5.314 to 15.555 µg/mg dw relative to 2.926 in control). After 720W (30s) and 900W (30s) exposures, PBP synthesis was almost inhibited when compared to control values (0.870 and 0.384 µg/mg dw, respectively, 2.926 µg/mg dw).

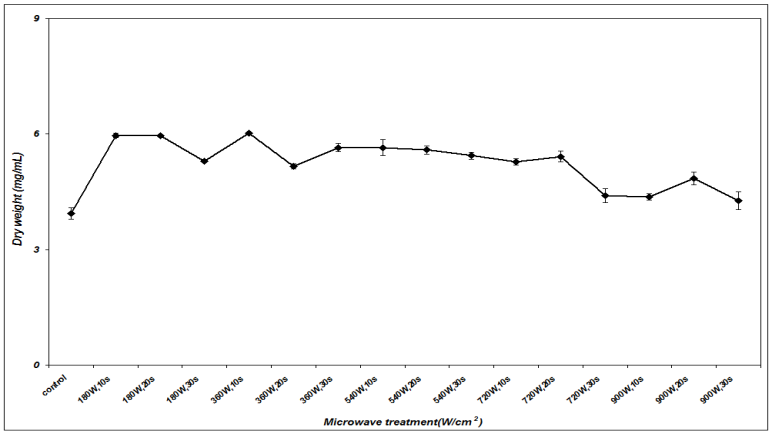

Fig. 1. Variation of dry weight in the cyanobacterium *Phormidium* sp. ISC31 grown under various microwave treatments. (Data are mean values of three experiments±SE).

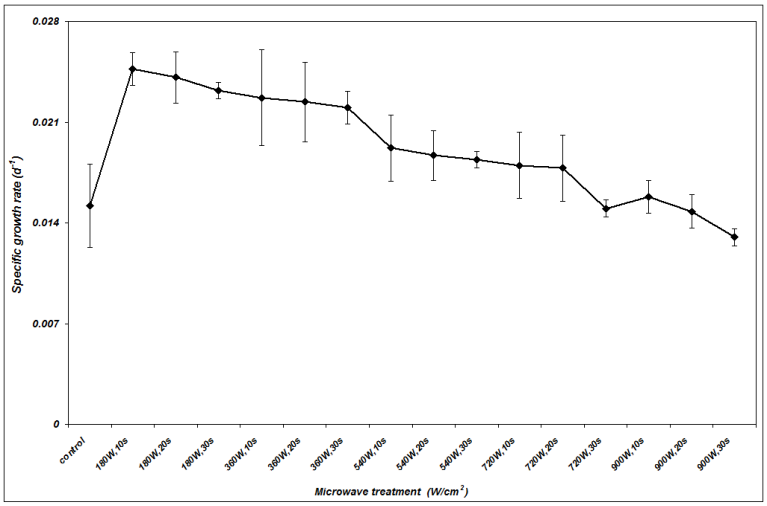

Fig. 2. The effect of various microwave treatments on the specific growth rate in the cyanobacterium *Phormidium* sp. ISC31 (Data are mean values of three experiments±SE).

In *Phormidium*, phycoerythrin (PE) is the main component of phycobiliproteins. Incubation in various microwave treatments induced a significant (ANOVA, $P<0.05$) increase in the PE content (Fig. 3). After 720W (30s) and 900W (30s) exposures, the content of this pigment in *Phormidium* sp. ISC31 cells decreased (0.430 and 0.306 µg/mg dw, respectively, in comparison to control, 2.710 µg/mg dw). A allophycocyanin (APC) is a component of the core of phycobilisomes, and the core remains constant, so a change in APC content reflects a change in the number of phycobilisomes. In *Phormidium* sp. ISC31 APC content was significantly (ANOVA, $P<0.05$) affected by microwave (Fig. 3). Total APC accumulation was increased at all exposures except 720W (30s) and 900W (30s) treatments. The highest APC content was shown by cells grown at 360W (30s) treatment (1.739 µg/mg dw). The effect of all tested exposures on total phycocyanin (PC) was equal to PBP, APC and PE contents. Overall, the total PBP, APC, PC and PE concentrations of *Phormidium* sp. ISC31 cells increased significantly (ANOVA, $P<0.05$), but after 720W (30s) and 900W (30s) exposures, synthesis of these compounds were almost inhibited when compared to control values (Fig. 3).

Phycobilisomes can exhibit a high sensitivity to variation of microwave. Transfer of energy within these additional pigments follows the path from phycoerythrin to phycocyanin to allophycocyanin to the long-wavelength pigment (Mimuro *et al*., 1986). In this study, the variability of phycobilisome size and structure was examined. The size of phycobilisomes can be usually represented by the ratio (PE (when present) +PC/APC). The size of phycobilisomes (by elongation of the phycobilisome rods) in *Phormidium* sp. ISC31 decreased significantly ($P<0.05$) (Fig. 4). Therefore, microwave treatment caused to increase PBP, APC, PC and PE contents and decrease PC+PE/APC ratio.

The ratio PBP/chlorophyll a is usually used to quantify the relationship between PSII and PSI (Yamamaka and Glazer, 1981). The ratios of PBP/chlorophyll a and PE/ chlorophyll a significantly ($P<0.05$) increased at all exposures except in 720 (30s) & 900W (30s) exposures. Also, the ratios of APC/ chlorophyll a and PC/ chlorophyll a (except for 900W (30s))significantly ($P<0.05$) increased at all exposures. So it seems that the photosynthetic apparatus of *Phormidium* sp. ISC31 is affected by changes in intensity and duration of exposure with microwave (Fig. 4).

We evaluated nitrogen-fixing rates under microwave treatment, as cyanobacteria are capable of fixing the atmospheric nitrogen (Steward, 1980) and their abundance in rice field soils has been proved to be of significant in rice growing Asian countries (Venkataraman, 1981). The effect of various microwave exposures on nitrogenase activity of *Phormidium* sp. ISC31 are

shown in Fig. 5. The results revealed microwave treatment significantly ($P<0.05$) suppressed nitrogenase activity. Photosynthetic oxygen evolution significantly increased ($P<0.05$) after various microwave exposures (Fig. 6). After 180 W (10s) and 720 W (10s) intensities, photosynthetic oxygen evolution was reduced when compared to control (0.121.57 and 152.20, respectively, in comparison to control, 167.92 nmol O_2 evolved/ µg chlorophyll a hour).

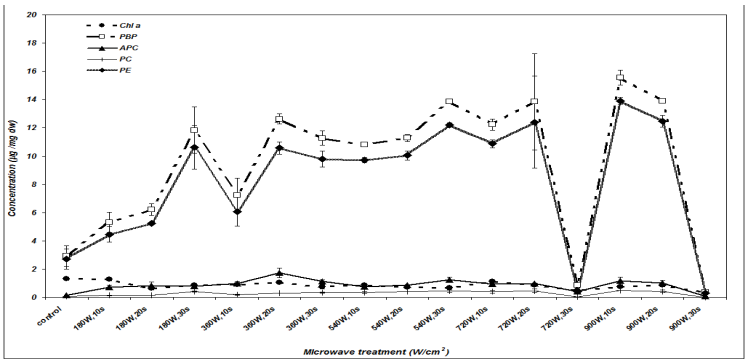

Fig. 3. Effect of microwave exposures on pigment contents of *Phormidium* sp. ISC31 grown for five days.(Data are mean values of three experiments±SE). Chl a-Chlorophyll a; PBP-Phycobiliproteins; APC-Allophycocyanin; PC-Phycocyanin; PE-Phycoerythrin.

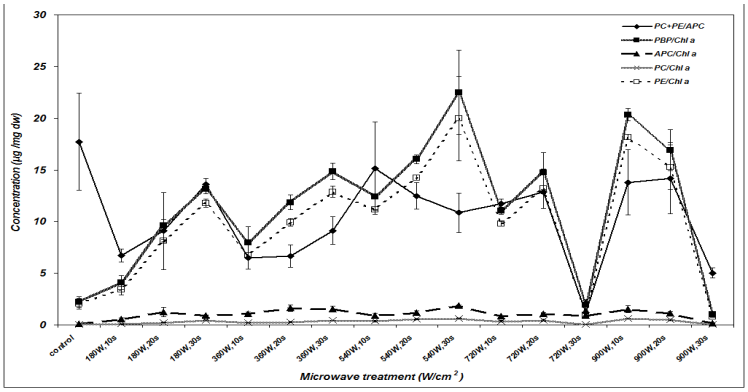

Fig. 4. Effect of microwave exposures on PC+PE/APC, PBP/Chlorophyll, APC/Chlorophyll, PC/Chlorophyll and PE/Chlorophyll ratios of *Phormidium* sp ISC31.(Data are mean values of three experiments±SE). Chl a-Chlorophyll a; PBP-Phycobiliproteins; APC-Allophycocyanin; PC-Phycocyanin; PE-Phycoerythrin.

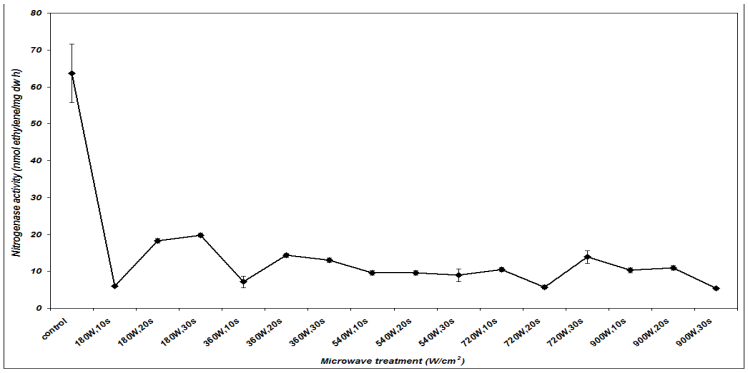

Fig. 5. Maximal nitrogenase specific activity in cells *Phormidium* sp. ISC 31under effect of microwave exposures .**(**Data are mean values of three experiments±SE).

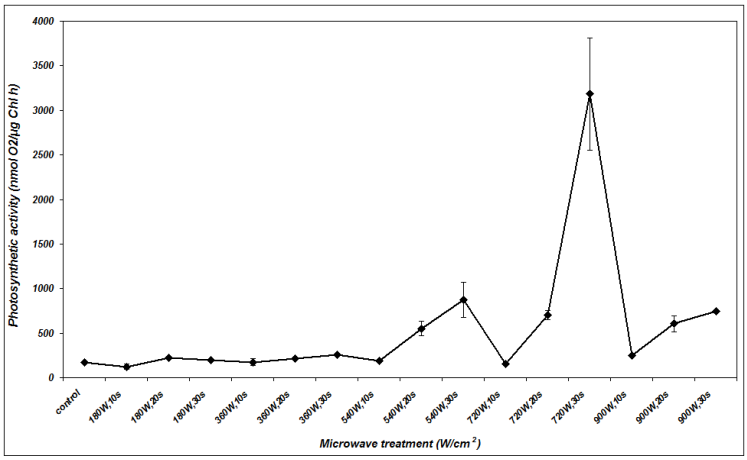

Fig. 6. Photosynthetic activity in cells *Phormidium* sp. ISC 31under effect of microwave exposures .(Data are mean values of three experiments±SE).

The sequence of the 16S rRNA gene was determined for *Phormidium* sp. ISC31. The sequences were compared with those of representative non-heterocystous *(Phormidium)* cyanobacteria available in GenBank (http://www.ncbi.nlm.nih.gov/BlAST). The 16S rRNA sequences were combined with other *Phormidium* species available in the database (Casamatta *et al*., 2003; Ezhilarasi and Anand, 2009). 16S rRNA gene sequence similarities of 97% within *Phormidium* sp. were observed. The nucleotide sequences described in this study have been submitted to the NCBI under the accession number NCBI:GU138682.

Discussion

Our results revealed that samples exposed to microwave various intensities for 10, 20 and 30 second showed significantly higher growth rate and biomass than non-irradiated controls (Figs. 1,2). The higher intensities seem to be more effective in decreasing the growth rate and biomass of this strain. Clearly, obtained results provide evidence that various microwave exposures were effective to *Phormidium* sp. ISC31. Data obtained in the present investigation revealed that *Phormidium* sp. ISC31 growth, expressed as dry weight and specific growth rate, was susceptible to microwave. Pakhomov *et al*. (2001) reported that exposure for 30 min at 2.2 mW/cm^2 and 7.1 mm wavelength enhanced the growth of blue-green algae *Spirulina platensis* by 50%. There was a different pigmentation response to the microwave treatments (Fig. 3). The levels of chlorophyll a in *Phormidium* sp. ISC31 cultures exposed to various microwave intensities clearly demonstrated that this treatment had effect on this photopigment. In *Phormidium* sp. ISC31, phycoerythrin is the major biliprotein and approximately occupies half of PBP. Microwave treatments similarly influenced the phycobiliprotein composition of phycobilisomes, the major light harvesting antennae. Taking into account all treatments, the amount of APC, PC and PE were increased except in 720 and 900W (30s) exposures. According to different strategies of adaptation of photosynthetic apparatus by irradiance (Reuter and Müller, 1993), it seems that *Phormidium* sp. ISC31 modulate the number and the size of phycobilisomes. The observed changes in cell pigmentation are reminiscent of the phenomenon of complementary chromatic adaptation. The PBP/Chl a, APC/Chl a, PC/Chla and PE/Chl a ratios in cells exposed to microwave significantly increased as compared with the control (Fig. 4). The external localization of PBP on intracellular thylakoid membranes might be a possible reason for a increasing effect of microwave on PBPs, because they are more exposed to the action of microwave irradiations. Increase of growth rate, phycobiliproteins contents and photosynthetic activity and decrease of chlorophyll a concentration and nitrogenase activity in present study showed that these effects might be an adaptative mechanism of *Phormidium* sp. ISC31 under various microwave intensities. In light of this, it may be suggested that the various microwaves intensities caused the bio-effects by differentially altering the structural chemistry of the nutrient solution (Feseno and Gluvstein, 1995; Singh *et al*., 1994; Singh *et al*., 1996). Singh *et al*. (1994) athermal physiological effects of continuous waves and modulated microwaves studied on a cyanobacterium *Nostoc muscorum*. The study showed that microwave different frequencies in continuous waves and modulated modes significantly showed different physiological effects on the algae. Water mediated bio-effects further presented

additional proof that water had the capability to remember the imposed electromagnetic field characteristics for an extended period of time. The effect of microwave modulated with square wave of different pulse repetition frequency was studied on physiological behavior of the cyanobacterium *Anabaena dolilum* by Samarketu *et al*. (1996). The study revealed that microwaves induced different biological effects by changing the structures by differentially partitioning the ions, altering the rate and/or direction of biochemical reactions and thereby pigmentation and nitrogenase activity affected (Singh *et al*., 1994; Rai, 1997; Rai *et al*., 1996). Low intensity of microwave has been found to modify behavior without modifying the core temperature of experimental subjects (Banik *et al*., 2003). Rai *et al*. (1999) suggested that the microwave exposures caused these thermal and athermal physiological effects by differentially changing the structural chemistry of the cyanobacterium's "live water" (i.e., water taking part in different biologic activities) and of the nutrient solution (Rai *et al*., 1999a,b). The various microwave intensities-dependent water structures might have induced the effect by partitioning the ions in the rank order of a Hofmeister series between and among the cyanobacterium's "live water" and the medium, changing the rate and/or direction of biochemical reactions, etc (Rai *et al*., 1999a,b).

This study revealed that various microwaves intensities could induce different physiological effects perhaps by changing the structure of water in cyanobacterium *Phormidium* sp. ISC31.

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