

FREEZE RECOVERY AND NITROGENASE ACTIVITY IN ANTARCTIC CYANOBACTERIUM *NOSTOC COMMUNE*

Rajan Kumar Gupta

¹Department of Botany, Pt. L.M.S. Govt. P.G. College, Rishikesh 249201 (Dehradun), Uttarakhand, India
e-mail: rajankgupta1@rediffmail.com

Abstract. Cyanobacteria, especially terrestrial forms, are exposed frequently to alternating freeze-thaw cycles during the early spring and late fall seasons in Antarctic regions. However, the question of how cyanobacteria respond to freezing has received little attention. In order to study the freezing recovery and its impact on Nitrogenase activity in the dominant cyanobacterium in the region *Nostoc commune* was cultured and propagated on sand with aqueous N-free BG-11 medium. Laboratory experiments were conducted to characterize the *in vivo* freeze recovery physiology of nitrogenase activity. Nitrogenase activity was monitored by the acetylene reduction activity. Frozen *Nostoc* mats were thawed and warmed to 2,4,6,8,10,20, or 25°C, nitrogenase activity was detected within 6h after thawing. Optimum thawing temperature with respect to the recovery of nitrogenase activity was 20°C. In Subsequent experiments, laboratory grown *Nostoc* mats were used along with the following conditions : prefreezing treatment of 3d of exposure to light or darkness, freezing and then thawing to 20°C in light or darkness, with or without DCMU or chloramphenicol. Approximately 25% of the energy in the initial recovery of nitrogenase activity (up to 12 h after thawing) appear to be supplied via the utilization of carbon compounds stored before freezing. Photosynthetic condition (i.e., light and without DCMU) were necessary for maximum recovery of nitrogenase activity. In the presence of protein synthesis inhibitor chlormphenicol, nitrogenase activity was still detected at 12 to 48 h after thawing. Although damage may occur to nitrogenase, some of the enzyme was capable of surviving the freeze-thaw period *in vivo*. However, complete recovery of nitrogenase activity (equal to prefreezing activity) may entail some *de novo* synthesis of nitrogenase. This work is supported by the Department of Ocean Development, New Delhi and NCAOR, Goa, India by providing me an opportunity to visit the Antarctic field during XIth and XIVth Indian Scientific Expeditions to Antarctica and collect the samples.

Key words: Cyanobacteria, Antarctica, , Nitrogenase, *Nostoc*

1. Introduction

Cyanobacteria, especially terrestrial forms, are exposed frequently to alternating freeze-thaw cycles during the early spring and late fall seasons in Antarctic regions. However, the question of how cyanobacteria respond to freezing has received little attention. Ono and Murata (1981 a, b) investigated the effect of chilling on the photosynthetic activities of *Anacystis nidulans*. They noted that both the dark reactions and primary photochemical reaction are damaged by chilling treatment. They correlated the chilling susceptibility with the fluidity of membrane lipids. Increased membrane permeability, as measured by leakage of potassium ions and other ions from the cytoplasm to the surrounding medium, was attributed to an altered state of cytoplasmic lipids at chilling temperatures (Ono and Murata 1982). This loss of ions then resulted in an inactivation of photosynthesis.

The effects of freezing on nitrogenase activity has been only studied with nitrogenase extract preparation . Haystead *et al* (1970) showed that nitrogenase activity in extracts of *Anabaena cylindrica* rapidly decreased when incubated at 0°C. After 12d at 0°C, there was 61.7% inhibition of nitrogenase activity when compared with control samples (20°C). In initial studies on crude extracts of *Clostridium* sp, Dua and Burris (1963) reported an inactivation of approximately 85% after 12 at 0°. Zumft and Mortenson (1975) have the data on the cold lability of nitrogenase (specifically the azoferodoxin protein component).

They postulated the possibility of structural changes in protein at lower temperature. There have been no reports describing the effects of freezing on *in vivo* nitrogenase activity in cyanobacteria or the ability of *in vivo* nitrogenase activity to recover from freezing.

In this study, we examined the recovery response of *in vivo* nitrogenase activity after a freeze period as to the energy source(s) involved, recovery of preexisting enzyme versus *de novo* synthesis, and the effect of prefreezing conditions.

2. Material and Method

2.1. Culture conditions

The cultures of *N. commune* was propagated on sand with aqueous modified BG-11 medium (N-free) in glass petri dishes (80 mm diameter). Several attempts were made to isolate heterotrophic diazotrophs associated with the colonies by plating the colonies on various media selective for heterotrophic diazotrophs. All these attempts proved negative. Furthermore no sustained nitrogenase activity occurred in the dark with the algal mats. From this we concluded that no contaminating heterotrophic diazotrophs were present. The cultures were grown on Petri dishes placed under a light bank of continuous cool day light fluorescent tubes (50 $\mu\text{E}/\text{m}^2/\text{s}$). Sand was used as a substratum because it is biologically inert material. Also, using sand, an aqueous medium could be added as needed and therefore eliminate the need for successive transfers (as with the use of an agar medium). The use of an aqueous medium in a simulated terrestrial system also facilitated the use of metabolic inhibitors.

2.2. Experimental conditions

All light treatments were performed in an incubator continuously illuminated with cool day light fluorescent tubes (50 $\mu\text{E}/\text{m}^2/\text{s}$) maintained at $14\pm 1^\circ\text{C}$. Dark treatments were performed in the same incubator in Petri dishes which were wrapped with aluminium foil. The plates were exposed to either light or darkness or 3 d before freezing. The algal mats were then frozen for 3-7 days at -14°C . Preliminary experiments showed that the duration of freezing between 1 and 20 d had no effect on the recovery of *in vivo* nitrogenase activity. Also our study was focussed on freeze recovery and not chilling injury. thus we wanted complete freezing of the *N. commune* mats. At the beginning of thawing, colonies were exposed to light or darkness alone or with exposure to metabolic inhibitor, the thawing time equilibration to ambient temperature was approximately 20 min.

2.3. Metabolic inhibitor

3-(3,4 dichlorophenyl)-1,1-dimethyl urea (DCMU) was used to inhibit photosynthesis . DCMU was added to experimental cultures to yield a final concentration of 2 μM . Protein synthesis was inhibited with chlormaphenicol added to the experimental cultures to yield a final concentration of 50 $\mu\text{g}/\text{ml}$.

2.4. Acetylene reduction assay for nitrogenase activity

Colonies were separated from substratum, rinsed in the medium and placed in vacutainer tubes (7.5ml). The tubes were fitted with rubber stopper and injected with acetylene to produce 10-12% acetylene atmosphere in the head space. The tubes were incubated in light at indicated temperatures (see results) for 1 h. Ethylene produced was quantified with Tracor model 540 gas chromatograph with dual columns (2mm by 2m) of Porapak R and flame ionization detector Column injector and detector temperature were maintained at 50°C .

3. Results

3.1. Freeze recovery of nitrogenase activity

The purpose of the experimentation (Fig 1) was to determine at what temperature the greatest recovery of nitrogenase activity could be achieved. the experiments were performed for maximum nitrogenase activity in subsequent experimentation. The amount of nitrogenase activity was greatest when colonies were thawed to 20°C . On the basis of these results, the mats were thawed to 20°C in all subsequent experiments.

Exposure to light during the thawing period enhances the recovery of nitrogenase activity (Fig 2). Colonies thawed during the light recovered detectable nitrogenase activity by 6 h, followed by a rapid increase in activity. As a control for each experiment shown in Fig 2 each set was analyzed for nitrogenase activity just before freezing (i.e, after the light or dark prefreeze treatment) to determine the time involved for freeze recovery nitrogenase activity to achieve the level of prefreeze nitrogenase activity. The control for the cultures pretreated in the light and thawed in light showed prefreezing nitrogenase activity of 1.6 μmol of C_2H_4 /mg chl a/h. Freeze recovery nitrogenase activity equaled this prefreeze activity by 24-36 h after thawing.

Cyanobacterial mat thawed in the dark (with a light prefreeze treatment) displayed nitrogenase activity at 12h (Fig 2) which was equal to 25% of the nitrogenase activity at 12h of mats thawed in light. Negligible nitrogenase activity was measured from 24-72 h after thawing. After exposure to light at 72 h, nitrogenase activity began to increase rapidly. The prefreeze (control) nitrogenase activity of these cultures was 1.63 μmol of C_2H_4 /mg chl a/h. Freeze recovery nitrogenase activity equaled this prefreeze activity by ca 36 h after exposure to light.

The above procedure was repeated except that colonies were kept in the dark for 72 h before freezing. This dark period was utilized to deplete the cells of stored carbon compounds (Lex and Stewart 1973), normally used as an energy or reductant source. After the 72 h dark prefreeze treatment, nitrogenase activity in the colonies was undetectable. Upon thawing, recovery of nitrogenase activity was much lower (Fig 2) than when cultures were exposed to light 3d before freezing and thawed in light.

3.2. Effect of metabolic inhibitor on recovery of nitrogenase activity

Fig 3 shows the effects of the metabolic inhibitors on the nitrogenase activity of unfrozen *Nostoc* mat. DCMU (an inhibitor of photosynthesis) was effective at 3 μM on *Anabaena cylindrica* (Lex and Stewart 1973) and also appeared to be effective at this concentration on *Nostoc*. Chloramphenicol (a translational inhibitor of protein synthesis in bacteria) is effective at 50 μg /ml on *Anabaena cylindrica* (Murray and Benemann 1979) and also appeared to be effective at this concentration on *Nostoc*.

After a prefreezing treatment of 3 d in the dark, followed by 3 d of freezing, mats were thawed in light in the presence of DCMU. At 48 h After thawing the DCMU was removed by rinsing the colonies six times with sterile distilled water. Within 12h after missing, nitrogenase activity was detected .

Nitrogenase activity through 96h after thawing, increase to more than double its rate of activity from 48 to 96 h after thawing

Table 1 shows the effect of chloramphenicol on the recovery of nitrogenase activity from freezing under various prefreezing and thawing conditions. When algal mats were thawed in the presence of chloramphenicol, the response of nitrogenase activity was similar for the various prefreezing and thawing treatments. When mats were exposed to light before freezing and thawed in the light with chloramphenicol , nitrogenase activity was detected 48h after thawing. However, the activity was much lower than that of controls. The mats thawed in dark with chloramphenicol showed nitrogenase activity similar to that of controls, although the peak in activity was at 24 h rather than at 12 h after thawing as in controls. By 48h, nitrogenase activity had declined to equal that of controls, both of which were detected at very low levels. Mats kept dark before freezing and thawed in the light again showed nitrogenase activity to 48 h after 48 h thawing , and this activity was also much less than that of controls . Nitrogenase activity was detected in these colonies again at 96 h after thawing.

4. Discussion

Prefreezing conditions (light or dark) appear to have a major impact on the freeze recovery of nitrogenase activity. Exposure to light before freezing promotes a more rapid rate of recovery of nitrogenase activity after thawing (Fig 2). When mats are kept dark before freezing. The rate of recovery is much slower. The light pretreatment would increase the potential for storage of carbon compounds (i.e. carbohydrates, cyanophycean granules), whereas the dark pretreatment would promote the utilization of stored carbon compounds (Lex and Stewart 1973) Many reports have shown that freeze protection in higher plants can be afforded by carbohydrates (Lineberger and Steponkus 1980, Santarius 1971), amino acids (Heber *et al* 1971),

organic acids (Santarius 1973), and proteins (Volger and Heber 1975). This storage of organic compounds may raise osmotic potential and hence play an important role in freeze protection. This study shows that conditions enabling cells to store carbon compounds before freezing (assuming compound storage during light pretreatment) have a beneficial effect on the freeze recovery rate of nitrogenase activity in *Nostoc commune*.

The ATP requirement of nitrogenase activity in cyanobacteria is normally supplied by photophosphorylation (Lex and Stewart 1973) and possibly by oxidative phosphorylation. The oxidation of pyruvate via Krebs cycle activity, usually supplies the majority of reductant (Eady and Postgate 1974, Lex and Stewart 1973). Reductant may be supplied by via the pentose phosphate pathway (Haystead and Stewart 1972) involving a glucose -6- phosphate-NADP⁺ complex. Energy requirements during freeze recovery of nitrogenase activity appear to be met via photosynthetic activity. Utilization of stored carbon compounds (under dark conditions) can supply energy for nitrogenase activity during the first 12 h after thawing (Fig 2). However, this source of energy can only support nitrogenase activity equal to about 25% of that in mats exposed to light upon thawing.

When mats were treated with chloramphenicol immediately upon thawing. Nitrogenase activity was still detected 12 to 48 h after thawing (Table 1). Murray and Benemann (1979) showed a decrease in nitrogenase activity within 1 h after the addition of chloramphenicol to air grown cultures of *Anabaena cylindrica*. They attributed this decrease to an oxygen inactivation of existing nitrogenase in the aerobically grown cultures and no *de novo* synthesis of nitrogenase to replace inactivated enzyme. When air grown *Nostoc commune* mats were treated with chloramphenicol, nitrogenase activity decreased substantially after 24 h and continued to decrease through 72 h. On the basis of the results of Murray and Benemann (1979), this decrease in nitrogenase activity of *Nostoc* mats may be due to oxygen inactivation or normal turnover of existing nitrogenase activity of *Nostoc* mats may be due to oxygen inactivation or normal turnover of existing nitrogenase. In the present study, low rates of nitrogenase activity were detected between 12 and 48 h after thawing in the presence of chloramphenicol. This implies that some nitrogenase is capable of surviving a freeze period *in vivo*. However, *de novo* synthesis of nitrogenase or other critical proteins is required for complete recovery of nitrogenase activity from freezing.

In the field, although temperature become limiting from late fall until early spring, *Nostoc commune* mats can recover from freezing with little physiological damage. When condition are favourable it appears that *N. commune* can resume growth and nitrogen fixation activity in existing colonies rather than relying solely on the formation of new colonies. This tolerance to freezing allows the mats to persist and possibly contribute substantial levels of nitrogen to localized field sites.

5. Acknowledgements

The author wish to thank the Head of Department, Centre of Advanced study in Botany for providing the laboratory facilities and all necessary help and UGC, New Delhi and UCOST, Dehradun for financial support.

6. References

- [1] Dua RD and Burris RH (1963). Stability of nitrogen fixing enzymes and the reactivation of a cold labile enzyme. Proc Natl Acad Sci USA. 50: 169-174
- [2] Eady RR and Postgate JR (1974). Nitrogenase. Nature (London). 249: 805-810
- [3] Haystead A and Stewart WDP (1972). Characteristic of the nitrogenase enzyme of blue –green alga *Anabaena cylindrica*. Arch Microbiol. 82: 325-336
- [4] Haystead A, Robinson R and Stewart WDP (1970). Nitrogenase activity in extracts of heterocystous and non–heterocystous blue–green algae. Arch Mikrobiol. 74: 235-243
- [5] Heber U, Tyankova L and Santarius KA (1971). Stabilization and inactivation of biological membranes during freezing in the presence of amino acids. Biochim Biophys Acta. 241: 587-592
- [6] Lex M and Stewart WDP (1973). Algal nitrogenase, reductant pools and photosystem I activity. Biochim Biophys Acta. 292: 436-443

- [7] Lineberger RD and Steponkus PL (1980). Cryoprotection by glucose, sucrose and reffinose to chloroplast thylakoids. *Plant Physiol.* **65**: 298-304
- [8] Murrey MA and Benemann JR (1979). Nitrogenase regulation in *Anabaena cylindrica*. *Plant Cell Physiol.* **20**:1391-1401
- [9] Ono T and Murata N (1981a). Chilling susceptibility of the blue green alga *Anacystis nidulans*. I. Effect of growth temperature. *Plant Physiol.* **67**: 176-181
- [10] Ono T and Murata N (1981b). Chilling susceptibility of the blue green alga *Anacystis nidulans*. II. Effect of growth temperature. *Plant physiol.* **67**: 182-187
- [11] Ono T and murata N (1982). Chilling susceptibility of the blue green alga *Anacystis nidulans*. III. Effect of growth temperature. *Plant Physiol.* **6**: 25-129
- [12] Santarius KA (1971). The effects of freezing on thylakoid membranes in the presence of organic acids. *Plant Physiol.* **48**: 156-162
- [13] Santarius KA (1971). The protective effect of sugars on chloroplast membranes during temperature and water stress and its relationship to frost, desiccation and heat resistance. *Planta.* **113**: 105-114
- [14] Volger HG and Heber U (1975). Cryoprotective leaf proteins. *Biochim Biophys Acta.* **412**: 335-345
- [15] Zumft WG and mortenson LE (1975). The nitrogen fixing complex of bacteria. *Biochim Biophys Acta.* **416**: 1-52

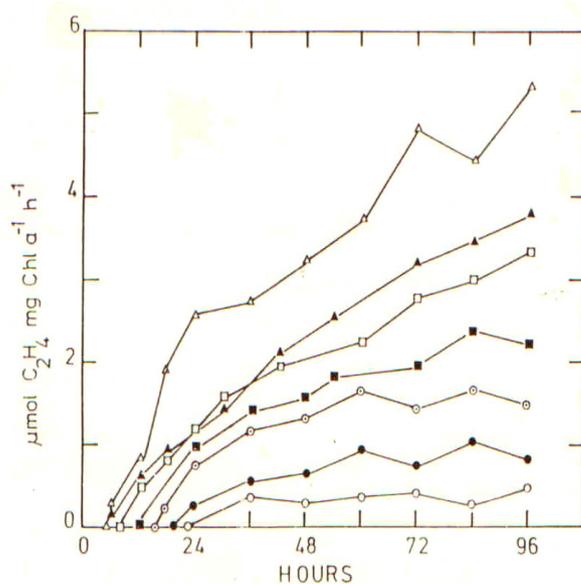


Fig. 1: Recovery of nitrogenase activity from freezing by *Nostoc commune* thawed to 2° (○), 4° (●), 6° (⊙), 8° (■), 10° (□), 20° (△) and 25° (▲) centigrate.

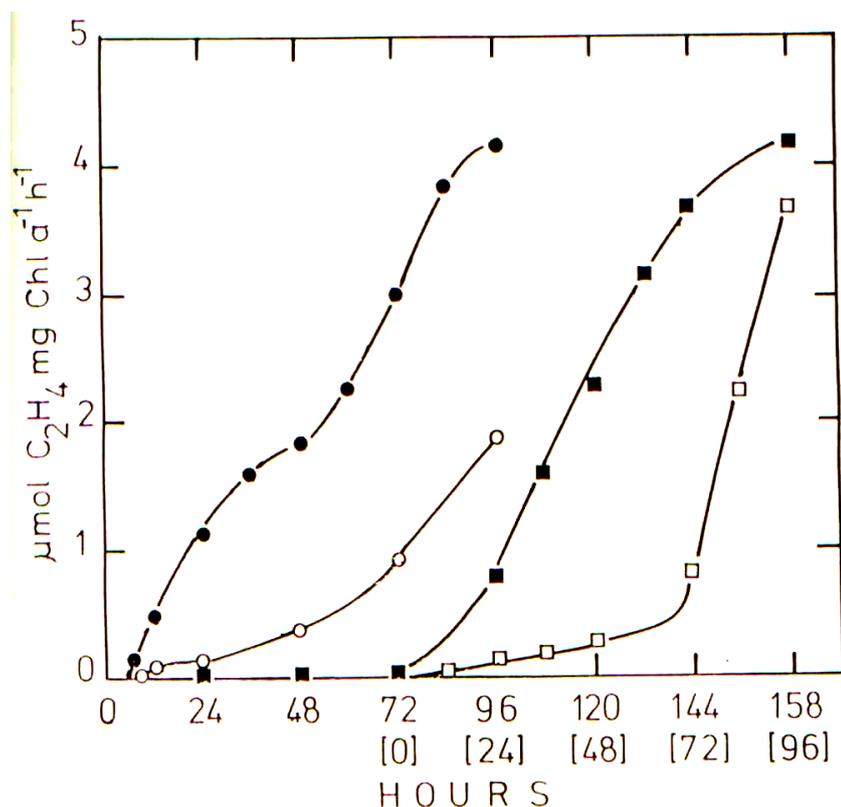


Fig. 2: Recovery of nitrogenase activity from freezing by *N. commune* grown under laboratory conditions and thawed to 20°C. Symbols: ●, cultures exposed to light for 3 d before freezing and thawed in light; ■, cultures exposed to light for 3 d before freezing and thawed in dark followed by exposure to light at 72 h; ○, cultures kept in dark for 3 d before freezing and thawed in light; □, cultures kept in dark for 3 d before freezing and thawed in dark followed by exposure to light at 72 h.

Table 1: Effect of chloramphenicol (50 µg/ml) on the recovery of nitrogenase activity from freezing in laboratory grown *Nostoc commune* mat^a.

Time (h)	Nitrogenase activity ^b					
	Light ^c				Dark ^c then light ^d	
	light ^d		Dark ^c		Control	Cm
	Control	Cm	Control	Cm	Control	Cm
0	0	0	0	0	0	0
12	630	20	190	40	20	50
24	1040	110	20	120	70	10
48	1460	110	20	30	260	60
72	1990	0	20	0	510	0
96	2790	0	20	0	1220	110

- a. *Nostoc* mat was exposed to light or kept dark before freezing and were thawed to 20°C in the light or dark. Colonies kept dark before freezing and thawed in dark showed no detectable nitrogenase activity in controls or with chloramphenicol (cm) treatment .
 b. Nitrogenase activity expressed as nmoles of acetylene reduced /mg chl a per hour
 c. Prefreezing treatment.
 d. Thawing treatment .

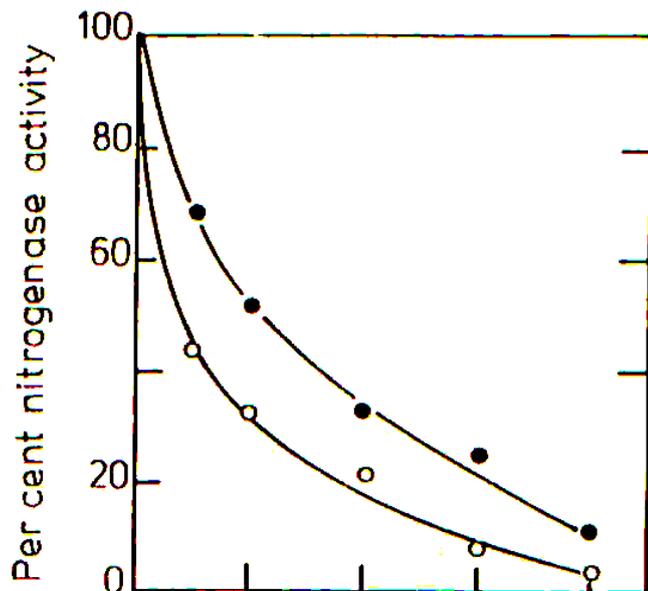


Fig. 3

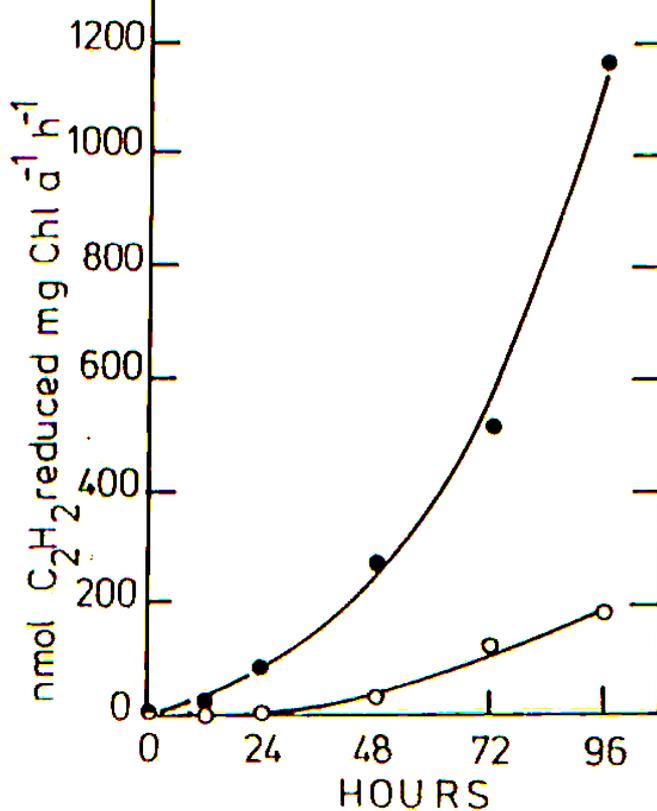


Fig. 4

- 3: Effect of DCMU (●) or chloramphenicol (○) on the nitrogenase activity of unfrozen laboratory grown Nostoc commune mats. Nitrogenase activity in controls were 480 and 5,600 nmol of acetylene reduced per milligram of chlorophyll a per h for (●) and (○), respectively.
- 4: Effect of DCMU (○) on the recovery of nitrogenase activity from freezing in laboratory grown Nostoc commune mats. (control - ●).

Author Index

A			
A. Fereidoon	49	K. Vengatajalabathy Gobi	25
A.R. Sabet	49	M	
Abdolhossein Fereidoon	11	M. A. Heydarlou	1
Abdolkarim Chehregani Rad	16,21	M. Bahrami	1
Abdolmajid Alipour	44	M. M.Zamani	49
Abdorahim Zahedi	54	Mahdi Goharrokhi	100
Alireza Kashaninia	54	Mahsa Mobarraz	94
Amin Karimi Zindashti	94	Majid Ghashang	90
Ananda Sankar Kundu	69	Mansoureh Pashangpour	64
Azalia Azlegini	111	Maryam Otadi	100
C		Marzieh Cheraghipoor	90
Chao Wu	39	Mashitah Binti Mohd Yusoff	59
E		Mehrangiz Akbari	21
Ebrahim Asl-Soleimani	30	Mohammad Hadi Pirahmadian	84
Etrat Lalbakhsh	80	Mohammad Javad Chaichi	94
F		Mohammad Reza Ganjali	94
Fardad Farrokhi	54	Mohammad Reza Mohammad Shafiee	90
Fariba Mohsenzadeh	16,21	MohammadHossein	75
Farnaz Montazer	106	Ghasempourabadi	
Fatemeh Dehghan Nayeri	30	Mohammadjavad	75
Fathima shahitha Jahir Hussain	59	Mahdavinejad	
G		Mojtaba Nasr-Esfahani	111
Guoguang Rong	39	Mojtaba Saeedi	34
Guoxing Wang	39	Morteza Rajabpour	11
H		N	
Hedayat Shahbazi	106	Negin Nikhoosh	75
Hojat Ghaedi	75	O	
Homayun Ahmad Panahi	100	Oishee Mazumder	69
Hossein Hemmatian	11	P	
I		Pegah shirazpour	80
I. Odeh	6	R	
I.Jassim	6	R. Pakdaman Zangabad	1
J		Rajan Kumar Gupta	116
Jinan B. Aldabbagh	6	S	
Junteng Xu	39	S. Pourang	1
K		Salman Jiryaiie sharahi	34
K. Koteswara Reddy	25	Seyed manouchehr Hosseini	30
K. Pourang	1	Pilangorgi	
		Sh. Dolati	49
		Subhasis Bhaumik	69

V
Vahid Ghaffari

64

Z
Zeinab Niroomandi

100