POLYGENETIC DIVERSITY OF NITROGEN FIXING CYANOBACTERIA ISOLATED FROM DIVERSED ECOSYSTEMS IN THAILAND

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CYANOBACTERIA/N_2-FIXING EFFICIENCY/PCR

One-hundred and two cyanobacterial strains were isolated from soils in Northen, Central and North-eastern parts of Thailand. All isolates were belonged to heterocystous filamentous cyanobacteria cell. Preliminary identification under microscope equipped with 400X magnification, 94.12% of total isolates were in the family Nostocaceae, 4.90% were branching cyanobacteria and 0.98% were unidentified. For study of N_2-fixing efficiency, as determined by acetylene reduction assay found that they were able to fix N_2 in the range of 0.073 to 2.715 / μ mol C_2H_4/mg of chlorophyll a/h and 0.0 to 2.724 μmolC_2H_4/mg of chlorophyll a/h under both light and dark conditions, respectively. Genetic diversity was determined by using PCR technique, when *nifH* -PCR products were generated the results showed relationship of almost cyanobacterial strians shared major band of PCR product in size of 330 bp. Tthree sets of random primers; STRR, DAF8.7b and DAF10.6e were conducted and PCR products from these primers were combined to generate the phylogenic tree. It was found that this approach able to clearly distinguished each strain even in intraspecies level.

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ลายมือชื่อนักศึกษา…….
ลายมือชื่ออาจารย์ที่ปรึกษา…….
ลายมือชื่ออาจารย์ที่ปรึกษาร่วม……..
ลายมือชื่ออาจารย์ที่ปรึกษาร่วม……..

ศศิธร อินทร์นอก : ความหลากหลายทางพันธุกรรมของไซยาโนแบคทีเรียที่ตรึงไนโตรเจนในระบบนิเวศต่างๆในประเทศไทย
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จากการศึกษาไซยาโนแบคทีเรียจำนวน 102 สายพันธุ์ที่ทำการแยกได้จากดินตามระบบนิเวศต่างๆ ในภาคเหนือ ภาคกลางและภาคตะวันออกเฉียงเหนือ พบว่าไซยาโนแบคทีเรียที่พบในพื้นที่ดังกล่าวมีลักษณะเป็นเส้นสายที่มีเซลล์เฮทเทอโรซีสต์ ในการจำแนกเบื้องต้นภายใต้กล้องจุลทัศน์โดยกำลังขยาย 400 เท่า พบว่าเป็นจำนวนสายพันธุ์ที่อยู่ในวงศ์ Nostocacaea ประมาณ 94.12%, อยู่ในกลุ่ม branching cyanobacteria 4.90% และยังไม่สามารถจำแนกได้อีก 0.98% เมื่อนำมาทดสอบประสิทธิภาพการตรึงไนโตรเจนพบว่าในสภาวะที่มีแสงสว่างและในที่มืดอยู่ในช่วง 0.023-2.715 และ 0.0-2.724 ไมโครโมล C_2H_4/มก.ของคลอโรฟิลเอ/ชั่วโมง ตามลำดับ ในการศึกษาความหลากหลายทางพันธุกรรมด้วยเทคนิคพีซีอาร์โดยใช้ชิ้นส่วนของ *nifH* เป็นไพรเมอร์ พบว่าไซยาโนแบคทีเรียส่วนใหญ่มีความสัมพันธ์กัน กล่าวคือมีผลิตภัณฑ์พีซีอาร์หลักประมาณ 330 bp หากแต่เมื่อนำมาศึกษาด้วยไพรเมอร์ 3 ชนิดได้แก่ STRR, DAF8.7b และ DAF10.6e อีกทั้งนำผลิตภัณฑ์จากไพรเมอร์ดังกล่าวมารวมกันเพื่อจัดกลุ่มความสัมพันธ์ พบว่าสามารถแยกความแตกต่างของแต่ละสายพันธุ์ในระดับสปีชีส์ได้อย่างชัดเจน

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List of Abbreviations

ARA	Acetylene Reduction Assay
BGA	blue green algae
bp	base pair
^{o}C	degree celcius
cm	centimeter
DAF	DNA amplification fingerprinting
dATP	deoxyadenosine 5' triphosphate
dCTP	deoxycytidine 5' triphosphate
dGTP	deoxyguanosine 5' triphosphate
DNA	deoxyribonucleic acid
dTTP	deoxythymine5' triphosphate
EDTA	Ethylene diamine tetraacetic acid
g	gram
h	hour
HIP	Highly Iterated Palindrome
l	litre
LTRR	Long Tandemly Repeated Repetitive Sequence
M	Molar
μm	Micrometer
μmol	Micromole
μl	Microlitre
μg	Microgram
mg	Milligram
min	Minute
ml	Millilitre
mM	Millimolar
nm	nanometer
OD	optical density
RAPD	Random Amplification Polymorphism DNA
RNAse A	ribonuclease A

rpm	revolution per minute
STRR	Short Tandemly Repeated Repetitive
vol/vol	volume per volume

Chapter I

INTRODUCTION

Nitrogen is an essential element for every living organisms. Eventhough, the large nitrogen source that can be made aviable is in the form of gas N_2 constituent 78 % of atmosphere but neither plants nor animals able to directly utilize N_2. Therefore, it has to be transformed to be a compound that plants can use as fertilizer. This transformation process is called nitrogen fixation. There are two processes of nitrogen fixation, industrial and biological processes. The industrial process reqiure high energy and high pressure for combining N_2 and H_2 to NH_3 resulted in the form of fertilizer which is relatively expensive. The biological process is the enzymatic catalyzation of N_2 and H_2 to NH_3 (as indicated in equation 1). This process occurs only in the prokaryotic microorganisms having nitrogenase enzyme. These group of organisms are called nitrogen fixing microorganisms, encompassing bacteria, actinomycetes and cyanobacteria (Sprent , J. I. and Sprent, P., 1990).

$N_2 + 8\ \text{ferridoxin}^- + 8\ H^+ + 16\ MgATP_2^- + 18\ H_2O \rightarrow 2\ NH_4 + 2OH^- + 8\ \text{ferridoxin} + 16\ MgADP^- + 16\ H_2PO_4^- + H_2$

Cyanobacteria as one of N_2-fixing microorganisms

Cyanobacteria have an ancient history which can be traced back almost 3×10^9 years (Mazel, Houmard, Castets and Marsac, 1990, quoted in Schopf and Watler, 1982), they were an ancient group of prokaryotic microorganisms exhibiting the general characteristics of gram negative bacteria (Rasmussen and Svenning, 1998). They were presumably the first oxygen-evolving photosynthetic organisms during the Precambrian and were thought to be responsible for the transition of the atmosphere of the earth from its primodial anaerobic state to the current aerobic condition (Mazel et al., 1990, quoted in Fogg, Stewart, Fay and Walsby, 1973). Ample evidences indicated that chloroplasts, which confer photoautogharphy to plants and algae, were derived from symbiotic cyanobacteria that were engulfed by primitive eukaryotic cells. Because of the varity of their physiological, morphological

and developmental features, cyanobacteria constitute an extremely diverse groups of prokaryotes which have colonized a wide range of habitats. they occured in almost all environmentals, including freshwater, seawater, non-acidic hot spring and deserts, where the often occur in such abundance that they were readily visible by eyes (Mazel et al, 1990).

Morphology of cyanobacteria

Cyanobacteria can be made of two broad morphological categories of thallus organization as following:

1. Non-filamentous forms

The non-filamentous forms were mainly coccoid form which were either single cell or grouped in palmelloid colonies (fig.1.1). The coccoid forms have different shapes, spherical, cylindrical and fusiform etc. Cell division (and multiplication) may be in one, two or three directions. In some daughter cells were separated away from each other but in many others still remained, for a shorter or longer period, united or attached to each other leading to formation of a colony made up of cells of difference generations. These cells may be developed by one or more different or firm mucilagious envelopes, the daughter cells of the same generation generally being surrounded by a common envelope.

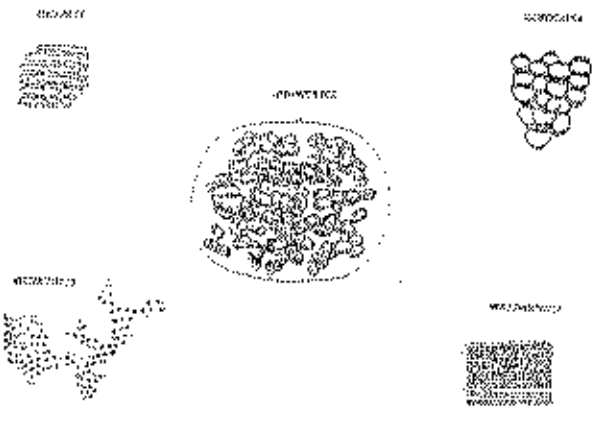


Fig. 1.1 Morphology of non-filamentous form of cyanobacteria

2. Filamentous forms

The simplest filamentous arrangement (fig. 1.2) was observed in the genus *Oscillatoria* which was made up of a long series of cells placed one over the other to form a "trichome". In *Arthrospira* and *Spirulina* the trichome is more or less permanently spirally coiled. The trichomes often secret mucilaginous material of varying consistency but in forms like *Lyngbya* a distinct, firm tube-like mucilaginous sheath is formed. The term "filament" is applied to denote the trichome and the sheath together. These forms were either straight or in some the tips may be lamellated or striated, the lamellation being either obviously parallel or divergent at various degrees from the trichome. In both of *Oscillatoria* and *Lyngbya*, all the cells of the trichomes are uniform or "homocystous", while in many others the trichomes have at one or both ends or was interrupted at more or less regular intervals by special thick-wall cells, namely "heterocyst". These latter forms are so called "heterocystous forms".The trichomes are either unbranched as in *Oscillatoria* and *Lyngbya* or may be branched as in *Scytonema*, *Tolypothrix* and *Plectonema* etc.

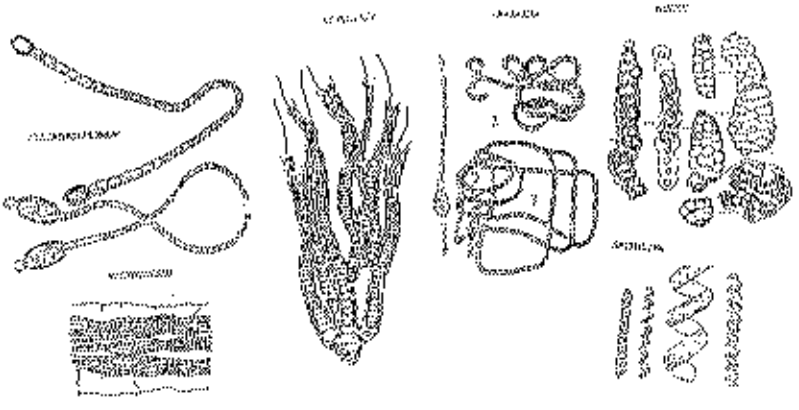


Fig.1.2 Morphology of filamentous cyanobacteria

Identification of cyanobacteria

The traditional characters employed for the identification of cyanobacterial in genera and species levels were those determinable on field materials: structural properties of the cells or filaments, colour, shape and structural of colonial aggregates such as (Rippka, 1988):

1. Growth formation; unicellular, colonial or filamentous strain
2. Colony formation; morphology of colony on solid media surface
3. Filament structure;

    3.1 Simple filament: *Oscillatoria* sp. and *Lyngbya* sp.

    3.2 Branching filament:

        (a) false branching: *Plectonema* sp., *Scytonema* sp. etc.

        (b) true branching: *Fischerella* sp., *Stigonema* sp. etc.

4. Cell differentation; differentation of having heterocyst or akinete cell such as *Anabaena* sp. were normally appeared, akinete cell nearby heterocyst cell.
5. Polarity; for example *Gloeotrichia* sp. has heterocyst cell on the tip of filament.
6. Sheath; for example *Lyngbya* sp., *Phormidium* sp. etc
7. Size and morpology of vegetative cell, heterocyst cell and akinete form

Cyanobacteria one of N_2-fixing microorganisms found in several patterns and forms of growth such as, free-living, symbiosis with plants, unicellular and filamentous. N_2-fixing cyanobacteria containing 23 genera, mostly were aerobic cyanobacteria (Burn and Hardy, 1973). For example in genera *Nostoc* sp., *Anabaena* sp. and *Calothrix* sp. can fix nitrogen in high humidity condition. Two well known genera of symbiotic cyanobacterial were *Anabaena azollae* symbiosis with *Azolla* as well as *Nostoc* sp. symbiosis with cycad and lichens. However, the efficiency of N_2-fixing by cyanobacteria was depend on genus/species and physical conditions such as day/night cycle. Nitrogen has been fixed by enzyme nitrogenase, which catalyzes the reduction of dinitrogen to ammonium, however this was rapidly inactivated by oxygen. Oxygenic photoautotrophs which were fix nitrogen must protect their nitrogenase from the oxygen evolved during photosynthesis (fig. 1.3) by the mechanisms in

concentration of oxygen condition (Zerh and McReynolds, 1989, quoted in Haselkorn, 1978). A few species of cyanobacteria had been isolated which could fix nitrogen during oxygen was evolved in the same cell. For example, the marine single-celled cyanobacteria *Synechococcus sp.* appeared to limit nitrogen fixation activity to specific time during the growth cycle (Zehr and McReynolds, 1989 quoted in Mitsui et al., 1986). *Gloeothece* sp., *Oscillatoria* sp. and *Synechococcus* sp. strain RF-1, although capable of fixing nitrogen under constant illumination, fix nitrogen mainly during the period of darkness when grown under alternating light and dark conditions, regardless of the growth phase (Zehr and McReynolds, 1989, quoted in Gallon et al., 1988, Grobelaar, Huang, Lin and Chow, 1986).

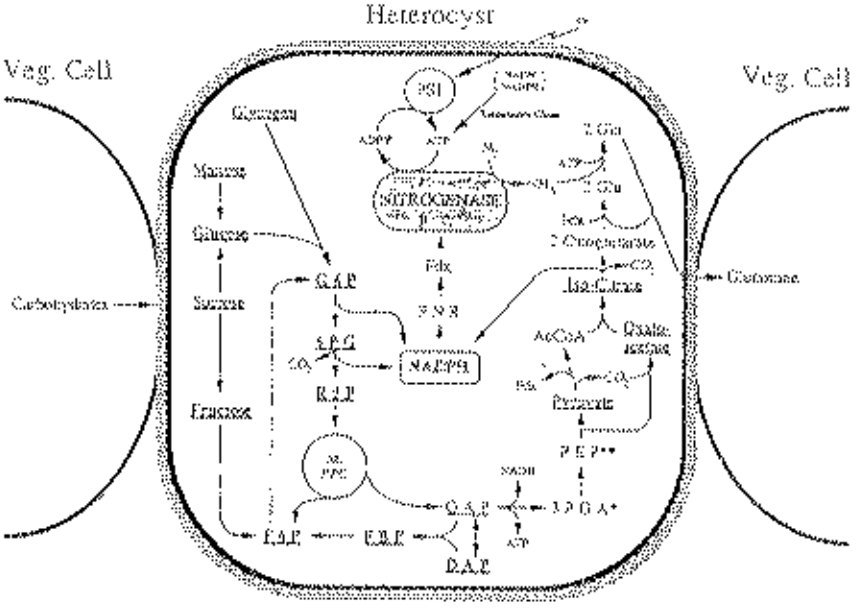


Fig.1.3 Heterocyst metabolism and electron donation to nitrogenase in *Anabaena*

However, the classically distinguishing of different species of cyanobacteria has relied upon identifying morphological, developmental and biochemical characteristic (Mazel et al.,1990). Tentative identification is greatly aided by thier colour characteristics, which may varied form green, blue-green or olive green to various shades of red to purple or even black; water-soluble pigment phycocyanin and phycoerythrin, minor colour commbunions being due to chlorophyll a and carotenoids. Although such

or olive green to various shades of red to purple or even black; water-soluble pigment phycocyanin and phycoerythrin, minor colour contributions being due to chlorophyl1 a and carotenoids. Although such colours are very indicative of cyanobacteria, final proof that field specimens do belong to this group of organisms will only come from; 1) critical examination of the samples under the microscope, to ensure that they are prokaryotes; 2) a demonstation by growth requirement or O_2-evolution measurements, to prove that they are not photosynthetic bacteria (which may be similar in colour due to carotenoids but lack phycobiliprotiens and an O_2-evolution photosystem II, and therefore need an electron donor other than H_2O for growth); and 3) an analysis of thier pigment contents, to exclude the possibility that they may be *Prochloron*-like organisms (which, like cyanobacteria, are oxygenic photosynthetic prokaryotes, but do not contain phycobiliproteins, their yellow-green colour resulting uniquely from chlorophyll a and b) (Rippka, 1988).

The colour of cyanobacteria in the natural environment, however, can be deceptive: starvation of nitrogen or sulfer, or conditions leading to photooxidation (high light intensity together with low CO_2 concentrations), cause a reduced phycobiliprotien content, and cyanobacteria exposed to such limitations will display the yellow-green colour of chlorophyll a rather than typical colours mentioned above (Rippka, 1988). Furthermore, certain cyanobacteria can be dark brown in appearance colour, owning either to massive akinete formation or to the production of brown sheaths that mask the typical coloration of cells or filaments. Considerable expertise was required to identify to the species level, in some cases it was not difficult to identify into the genera level such as *Calothrix*. Whereas, for many genera including *Oscillatoria*, *Lyngbya* and *Phormidium* is often difficult for non-expert to be confident of their diagnosis. Recently, molecular genetic approches have been developed to supplement or supplant conventional methods such as serotyping, bacteriophage-susceptibility characteristics and DNA-fingerprinting. DNA base composition was also contributed as the supporative data for taxonomical study (table 1). The methods using the PCR reaction have been extensively used nowadays such as amplifying of *nifH* gene for detect and characterize N_2-fixing cyanobacterial group (Porath and Zehr, 1994), sets of random amplified polymorphic DNA (RAPD) DNA amplification fingerprinting (DAF) which able to group the *Azolla* symbiosis cyanobacteria as well as primers for fingerprinting axenic cyanobacterial cultures (West and Adams, 1997, quoted in Anolles, 1994). Moreover, short tandemly repeated repetitive sequences (STRR) and highly iterated palindrome (HIP1) interspersed repetitive sequences have been achieved to distinguish symbiotic *Nostoc* with cycads and *Anabaena*, respectively (Robinson et al., 1995).

Table 1.1 DNA Base Compositional Spans and Reference Strains of Genera or Group

Section	Genus for groups	Mean DNA base Composition (mol % G+C)[a]	Reference strain[b]
Section I			
	Synechococcus		
	Type I	39-43 (5)[c]	PCC7202[d]
	Type II	47-59 (15)[c,e]	PCC6301[d]
	Type III	66-71 (9)[c]	PCC6307[d]
	Gloeothece	40-43 (5)[c]	PCC6501[d]
	Synechocystis		
	Type I	35-37 (5)[c]	PCC6308
	Type II	42-48 (12)[c]	PCC6714
	Gloeocapsa	40-46 (4)[c]	PCC73106
	Gloeobacter	64(1)[c]	PCC7421
	Chanaesiphon	47 (2)[c]	PCC7430
Section II			
	Dermocarsa	38-44 (6)[c]	PCC7301
	Xenococcus	44 (2)[c]	PCC7305
	Dermocarpella	45 (1)[c]	PCC7326
	Myxosarcina	43-44 (2)[c]	PCC7312
	Chroococcidiopsis	40-46 (8)[c]	PCC7203
	Pleurocapsa group	39-47 (11)[c]	
	Type I[f]		PCC7319
	Type II[g]		PCC7516
Section III			
	Spirulina	53 (1)[c]	PCC6313
	Arthrospira	44 (1)[c]	PCC7345
	Oscillatoria	40-50 (10)[c]	PCC7515
	Pseudonabaena	44-52 (8)[c]	PCC7429
	LPP group A[h]	43 (1)[c]	PCC7419
	LPP group B[i]	42-67 (19)[c]	
	Plectonema baryanum" type[j]	46-48 (5)[c]	PCC6306
Section IV			
	Anabaena	38-44 (3)[c,k]	PCC7122
	Nodularia	41-45 (2)[c,k]	PCC73104
	Cylindrospermum	43-45 (3)[c,k]	PCC7417
	Nostoc	39-47 (21)[c,k]	PCC73102

Section	Genus for groups	Mean DNA base Composition (mol % G+C)[a]	Reference strain[b]
	Scytonema	44 (1) [c,k]	PCC7110
	Calothrix	40-45 (15)[c,k]	PCC7102
	Tolypothrix tenuis" type[l]	41-46 (5)[c,k]	PCC7101
Section V			
	Chlorogloeopsis fritschii	42-43 (2)[c]	PCC6912
	Fischerella	42-46 (8)[c,k]	PCC7414

[a] Number in parentheses indicates the number of strains examined.

[b] Reference strains taken from R. Rippka, J. Deruclles, J. B. Waterbury, M. Herdman, and R. Y. Stanier, *J. Gen Microbiol*. **111**, 1 (1979).

[c] M. Herdman, M. Janvier, J. B. Waterbury, R. Rippka, R. Y. Stanior, and M. Mandel, *J. Gen. Microbiol*. **111**, 63 (1979).

[d] These reference strains were proposed as types or neotypes of the following genera: PCC 7202, *Cyanobacterium stanieri*; PCC 6301, *Synechococcus elongatus*; PCC 6307, *Cyanobium gracile*; PCC 6501, *Gloeothece membranacea*. See R. Rippka and G. Cohen-Bazire, *Ann. Microbiol. (Paris)* **134B**, 21 (1983).

[e] Data fpr one strain (PCC 7942) taken from A. M. R. Wilmotte and W. T. Stam, *J. Gen. Microbiol*. **130**, 2737 (1984).

[f] Typical of members with symmetric baeocyte enlargement.

[g] Typical of members in which the baeocyte develops early polarity.

[h] "*Lyngbya aestuarii*" type, see comments to Section III and Key 4.

[i] This group is very heterogeneous, and designation of a reference sstrain is therefore meaningless.

[j] See comments to Section III and Key 4.

[k] M.-A. Lanchance, *Int. J. Syst. Bacteriol*. **31**, 139 (1981).

[l] See comments to Section IV and Key 5. **(Rippka, 1988)**

Ecological factors influencing on cyanobacteria

1. Light. Algae, as phototrophic microorganisms, are restricted to the photic zone and usually located in the upper 0.5 cm horizon. Yet algae also exist in deeper horizons, in a dormant condition as spores or filament fragments (Roger and Reynaud, 1982, quoted in V. J. Chapman and D. J. Chapman, 1973). Light availability for soil algae depends upon the season and latitude, the cloud cover, the plant canopy, the vertical location of the algae in the photic zone and the turbidity of the water. Light intensity reaching the soil may vary from too low to excessive levels (10 to 110,000 lux).

In cultivated soil the screening effect of a growing crop canopy appears to cause a rapid decrease of light reaching the algae. Thus the canopy of transplanted rice decreased light by 50% when plants were 10 days old, 85% after one month and 95% after two months (Roger and Reynaud, 1982, quoted in Kurasawa, 1956). In Senegal, diatoms and unicellular green algae developed first and blue green algae (BGA) developed later when the plant cover was dense enough to protect them from excessive light intensities, higher than 80 klux at 13:00 h ; the N_2 - fixing algae biomass and the density of the plant cover were positively correlated (Roger and Reynaud, 1982, quoted in Roger and Reynaud, 1976).

In the laboratory, after one month of incubation of a submerged unplanted soil under a range of screens, BGA were dominant in the most heavily shaded one and green algae and diatoms were dominant in the soil exposed to full sunlight (Roger and Reynaud, 1982, quoted in Reynaund and Roger, 1978). A beneficial effect of the plant canopy shading the algae was also reported by Singh (Roger and Reynaud, 1982, quoted in Singh, 1961) in sugarcane fields, maize fields and grasslands in India. As BGA are generally sensitive to high light intensities, they develop various protective mechanisms against it such as

– vertical migrations in the water of submerged soil

– preferential growth in more shaded zones like embankments, under or inside decaying plant material (Roger and Reynaud, 1982, quoted in Kulasooriya , Roger, Barraquic and Watanabe., 1980) or a few millimeters, below the soil surface (Roger and Reynaud, 1982, quoted in Fogg, 1973)

– migration into shaded zones (photophobotaxis) and aggregation providing a self-shading effect (photokinesis) (Roger and Reynaud, quoted in Reynaud and Roger, 1978)

– stratification of the strains in algae mats where N_2-fixing strains grow under a layer of eukaryotic algae more resistant to high light intensities (Roger and Reynaud, 1982, quoted in Reynaud and Roger, 1981).

However, some strains of BGA seem more resistant to high light intensities such as *Cylindrospermum* sp. developed large biomasses in a harvested paddy field in Mali where light intensity was higher than 100 klux at 13:00 (Roger and Reynaud, 1982, quoted in Traore, Roger, Reynaud and Sasson, 1978). *Oscillatoria princeps* was also reported to grow profusely in full sunlight (Roger and Reynaud, 1982, quoted in Reynaund and Roger, 1978).

On the other hand, light deficiency may also be a limiting factor. In Japan, avialable light under the canopy was below the compensation point of the phytoplankton during the second part of the cycle (Roger and Reynaud, 1982, quoted in Ichimura, 1954). In the Philippines, during the wet season when light was moderate acetylene reducing activity (ARA) was higher in bare soil than in plant soil (Roger and Reynaud, 1982, quoted in Watanabe et al., 1977).

2. Temperature. The optimal temperature for BGA growth is about 30-35 °C which is higher than that for the growth of eukaryotic algae. In submerged soils daily variations in the temperature are moderated by the buffering effect of flood-water; temperature is rarely a limiting factor for BGA in paddy fields, because the range of temperatures permitting the growth of BGA is larger than that required by rice; however, it influences both algal biomass composition and productivity. Low temperatures favour both the phytoplankton productivity and BGA (Roger and Reynaud, 1982, quoted in Roger and Reynaud, 1979).

Daily changes in the temperature are more drastic in terrestrial habitats than in aquatic environments (Roger and Reynaud, 1982, quoted in Raghu and MacRae, 1967). An inhibitory effect of high temperature was observed by Jones (Roger and Reynaud, 1982, quoted in Jones, 1977) in the Kikuyu grasslands in Africa where algal N_2 fixation was higher on overcast days than on hot sunny days. Stewart (Roger and Reynaud, 1982, quoted in Stewart, 1977) indicated a correlation between the algal ARA response to temperature and the temperature of the habitats from which the algae were collected. For many tropical species, ARA is optimum between 30-35°C, but a *Nostoc* sp. isolated from the algal crust on a sandy soil in Senegal still exhibited significant ARA at 60°C. High temperatures occurring in the surface of tropical upland soils may have a selective action on the algal flora, favouring BGA which are more tolerant to high temperatures than eukaryotic algal. For example, the dry spores of *Nostoc* sp. can tolerate 2 minutes at 100 °C, the wet spores 20 minutes at 60-70 °C, and the vegetative filaments 10 minutes at 40 °C (Roger and Reynaud, 1982, quoted in Chapman, V.J. and Chapman, D.J., 1993).

3. Desiccation and remoistening. Algal growth is hindered by intermittent desiccation periods which occur during the dry season and even during drought periods that occur in the rainy season. BGA have a high capacity to withstand desiccation. *Nostoc muscarum* and *Nodularia harveyana* were

isolated from a soil that had been dried for 79 years (Roger and Reynaud, 1982, quoted in Chapman, V. J. and Chapman, D.J.). Resistance to desiccation has been attributed to various characteristics (Roger and Reynaud, 1982, quoted in Prescott, 1968) with respect to fatal plasmolysis, the lack of cell vacuoles, the ability of some genera to quickly take on an encysted form, the presence in some genera of a mucilaginous sheath that absorbs water quickly and retains it. This latter characterisric could explain the dominance of mucilaginous colonies of *Nostoc* spp. and *Cylindrospermum* spp. in the paddy field during the last part of the cultivation cycle when the soil dries (Roger and Reynaud, 1982, quoted in Pandey, 1965, Reynaund and Roger, 1978 and Traore et al., 1978). The dominance of BGA comprised only about 30% of the algal flora (Roger and Reynaud, 1982, quoted in Materasi and Ballani, 1965) whereas in Senegal, where the dry season lasts about 8 months, spores of heterocystous BGA consituted more than 95% of the algalflora at the end of the dry period. In Uttar Pradesh (India), a large number of Chlorophyceae occurred in low-lying fields, whereas BGA were found in larger numbers in paddies at higher elevations (Roger and Reynaud, 1982, quoted in Pandey, 1965). In arid soils, BGA have been reported as dominant species (Roger and Reynaud, 1982, quoted in Marathe and Anantani, 1972) and sometimes as the only species present (Roger and Reynaud, 1982, quoted in Barbey and Coute, 1976 and Chapman, V.J. and Chapman, D.J.. 1993)

4. pH. Among the soil properties, pH is the most important factor determining the algal flora composition. In culture media the optimal pH for BGA growth seems to range from 7.5 to 10.0 and the lower limit is about 6.5 to 7.0 (Roger and Reynaund, 1982, quoted in Holm-Hansen, 1968). Under natural conditions BGA grow preferentially in environments that are neutral to alkaline. The beneficial influence of high pH on BGA growth was further demonstrated by the fact that the addition of lime increases BGA growth and N_2 fixation (Roger and Reynaund, 1982, quoted in Roger and Kulasooriya, 1980). However, the presence of certain strains of BGA in soils with pH values between 5 and 6 have been reported. Durrel (Roger and Reynaund, 1982, quoted in Durrel, 1964) demonstrated the presence of *Nostoc muscorum* and *Anabaena torulosa* in soils with pH ranging from 5 to 7. *Aulosira fertilissima* and *Calothrix brecissima* have been reported to be ubiquitous in Kerala rice fields with pH from 3.5 to 6.5 (Roger and Reynaud, 1982, quoted in Aiyer, 1965). The development of a dense algal bloom on an acidic soil (pH 5.5) was observed after the surface application of straw (Roger and Reynaund, 1982, quoted in Matsuguchi and Ick-Dong Yoo, 1979). Stewart (Roger and Reynaund, quoted in Stewart et al.) also reported that some tropical BGA exhibited ARA even at pH 4. The poor growth of N_2-fixing

BGA, frequently observed in acidic soils, is probably due to the inability of BGA to compete with Chlorophyceae, which are favoured by acidic conditions.

5. Inorganic ions. Fe, P, Mo, Mg, Co and K, which were essential component of growth as well as necessary for synthesizing the nitrogenase enzyme (Heselkorn and Buikema, 1992).

Since the ecological and agricutural importance of the organisms depend on their ability to fix N_2, thus application of biofertilizer by using cyanobacteria, in stead of chemical fertilizer, has been implemented. Therefore, this study was to isolate and examine the indigenous N_2-fixing cyanobacteria with regard to their distribution in various types of ecosystem in conjunction with the investigation of their genetic diversities for further sustainable maintaining and appropriately manipulating them under their ecosystems.

OBJECTIVE

1. **To identify and examine diversification of the indigenous N_2-fixing cyanobacteria with regard to their distribution in various representative soil types in North, Central and North-eastern part of Thailand.**
2. **To characterize some properties of isolated N_2-fixing cyanobacteria.**
3. **To investigate the genetic diversity of isolated N_2-fixing cyanobacteria.**

Chapter II

METERIALS AND METHODS

MATERIALS

1. Cyanobacterial strains

The cyanobacterial strains used as reference strains were as follow : *Nostoc sp.*, *Anabaena cylindrica*, *Hapalosiphon sp*. DASH 5101, *Calothrix sp*. DASH 02101 and *Scytonema sp*. were obtained from Department of Soil Science, Faculty of Agriculture, Chiangmai University.

All cyanobacterial isolates were randomly selected from soil isolation. Soil samples from the mountain, flat area of agriculture practice (field crop cultivation, rice cultivation, rice in rotation with other crops) and uncultivation areas from Northern, Central and North-eastern part of Thailand were choosen as sampling sites. They were isolated from Department of Soil Science, Faculty of Agricuture, Chiengmai University. Prior conducted experiment, they were purified by streaking on agar plates, and new single colony isolates were cultivated and maintained for further used.

2. Culture media and Cultivation

BG11 medium

Composition per litre

$MgSO_4 . 7H_2O$	0.076 g
Na_2CO_3	0.020 g
$CaCl_2 . 2H_2O$	0.035 g
$C_6H_8O_7 . H_20$	0.006 g
$FeNH_4$ citrate	0.006 g
$C_{10}H_{14}N_2Na2O_8 . 2H_2O$	0.001 g
K_2HPO_4	0.038 g

A micronutrient *	1 ml
pH 7.4	
agar (for solid medium)	15 g

Composition per litre of A micronutrient *

H_3BO_3	2.8 g
$MnSO_4.H_2O$	1.56 g
MoO_3	0.15 g
$ZnSO_4.7H_2O$	0.22 g
$CuSO_4.5H_2O$	0.08 g
$CrKO_8S_2.12H_2O$	0.10 g
$NiSO_4.6H_2O$	0.045 g
$Co(NO_3)_2.6H_2O$	0.05 g
$Na_2WO_4.2H_2O$	0.018 g
TiO_2	0.017 g
NH_4VO_3	0.02 g

BG11 solid medium were used for strains purification whereas liquid medium were used for cultivation before DNA extraction and detection of efficiency in N_2 fixing efficiency.

3. Chemicals

All chemicals used were laboratory grade, or otherwise specified.

3.1 Acetylene reduction assay (ARA) measurement

3.1.1 Gases

Compressed air, hydrogen (H_2), ethylene standard (C_2H_4), nitrogen (N_2) were obtained from Thai Industrial Gas and Casting. Acetylene (C_2H_2) were obtained from calcium cabide added with H_2O.

3.1.2 Packing material

Porapak N (Pack column) No. 530-5014, Autosystem XL, Perkin Elmer, USA

3.2 Reagent for chlorophyll a extraction

ethanol, 95 %

3.3 Reagent for DNA extraction

Modified *Azolla* extraction buffer : 100 mM Tris-HCl (pH 8.0), 250 mM NaCl, 100 mM EDTA, 0.4 % 2-mercaptoethanol *

CTAB stock : 2 % hexadecytltrimethylammonium bromide (CTAB), 1.4 NaCl

TE buffer : 0.01 M Tris-HCl, 0.001 M EDTA, pH 7.4

lysozyme solution : 1 mg/ml in TE buffer

sarcosyl , 1 % (v/v)

phenol

chloroform - isoamyl alcohol, 24:1 (v/v)

isopropanal

absolute ethanol

ethanol , 70 %

Rnase A - solution; 100 μg/ml

* add just before using buffer

3.4 Reagent for detection of DNA by agarose gel electrophoresis

TBE buffer : 0.089 M Tris-base, 0.088 M Boric acid , 2.5 mM Na_2EDTA (3.18 mM EDTA pH 8.3)

Loading dye : 0.25 % bromphenol blue, 0.25 % xylene cyanol FF, 0.4 % orange G, 10 % Ficoll 400, 10 mM Tris-HCl (pH 7.5) and 50 mM EDTA

Staining solution : 2.5 μg/ml ethidium bromide

DNA marker : 1 kb ladder DNA (purchased from Promega)

: 100 bp DNA (purchased from GIBCOBRL®)

3.5 Reagent for Scanning Electron Microscope

0.1 phosphate buffer solution : solution A : 0.2 M sodium phosphate monobasic ($NaH_2PO_4.H_2O$) 27.6 g/l, solution B : 0.2 M sodium phosphate dibasic (Na_2HPO_4) 28.4 g/l. 28.0 ml solution A and 72.0 ml solution B were mixed for 100 ml and adjusted to pH 7.2.

METHODS

1. Identification of cyanobacteria

Cyanobacterial strains were restreaked onto BG11 medium after obtained from Department of Soil Science, Faculty of Agriculture, Chiangmai University. Colony-forming characteristics of each cyanobacterial isolates were observed and recorded as the data for next analyses. Microscopic study of all strains were employed under 400X magnification microscope. The results of cell morpology (characterzation of heterocyst, vegetative and akinete cells) were interpreted along with Desikachary, T.V., 1958 and Rippka, R., 1988 'S description.

2. Scanning Electron Microscope

Cyanobacterial colony on BG11 solid medium was cut for small cubic sample, then soaked in 3% glutaraldehyde resuspended at 4°C overnight (3-24 h) in phosphate buffer solution. Solution was poured carefully to avoid contact with air. The sample was washed twice (15 min per time) with 0.1 M phosphate buffer solution. After that, sample was serially dehydrated by ethanol concentration varied from 30%, 50%, 70%, 90%, 95% and 100% twice for 15-20 min in each step, then the sample was dried by critical point dryer (CPD). Dried sample was attached on a grid and coated with gold by Ion Sputtering device, JFC 1100E for 4 min. Sample was determined by Scanning Electron Microscope (JSM 6400, Japan) with black-and-white negative film (verichrome pan film, VP 120, kodak).

3. Detection of Acetylene Reduction Assay (ARA)

The 50 ml tubes containing cyanobacterial cultures with 25 ml of BG11 liquid medium were incubated at 25°C under a 12 h/12 h light/dark cycle. After cultivation for 30 days, tubes were plugged with double septum before 10 % of the head space air volume was replaced with acetylene. Allow the incubation to proceed at 25°C for 1 h under 400 μE. S^{-1}. M^{-2} light intensity. To deyermine N_2-fixing efficiency in the dark, the incubation period was 12 hr. One ml of gas mixture was withdrawn and analyzed by Gas chromatography (GC) equiped with porapak N column (Pack column No. 530-5014, AutoSystem XL, Perkin Elmer, USA). Ethylene production per tube per 1 hr was determined by comparing the peak height in cm. with that of known amount of standard ethylene efficiency of N_2-fixing cyanobacteria has compared with amount of chlorophyll a. Then, chlorophyll a from each cyanobacterial isolates were extracted after harvested with centrifugation at 9,000 rpm for 5 min, resuspended cyanobacterial pellet with 10 ml 95 % ethanol. Cyanobacterial pellet were homogenized by using homoginizer (Ace Homoginizer No. 10-717, Nissei-AM-8 Japan, Nihonseiki kaisha, LTD) at 13,000 rpm for 5 min, then incubated for 15 min in the dark, centrifugated at 9,000 rpm for 5 min and supernatant was collected and measured at the optical density at absorbance of wave length 665 nm. Amount of chlorophyll a was calculated by using the equation :

$$\frac{\text{vol. of 95 \% ethanol} \times OD_{665}}{83.4} = \text{mg. of chlorophyll a}$$ (Wintermans and DeMots, 1965).

4. DNA extraction

Cyanobacterial strains were cultured in 250 ml Erlenmeyer flask containing 100 ml of BG11 liquid medium. Cell pellets were harvested by centrifugation at 9,000 rpm for 10 min, resuspended with 3 ml of *Azolla* extraction buffer (100 mM Tris-HCl [pH 8.0], 250 mM NaCl, 100 mM EDTA and 0.4% 2-mercaptoethanol), then added 1 mg lysozyme / 1 ml TE buffer (10 mM Tris-HCl [pH8.0] and 1 mM EDTA) and incubated at 37°C for 1 h (occasionally mixed). Two-hundred μl of 1% v/v sarcosyl was added, vortexed and extracted twice with equal volume of phenol. Aqueous phase was transferred into an another microcentrifuge tube, equal volume of CTAB stock (2 % CTAB/1.4 M NaCl) was added before incubated at 65°C for 30 min then, extracted with equal volume of chloroform/isoamylalcohol (24/1, vol/vol). DNA was precipitated for overnight with 2.0 volumes of cold isopropanol. The

solutions were centrifuged, washed with 70% ethanol, dried and the DNA pellet was resuspened in TE buffer with 1/10 Rnase A, then incubated at 55°C for 10 min and collected at 4°C for further analysis.

5. PCR analyses

Primer 1: 5'-GGAATTCCTGYGAYCCNAARGCNGA-3' and primer 2: 5'-CGGATCCGDNGCCATCATYTCNCC-3' (Y was T or C; N was A, C, G or T; R was A or G and D; A, G or T) were used for the PCR amplification of the *nifH* gene. The *nifH* gene was amplified from 50 ng of DNA template by using 2.5U of Taq polymerase (GibcoBRT®, Brasil) which carried out in the total reaction volume of 50 μl, 0.6528 pmol and 0.8125 pmol of primer 1 and 2, respectively, 200 μM deoxyribonucleoside triphosphate, 1.5 mM $MgCl_2$ and 1 cycle at 93°C for 5 min; 35 cycles of denaturation (93°C, 1.2 min), annealing (50°C, 1.0 min) and extension (70°C, 1.5 min); 1 cycle at 70° C for 10 min in PCR Sprint Temperature Cycling System (Hybaid Limited, UK). The two sets of primer were originally used to amplify *Anabaena* sp. and *Azolla* sp. DNA (Eskew, Anolles, Bassam and Gresshoff, 1993) sequence [5'-3'] were (GCTGGTGG (DAF8.7b) and GTGACGTAGG (DAF10.6e)). Fifty ng of genomic DNA was used as the template for PCR amplification which carried out in the total reaction volume of 50μl, 1.0895 pmol primer 8.7b or 1.7480 pmol primer 10.6e, 200 μM deoxyribonucleoside triphosplate, 1.5 mM $MgCl_2$ and 2.5 U Taq polymerase (GibcoBRT®, Brasil) in reaction buffer (10 nM Tris, 50 mM KCl, 0.01 % gelatin, 0.1 % TritonX-100 [pH9.0]). Amplification was performed in PCR Sprint Temperature Cycling System (Hybaid Limited, UK) for 30 cycles of 30 s at 95°C, 1 min at 30°C and 1 min at 72°C, followed by a final extension period of 10 min at 72°C. For the STRR primer (Rasmussen and Svenning, 1998)[5'-3'] sequences of primer were as follow: CCARTCCCCARTCCCC the cycles were follows : 1 cycle at 95°C for 6 min ; 30 cycles of 94°C for 1 min, 56°C for 1 min and 65°C for 5 min; 1 cycle at 65°C for 16 min and final step at 4.0°C. The PCR was carried out in a 50 μl volume containing 0.9670 pmol of primer, 200 μM deoxyriboucleoside triphosplate, 1.5 mM $MgCl_2$ and 2.5U Taq polymerase (GibcoBRT®, Brasil) in reaction buffer (10mM Tris, 50 mM KCl, 0.01 % gelatin, 0.1 % Triton-100 [pH9.0]). After the amplification, 15 μl aliquots of the PCR products were resolved by gel electrophoresis at 80Vcm^{-} in 1.0 % agarose gel (Promega,USA) stained with the ethidium bromide

6. Phylogenetic analysis

Dendrogram were constructed from the similarrity matrix by the unweighted pair group method with arithmetic mean (UPGMA). In order to test the goodness of fit of cluster analysis, cophenetic value matrices were calculated and compared with the original similarity metrics that were UPGMA clustered by using the NTSYS-pc package (version 1.8; Exeter Software, Setauket, N.Y.).

Chapter III

RESULTS AND DISCUSSIONS

1. Identification and characterization of cyanobacteria

Both of cyanobacterial cultures on BG11 solid and liquid N-depleted medium at 25°C for 4 weeks were taken to examine colony and cell morphology under 400 magnification microscope (Table 3.1). The criteria of cyanophyta (Desikachary,1958) were modified for this study which identification based on colour of cyanobacteria on solid and liquid medium, size of vegetative cell and colony, morphology of heterocyst cell including position of cell, forms and edges of colony and growth in culture broth. One-hundred and two cyanobacterial isolates were identified in 4 genera for 5 groups as *Nostoc* sp. 45 isolates, *Anabaena* sp. 44 isolates, *Anabaenopsis* sp. 5 isolates, *Nodularia* sp. 3 isolates and branching group 5 isolates. Their colour characteristics were depended on each strains, which may vary from green, blue-green, or olive green to various shades of red to purple, or even dark brown. The colour is mainly determined by the relative amounts of the major light-harvesting, water-soluble pigments phycocyanin and phycoerythrin, minor colour contributions being due to chloropyll a and carotenoids (Rippka,1988) which this was similar to their study. Size of colony on solid medium were varied from 0.1 to more than 0.4 inches. They performed of many forms such as circular, irregular, filamentous, hairy and gas forming colony. Elevations of colony were flat and convex. For the edge characteristics were entire, erose, filamentous and curled. When cyanobacterial forming characteristics were observed from liquid medium, cell clumped in both over and under surface of medium and some cell clumped on tube surface for single colony or mat. Cell morphology under 400X magnification microscope, all of cyanobacteria were filamentous and found heterocyst cell on intercalary or terminal of trichome depended on strain while some strains were branching.

Comparison study is expected *Nostoc* sp. group (Table 3.1), three criteria as, morphology of cell under microscope, solid and liquid medium were not significantly distinguished for identifying each strains. However, most heterocystous cells were spherical form 50% and ellipsoidal form 44.12% and only 5.88% as quadrate form. For the expected *Anabaena* sp. group, morphology of this group similar to *Nostoc* sp. whereas some strains were filamentous or gas forming colony. In liquid medium, all of this group were clump-forming strain both over and under the surface of medium as well as clumped beside the tube. Morphology of heterocyst cell were mostly ellipsoidal and trichome were arranged in

formal filamentous, in contrast, *Nostoc* sp. were irregular filamentous including heterocyst cell were unaggregated on filament The expected *Anabaenopsis* sp. group, all of cyanobacteria were brownish colour and filament was spirally coiled. For expected *Nodularia* sp. group, colony on solid medium be able to distinguished from *Nostoc* sp. and *Anabaena* sp. obviously since their presence of hairy colony, heterocyst cells were quadrate form, vegetative cells were broader than long (Desikachary, 1958). The growth of branching group was found slower than other cyanobacteria and some strains had hairy colony and found branching orientation under microscope.

Table 3.1 shown that *Nodularia* sp., *Anabaenopsis* sp. and branching group could be distinguished from other groups based on colony forming characteristics on solid medium and cell morphology under microscope whereas *Nostoc* sp. and *Anabaena* sp., main genera found in soil, were similar and difficult to distinguish when using mentioned three criteria. Rippka,(1988) used hormogonia forming for distinguish among two genera. *Nostoc* sp., hormogonia gave rise to young trichomes that differentiable terminal heterocyst at both ends of the cellular chain. Hormogonia were composed of cell that were generally smaller in size, different in shape (often cylindrical or isodiametric) and may contain polar or irregularly distributed gas vacules. *Anabaena* sp., hormogonia gave rise to young trichomes that differentiate heterocysts from the terminal cells at only one and of the cellular chains. Hormogonia were composed of cells that are not markedly different in size or shape (although they are generally somewhat shorter than those of the mature filaments and do not contain gas vacuoles).

From this study, they were difficult to clearly distinguish in each genus and species. Recently, molecular genetic approaches were used to study cyanobacteria such as detection and characterization of cyanobacteria (Porath and Zehr, 1994), identification and phylogenetic analysis of toxigenic cyanobacteria (Neilan, 1995) and phenotypic and genotypic comparison of symbiotic and free-living cyanobacteria from single field site (West and Adams,1997). Therefore, next step aims to evaluate these strains on the basis of molecular genetic approaches.

Table 1 Morphology of cyanobacterial strain (modified from Desikachary, T V (1958) and efficiency of N_2- fixing cyanobacteria by Acetylene Reduction Assay (ARA)

A : cyanobacteria that expected to be *Nostoc sp*

	Cell Morphology																Colony on BG_{11} solid medium																		Clumping cell on BG_{11} liquid medium										ARA	
	Colour						Size of vegetative cell (um)			Ratio of size vegetative			Heterocyst cell			Location of heterocyst cell			Colour						Size of colony on 4 weeks (cm)			Form					Elevation		Edge				Colour						Broth						
Strains	Green	Pale-green	Olive-green	Brownish	Yellowish-brown	Reddish-purple	0-1	1-2	3-5	>5	W>L	W<L	W=L	Spherical	Elliptical	Quadrate	Terminal	Intercalary	Both	Green	Pale-green	Olive-green	Brownish	Yellowish-brown	Reddish-brown	0-1.5	1.5-3.5	4.0-7.5	>7.5	Circular	Irregular	Filamentous	Hairy	Gas forming	Flat	Convex	Entire	Erose	Filamentous	Curled	Green	Pale-green	Olive-green	Brownish	Yellowish-brown	Reddish-brown	Ring	Grassular	Viscid	Clumping as tufts	Mat	Light	Dark
ISP		/					/					/				/			/		/									/				/	/											1.020	0.090
ISNM 5-1-1				/				/				/				/			/			/				/			/				/	/											2.715	0.458
ISNB1-28		/						/				/				/			/			/				/			/				/	/											1.899	0.251
ISNB 5-3		/						/				/				/			/			/				/			/				/	/											0.467	0.388
ISNB 1-3		/						/				/				/			/			/				/			/				/	/											1.434	0.167
ISNB 4-2		/						/				/				/			/			/				/			/				/	/											0.660	0.629
INLCR 3-4		/						/				/				/			/			/				/			/				/	/											0.530	0.447
IVLCR 3-9	/						/					/				/			/			/				/			/				/	/											0.446	0.279
INBM 5-1-2			/				/					/				/			/			/				/			/				/	/											0.209	0.948
INAM 5-3-11		/					/					/				/			/			/				/			/				/	/											0.346	0.127
INEB 1-1		/					/					/				/			/			/				/			/				/	/											0.645	1.516
INEB 7-28		/					/					/				/			/			/				/			/				/	/											0.366	0.133
ICP 3-2				/				/				/				/			/			/				/			/				/	/											1.077	0.311
ICP 3-22		/						/				/				/			/			/				/			/				/	/											0.464	0.343
ICR 1-1		/						/				/				/			/			/				/			/				/	/											1.096	1.046
ICC 3-2		/						/				/				/			/			/				/			/				/	/											1.044	0.410
ICCR 3-22		/					/					/				/	/		/			/				/			/				/	/											0.183	0.080
ICM 3-3-7				/			/					/				/			/			/				/			/				/	/											1.689	0.936
ICD 1-4		/						/				/				/			/			/				/			/				/	/											0.475	2.724
ICD 1-1		/						/				/				/			/			/				/			/				/	/											1.114	0.436
ICD 3-2	/							/				/				/			/			/				/			/				/	/											0.664	0.184
IBNR 3-2		/						/				/				/			/			/				/			/				/	/											0.199	0.044
IBNR 4-8		/						/				/				/			/			/				/			/				/	/											1.157	0.826
IBNM 5-4-1		/					/					/				/			/			/				/			/				/	/											0.440	0.060
IBNM 5-1-9		/					/					/				/			/			/				/			/				/	/											1.172	0.480

Strains	Cell Morphology																Colony on BG_{11} solid medium																						Clumping cell on BG_{11} liquid medium																						ARA	
	Colour						Size of vegetative cell (um)				Ratio of size vegetative			Heterocyst cell			Location of heterocyst cell			Colour						Size of colony on 4 weeks (cm)				Form				Elevation		Edge				Colour						Brush						umol/C2H4/mg of Chlorophyll a/h	
	Green	Pale-green	Olive-green	Brownish	Yellowish-brown	Reddish-purple	0-1	1-2	2-3	>3	W>4	W=4	W<4	Spherical	Ellipsoid	Quadrate	Terminal	Intercalary	Both	Green	Pale-green	Olive-green	Brownish	Yellowish-brown	Reddish-brown	0-0.5	0.5-1.0	1.0-1.5	>1.5	Circular	Irregular	Filamentous	Hairy	Fuzz forming	Flat	Convex	Entire	Erose	Filamentous	Curled	Green	Pale-green	Olive-green	Brownish	Yellowish-brown	Reddish-brown	Ring	Granular	Viscid	Clumping on wire	Mat	Light	Dark
BSNM 2-4-1		/						/					/			/					/						/												/										/	0.856	0.123
BNB 1-5		/						/					/			/			/		/						/												/						/	/	/	/	0.091	0.060
BNCY 1-4	/						/						/	/				/		/	/						/												/						/	/	/	/	0.086	0.100
BNBF 2-4		/					/						/	/				/			/						/												/						/	/	/	/	0.108	0.201
BNEC 4-21		/						/	/				/	/				/			/						/												/						/	/	/	/	0.227	0.307
BDYEC 3-29	/							/					/	/				/	/	/							/												/						/	/	/	/	0.622	0.058
BNEC 2-13			/				/						/	/				/	/	/							/												/						/	/	/	/	2.357	0.293
BNEM 1-1-4	/							/					/	/				/	/		/						/												/						/	/	/	/	0.053	0.039
BDYEM 3-2-40		/		/			/						/	/			/										/												/						/	/	/	/	0.162	0.174
BNEM 3-1-3	/						/						/	/				/		/							/												/						/	/	/	/	0.677	0.398
BNEA 1-1			/					/					/		/			/		/							/												/						/	/	/	/	0.549	0.068
BNEA 2-1				/				/					/	/				/									/												/						/	/	/	/	0.595	0.095
BNEC*1-20		/					/						/	/				/	/	/							/												/						/	/	/	/	4.481	0.050
BDYEC*1-3		/					/						/	/				/	/	/							/												/						/	/	/	/	0.624	0.071
BECB 2-1		/					/						/	/			/										/												/						/	/	/	/	0.858	0.047
BECM 3-1					/		/						/	/			/										/												/						/	/	/	/	0.551	1.884
BNCA 1-33		/					/						/	/				/		/							/												/						/	/	/	/	0.476	0.124
BCB 3-27	/						/						/	/				/		/							/												/						/	/	/	/	0.233	0.047
BNCD 1-17		/					/						/	/			/			/							/												/						/	/	/	/	0.025	0.215
BNEA 3-11	/						/						/	/				/		/							/												/						/	/	/	/	1.551	0.351

B : cyanobacteria that expected to be *Anabaena* sp.

| Strains | Cell Morphology | | |

	Cell Morphology																				Colony on BG_{11} solid medium																								Clumping cell on BG_{11} liquid medium												ARA	
	Colour						Size of vegetative cell (um)				Ratio of size vegetative cell			Heterocyst cell			Location of heterocyst cell			Colour						Size of colony on 4 weeks (cm)				Form					Elevation		Edge				Colour						Broth						umolC2H4/mg of Chlorophyll a/h	
Strains	Green	Pale-green	Olive-green	Brownish	Yellowish-brown	Redish-purple	0-1	1-3	3-5	>5	W>L	W<L	W=L	Spherical	Elliptical	Quadrate	Terminal	Intercalary	Both	Green	Pale-green	Olive-green	Brownish	Yellowish-brown	Redish-brown	0-0.5	0.5-0.8	0.8-1.1	>1.1	Circular	Irregular	Filamentous	Hairy	Gas forming	Flat	Convex	Entire	Erose	Filamentous	Curled	Green	Pale-green	Olive-green	Brownish	Yellowish-brown	Redish-brown	Ring	Granular	Viscid	Clumping on tub	Mat	Light	Dark
III NM 3-3-22				/			/					/			/		/						/						/		/					/			/					/			/			/		0.980	0.076
III NM 3-3-13				/				/				/		/					/					/						/						/	/							/			/		/	/		0.285	0.061
III NA 3-2		/						/				/		/			/			/						/				/						/	/				/						/		/	/		0.038	0.000
III NB 3-3		/					/					/		/					/		/						/				/					/		/			/						/			/		0.492	0.231
III NBA 3-7		/						/				/			/		/					/										/							/			/					/				/	0.172	0.272
III NEC B 4-22				/					/			/			/			/					/						/		/					/			/					/								0.542	0.000
III NEA 2-6 1/2				/				/				/		/				/					/			/					/				/			/						/					/	/		0.698	0.043
III NBB 2-2	/						/						/		/			/			/											/							/		/							/		/		0.558	0.021
III NBD 2-9				/					/			/			/				/					/							/					/		/						/				/		/		0.052	0.000
III CF 3-26				/				/				/			/			/						/				/			/					/				/				/					/	/		0.215	0.117
III CR 1-2		/						/				/			/				/		/											/							/		/						/			/		0.671	0.037
III CC 1-32				/					/			/		/				/					/					/			/					/				/				/				/		/		0.061	0.096
III CCR 4-24	/								/			/			/			/		/									/		/				/				/			/					/	/				0.168	0.116
III CM 1-3-46				/					/			/		/					/				/			/				/				/		/	/							/			/	/				1.881	0.331
III CB 3-3		/						/				/			/				/			/							/		/				/					/			/					/	/			0.964	0.000
III CB 3-2	/							/				/			/		/			/						/					/				/					/							/		/	/		0.949	0.055
III CC 1-10		/						/				/			/				/	/						/				/					/			/				/					/		/	/		0.617	0.026

C : cyanobacteria that expected to be *Anabaenopsis* sp

	Cell Morphology																			Colony on BG_{11} solid medium																								Clumping cell on BG_{11} liquid medium											ARA	
	Colour						Size of vegetative cell (um)				Ratio of size vegetative			Heterocyst cell			Location of heterocyst cell			Colour						Size of colony on 4 weeks (cm)				Form					Elevation		Edge				Colour						Broth					umolC2H4/mg of Chlorophyll a/h	
Strains	Green	Pale-green	Olive-green	Brownish	Yellowish-brown	Redish-purple	0-1	1-3	3-5	>5	W>L	W<L	W>L	Spherical	Elliptical	Quadrate	Terminal	Intercalary	Both	Green	Pale-green	Olive-green	Brownish	Yellowish-brown	Redish-brown	0-0.5	0.5-0.8	0.8-1.1	>1.1	Circular	Irregular	Filamentous	Hairy	Gas forming	Flat	Convex	Entire	Erose	Filamentous	Curled	Green	Pale-green	Olive-green	Brownish	Yellowish-brown	Redish-brown	Ring	Granular	Viscid	Clumping on tube	Mat	Light	Dark
INEE 3-1				/					/			/			/							/				/				/						/	/							/			/	/				1.499	0.357
ICF - 2				/					/			/			/			/				/				/				/						/		/						/			/	/		/		0.521	0.396
IBNCR 4-1				/					/			/			/				/			/				/				/						/	/							/				/				0.284	0.000
IBHM 1-2-2				/				/				/		/				/					/		/					/					/		/						/					/	/		0.334	0.125
IBNEC 2-1				/				/				/		/			/						/									/												/			/		/	/		1.450	0.112

D : cyanobacteria that expected to be *Nodularia* sp.

	Cell Morphology																			Colony on BG_{11} solid medium																								Clumping cell on BG_{11} liquid medium											ARA	
	Colour						Size of vegetative cell (um)				Ratio of size vegetative cell			Heterocyst cell			Location of heterocyst cell			Colour						Size of colony on 4 weeks (cm)				Form					Elevation		Edge				Colour						Broth					umolC2H4/mg of Chlorophyll a/h	
Strains	Green	Pale-green	Olive-green	Brownish	Yellowish-brown	Redish-purple	0-1	1-3	3-5	>5	W>L	W<L	W>L	Spherical	Elliptical	Quadrate	Terminal	Intercalary	Both	Green	Pale-green	Olive-green	Brownish	Yellowish-brown	Redish-brown	0-0.5	0.5-0.8	0.8-1.1	>1.1	Circular	Irregular	Filamentous	Hairy	Gas forming	Flat	Convex	Entire	Erose	Filamentous	Curled	Green	Pale-green	Olive-green	Brownish	Yellowish-brown	Redish-brown	Ring	Granular	Viscid	Clumping on tub	Mat	Light	Dark
INEA 1-2-2	/							/					/			/		/			/					/							/								/										/	0.172	0.072
ICM 3-1-3		/						/			/					/		/			/					/							/								/							/	/		0.353	0.647
ICM 3-1-3					/				/				/			/		/					/						/			/			/				/					/			/				0.495	0.124

F : cyanobacteria that expected to be branching group

	Cell Morphology																			Colony on BG_{11} solid medium																								Clumping cell on BG_{11} liquid medium											ARA	
	Colour						Size of vegetative cell (um)				Ratio of size vegetative cell			Heterocyst cell			Location of heterocyst cell			Colour						Size of colony on 4 weeks (cm)				Form					Elevation		Edge				Colour						Broth					tumolC2H4/mg of Chlorophyll a/h	
Strains	Green	Pale-green	Olive-green	Brownish	Yellowish-brown	Redish-purple	0-1	1-3	3-5	>5	W>L	W<L	W=L	Spherical	Elliptical	Quadrate	Terminal	Intercalary	Both	Green	Pale-green	Olive-green	Brownish	Yellowish-brown	Redish-brown	0-0.5	0.5-0.8	0.8-1.1	>1.1	Circular	Irregular	Filamentous	Hairy	Gas forming	Flat	Convex	Entire	Erose	Filamentous	Curled	Green	Pale-green	Olive-green	Brownish	Yellowish-brown	Redish-brown	Ring	Granular	Viscid	Clumping on tub	Mat	Light	Dark
INER 4-4		/							/				/			/		/			/					/							/								/							/			0.503	0.504
ICM 1-2-21			/						/				/			/		/				/				/					/					/		/				/							/			1.182	0.826
ICM 3-1-1		/							/				/			/		/		/						/							/								/							/	/		0.358	0.437
ICM 3-4-2		/							/			/		/				/				/				/							/								/							/			0.475	1.690
IBCC* 3-1-1-2		/						/				/			/			/		/												/									/						/				0.750	0.042

F : reference strains

	Cell Morphology																			Colony on BG_{11} solid medium																								Clumping cell on BG_{11} liquid medium											ARA	
	Colour						Size of vegetative cell (um)				Ratio of size vegetative cell			Heterocyst cell			Location of heterocyst cell			Colour						Size of colony on 4 weeks (cm)				Form					Elevation		Edge				Colour						Broth					tumolC2H4/mg of Chlorophyll a/h	
Strains	Green	Pale-green	Olive-green	Brownish	Yellowish-brown	Redish-purple	0-1	1-3	3-5	>5	W>L	W<L	W=L	Spherical	Elliptical	Quadrate	Terminal	Intercalary	Both	Green	Pale-green	Olive-green	Brownish	Yellowish-brown	Redish-brown	0-0.5	0.5-0.8	0.8-1.1	>1.1	Circular	Irregular	Filamentous	Hairy	Gas forming	Flat	Convex	Entire	Erose	Filamentous	Curled	Green	Pale-green	Olive-green	Brownish	Yellowish-brown	Redish-brown	Ring	Granular	Viscid	Clumping on tub	Mat	Light	Dark
Nostoc sp		/							/			/		/			/			/											/									/								/			0.048	0.000
A. cylindrica		/							/			/			/		/				/										/									/						/					1.217	0.225
Aegolosiphon sp	/									/	/					/		/		/							/						/								/						/		/	/		2.152	0.467
Calothrix sp					/				/	/	/				/		/						/			/			/		/				/						/				/					/			1.108	0.212
Scytonema sp	/						/					/				/		/		/						/							/								/						/				/	0.386	0.000

2. Efficiency of N_2-fixing cyanobacteria

Acetylene reduction assay (ARA) of nitrogenase enzyme was used to determine efficiency of N_2-fixing cyanobacteria in light and dark condition. Most of 102 cyanobacterial isolates able to fix N_2 in both of light and dark conditions. All the major isolates were heterocystous cyanobacteria included, species capable of fixing N_2. However, as noted before, many filamentous nonheterocystous forms, such as *Plectonema boryanum*, can fix N_2 under anaerobic or microaerobic conditions (Haselkorn and Buikema, 1992, quoted in Rippka and Waterbury, 1977 and Stewart, 1980). Although this ability may be significant in nature (e.g., in particular layers of complex mats or during the night), it has not extensively been studied in the laboratory in recent years. Several unicellular species have attracted more attention. It was reported that marine *Synechococcus* could carry out photosynthesis and N_2 fixation in the same cell (Haselkorn and Buikema, 1992, quoted in Mitsui et al., 1986). However, N_2-fixing cyanobacteria isolated in this study, no any unicellular form was found.

Earlier work on *Gloeocapsa* (Haselkorn and Buikema, 1992, quoted in Wyatt and Silvey, 1969) and *Gloeothece*, which grow extremely slow, made the same phenomena (Haselkorn and Buikema, 1992, quoted in Gallon, 1980). Subsequently, it was shown that when the cells are synchronized with a light/dark cycle, photosynthesis is confined to the daytime as expected, but N_2 fixation occurs extensively at night (Haselkorn and Buikema, 1992, quoted in Mitsui et al. and Mullineaux, Chaplin and Gallon, 1980). The latter result was unanticipated because extensive studies of *Anabaena* showed that light (and accompanying CO_2 fixation and ATP production) was essential for N_2 fixation. The unicellular cyanobacteria, in contrast, accumulate enough carbohydrate during the day to fuel N_2 fixation most of the night. As dawn approaches, the carbohydrate reserve was exhausted and N_2 fixation stop. Cessation of N_2 fixation during the day was not due to inactivation of nitrogenase by photosynthetically generated O_2. However, in *Gloeothece*, O_2 is required for N_2 fixation both in the dark and in the light (Haselkorn and Buikema, 1992, quoted in Maryan, Eady, Chaplin and Gallon, 1986). It appears that oxidative phosphorylation is the major source of both ATP and reductant for nitrogenase in that organism. The circadian appearance of nitrogenase had also been observed in a fresh water *Synechococcus* isolated form a rice paddy. In this organism it had been shown further that the diurnal appearance of nitrogenase activity is accompanied by synthesis of *nifHDK* messenger RNA, so it most likely that nitrogenase was destroyed during the (oxygenic) daytime and must be synthesized a new each night (Haselkorn and Buikema, 1992, quoted in Huang, Chow and Hwang, 1988). *Trichodesmium thiebautii*, nonheterocystous cyanobacteria could fix nitrogen in the height rates during daylight hours,

when photosynthesis activity is maximal. This reasons might be due to 1) cells along the trichome of *Trichodesmium* spp. were differentiates with respect to oxygen evolution and nitrogen fixation activity or 2) the structure of individual cells were specially separated within the cells. Whereas, *Gloeothece* spp., *Oscillatoria* spp. and *Synechococcus* sp. strain RF-1 were fixed nitrogen in dark condition with low oxygen concentration.

Results obtained from this study indicated that in light condition 48.54% of total cyanobacterial isolates could fix N_2 in low concentration (0.0-0.5 $\mu molC_2H_4$/mg of chlorophyll a/h), 28.16% medium concentration (0.51-1.0 $\mu molC_2H_4$/mg of chlorophyll a/h) and 23.30% high concentration (more than 1.0 $\mu molC_2H_4$/mg of chlorophyll a/h). However, in dark condition, 83.50%, 10.68% and 5.82% of total isolates able to fix in low, medium and high nitrogen concentration, respectively. From Table 2 and 3 showed that cyanobacterial isolates could mainly fix N_2 in low concentration both of light and dark conditions. In light condition, 20.39% of *Nostoc* sp., 20.39% of *Anabaena* sp., 1.94% of *Anabaenopsis* sp., 1.94% of branching group and 3.88% of *Nodularia* sp. fixed nitrogen in low concentration. It was similar to dark condition which 36.89%, 37.86%, 4.85%, 1.94%, 1.94% of *Nostoc* sp., *Anabaena* sp., *Anabaenosis* sp., branching group and *Nodularia* sp., respectively. *Anabaena* sp. could fix N_2 in medium concentration as 14.57% in light condition, *Nostoc* sp. as 10.68%, branching group as 1.94 and *Anabaenopsis* sp. 0.97%. Under dark condition, *Anabaena* sp. was 3.88%, *Nostoc sp*. 2.91%, *Nodularia* sp. 1.94% and branching group also 1.94% in medium ranging of N_2-fixation. For high concentration of fixed N_2, *Nostoc* sp. was 12.62%, *Anabaena* sp., 7.77%, *Anabaenopsis* sp., 1.94% and branching group was 0.97% in light condition while in dark condition, *Nostoc* sp. was 3.88%, *Anabaena* sp. 0.97% and branching group was also 0.97%.

From this study, the groups that could fix N_2 more than 0.5 $\mu molC_2H_4$/mg of chlorophyll a/h were mostly belong to *Nostoc* sp. and *Anabaena* sp. (Table 3.2). 56% of all cyanobacteria at mountain area able to fix N_2 more than 0.5 $\mu mol/C_2H_4$/mg of chlorophyll a/h. For flat area of agricultural practice (field crop cultivation, rice cultivation, rice in rotation with other crops) were 52.94% of all cyanobacteria which could perform N_2-fixing in rather high range and 50% in uncultivated area. And cyanobacterial isolates from undisturb forest, forest clearance for crop cultivation for 1 and more than 2 years and in the area where intensive agricultural production using high rate of pesticides and fertilizers as vegetable planting area were 47.22%. Furthermore, some strain such as I NM3-1-1 could fix nitrogen under dark more than light condition might be accumulated ATP from day time for fix nitrogen on night time as well as some unicellular cyanobacteria. This study indicated that forest clearance for crop

cultivation more than 1 year without maintenance could decreased diversity of cyanobacteria. Roger, Zimmerman and Lumpkin (1992) found that ability of N_2 fixation based on condition such as *Anabana* sp, *Nostoc* sp and *Calothrix* sp fixed nitrogen very well on high humidity condition. Population of cyanobacteria about $1x10^2$-$8x10^6$ cell per 1 gram of soil, could fix nitrogen for rice about 10-20 kgN/ha, moreover pH and available P also influences to the population of cyanobacteria. From this study, cyanobacterial strains in high P available area were examined. Most of cyanobacterial strains in those areas were capable of fixing nitrogen higher than other areas such as cyanobacterial strain INC4-21 from field crop cultivation area in northern part found high amount of phosphorus (about 70 ppm) could fix nitrogen as high concentration as 1.443 and 0.626 $\mu molC_2H_4$/mg of chlorophyll a/h in light and dark condition, respectively, as well as IND1-3 from vegetable planting area in northern part (153 ppm of phosphorus rate) able to fix nitrogen 1.434 and 0.167 $\mu molC_2H_4$/mg of chlorophyll a/h in light and dark condition, respectively. On the other hand, INEM 3-3-11 from foot hill of mountain in north-eastern part contained 4 ppm of phosphorus could fix nitrogen with concentration as 0.346 and 0.127 μ $molC_2H_4$/mg of chlorophyll a/h in light and dark conditions, respectively. These might due to phosphorus was component of ATP which required for nitrogen fixation processes, it could activated nitrogenase enzyme for changing N_2 to NH_3.

Table 3 Ranking Number of cyanobacterial strains were separated depend on efficiency of N_2-fixation by Acetylene Reduction Activities (ARA's)

ARA (unol C2H4/mg of chlorophyll a/h)	Genus	No. of cyanobacterial strains		Habitat	No. of cyanobacterial strains					
					Light			Dark		
		Light	Dark		Northern	North-eastern	Central	Northern	North-eastern	Central
more than 1.0	*Nostoc sp.*	13	4	F	1	-	1	-	-	-
	Anabaena sp.	8	1	C	1	2	2	-	-	-
	Anabaenopsis sp.	2	-	R	2	1	1	-	-	1
	Nodularia sp.	-	-	CR	1	-	-	-	-	-
	Branching group	1	1	M1	1	-	2	-	-	-
				M2	1	-	1	-	-	-
				M3	1	1	1	1		2
				A	-	1	-	-	-	-
				B	1	-	-	-	1	-
				C*	-	-	-	-	-	-
				D	1	-	1	-	-	1
0.51-1.0	*Nostoc sp.*	11	3	F	-	1	1	-	1	-
	Anabaena sp	15	4	C	-	1	-	-	1	-
	Anabaenopsis sp.	1	-	R	1	1	1	-	1	-
	Nodularia sp.	-	1	CR	2	2	-	-	-	-
	Branching group	2	2	M1	-	-	-	1	-	1
				M2	2	-	-	-	-	1
				M3	2	1	1	-	1	1
				A	-	3	-	-	-	-
				B	-	2	2	-	-	-
				C*	1	1	2	-	-	-
				D	1	-	1	1	1	-
0.0-0.5	*Nostoc sp.*	21	38	F	1	1	2	2	1	4
	Anabaena sp	21	39	C	2	2	1	3	4	3
	Anabaenopsis sp.	2	5	R	2	1	1	5	2	2
	Nodularia sp	3	2	CR	3	1	3	6	3	3
	Branching group	2	2	M1	2	1	-	2	1	1
				M2	-	-	1	3	-	1
				M3	1	3	3	3	4	2
				A	2	3	2	2	5	2
				B	2	3	1	3	2	3
				C*	1	1	-	2	2	2
				D	3	2	2	4	1	3

F = uncultivation area
C = field crop cultivation
R = rice cultivation
CR = rice in rotation with other crop

M1 = the foot hill of mountain
M2 = the middle of mountain
M3 = the top of mountain

A = undisturb forest
B = forest clearance for crop cultivation for 1 year
C* = forest clearance for crop cultivation more than 1 year
D = vegetable garden

3. *nifH* gene profile analysis

Since nitrogenase known as the key enzyme in these N_2-fixing cyanobacterial isolates, thus to determine the diversification on the basis of *nif* genes profiles was conducted. *NifH* was recognized as the Fe protein production (Porath and Zehr, 1994). Therefore, *nifH* primer was used throughout this study. Two different sets of degenerate oligonucleotide primers were used for the PCR amplification of the *nifH* gene fragment. Primer 1 (5'-GGAATTCCTGYGAYCCNAARGCNGA-3'), located at positions 318 to 335 in reference to the *Anabaena* sp. strain PCC 7120 *nifH* sequence. Primer 2 (5'-CGGATCCGDNGCCATCATYTCNCC-3'), located in positions 659 to 674 of the opposite strand. Amplification of DNA sample from 107 cyanobacterial isolates (included 5 reference strains) produced one to six amplification products depend on each strains (fig. 4). The *Hapalosiphon sp*. DASH 05101 yielded a single major band, 330 bp. Two band products (830 and 330 bp) were generally observed with the *Calothrix sp*. DASH 02101, as in *Scytonema sp*., as well as, *Anabaena cylindrica*, but from *Nostoc* sp. gave five bands (830, 446, 330, 208 and 182 bp) (fig. 3.1). This main PCR product found in size about 330 bp fragment which nearly reported by Porath and Zehr (1994) as 359 bp fragment. All cyanobacterial strains were distinguished into 77 different groups and only four strains did not contain major band at 330 bp fragment such as I CF3-22 (*Nostoc* sp.), I CD1-1 (*Nostoc* sp.), I NM3-1-3 (*Anabaena* sp.) and III NCR4-1 (*Anabenopsis* sp.). When they were estimated relationship between the *nifH* gene, a similarity matrix, used to construct a dendrogram based on the UPGMA algolithm (fig. 3.2), the results were in agreement with the classification of the strains into 4 main groups and one most distinctive strain (III NCR4-1). From this results, every genera of cyanobacteria seemed to scattered in the main cluster. Each of genus was separately analyzed by using *nifH*-PCR products, *Nostoc* sp. group (fig. 3.2) was grouped into 2 main clusters from 46 strains (included *Nostoc* sp.), as well as *Anabaena* sp. group (fig, 3.2) was grouped into 2 main clusters from 45 strains (include *Anabaena cylindrica*). In addition *Anabaenopsis* sp. group (fig. 3.2), *Nodularia* sp. group (fig. 3.2) and branching group (fig. 5F) were also determined and found clearly distinguished among groups. Comparing between relationship of each cyanobacteria based on *nifH* fragment and efficiency of nitrogen fixation indicated that mostly member group of *nifH* fragment which similarity related with *Anabaena, cylindrica* and contained PCR products in size about 380, 330, and 290 bp. could fix N_2 more than 0.5 $\mu molC_2H_4$/mg of chlorophyll, a/h, thus, *nifH* fragment might used as prediction tool for efficiency of nitrogen fixation .

Then, efficiency of nitrogen fixing in each cluster was compared each other and detected that some cyanobacterial isolates in the same cluster could similarly fix nitrogen such as III NEF3-4; 0.108

and 0.201 and III NR2-2; 0.109 and 0.044 μmolC_2H_4/mg of chlorophyll a/h in light and dark condition, respectively, whereas some clusters could unlikely fix nitrogen such as III NEA 2-11; 1.851 and 0.351 and INA 2-1;0.201 and 0.005 μmolC_2H_4/mg of chlorophyll a /h in light and dark conditions, respectively. Although Porath and Zehr (1994) reported that *Anabaena oscillarioides* (M63686) , *Anabaena* sp. strain CA, *Nostoc* sp and *Nostoc muscorum* had similarly *nifH* sequences, thus, *nifH* fragment could not use to clusterize the diversification of N_2-fixation cyanobacteria. Moreover, Zehr and McReynold (1989) reported that the DNA sequence of *nifH* gene was 66% to 79% similar to the corresponding nucleotide sequence of *nifH* gene from an *Anabaena* sp. (Mevarech, Rice and Haselkorn, 1980), *Rhizobium meliloti* (Torok and Kondorosi, 1981), *Clostridium pasteurianum* (Chen, K.C.N., Chen, J.S. and Johnson, 1986), *Azotobacter vinelandii* (Brigle, Newton and Dean, 1985), *Klebsiella pneumoniae* (Scott, Rolfe and Shine, 1981 and Sundaresan and Ausubel, 1981) and *Rhodobacter* (*Rhodospseudomonas*) *capsulatus* (Schumann and Waitches, 1986). Furthermore, Zehr and Porath (1994) also suggested that DNA sequence of a *nifH* fragment amplified from several species of heterocystous and nonheterocystous cyanobacteria compared with corresponding sequences from other cyanobacterial, eubacterial and archaeobacterial sequences were closely strains which based on types of bacteria. Subsequently, *nifH* gene was difficult for distinguishing each genus of cyanobacteria thus, random primers were used for further investigation.

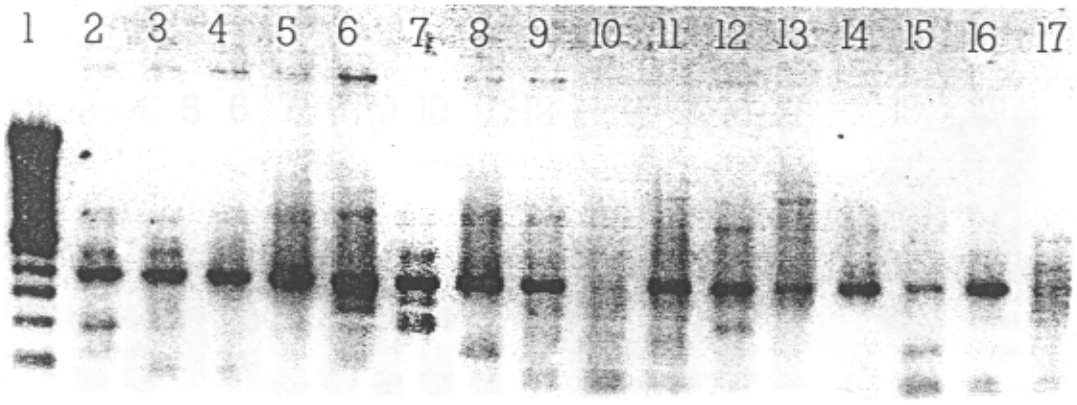


Fig. 3.1A *NifH*-PCR fingerprint patterns of *Nostoc* sp. group (Table 3.1A) with genomic DNA as the template. Lane 1-17; 100 bp DNA ladder marker, *Nostoc sp*, *Anabaena cylindrica*, *Hapalosiphon sp.* DASH 05101, *Calothrix sp.* DASH 02101, *Scytonema sp.*, III NM 1-4-1, II NEM 1-4-8, III NM 2-1-9, III NM 2-4-1, I CM 2-3-7, I NM 3-1-1, I NEM 3-1-3, I NEM 3-1-4, I NEM 3-3-11, II NEM 3-2-40 and III CM 3-1, respectively.

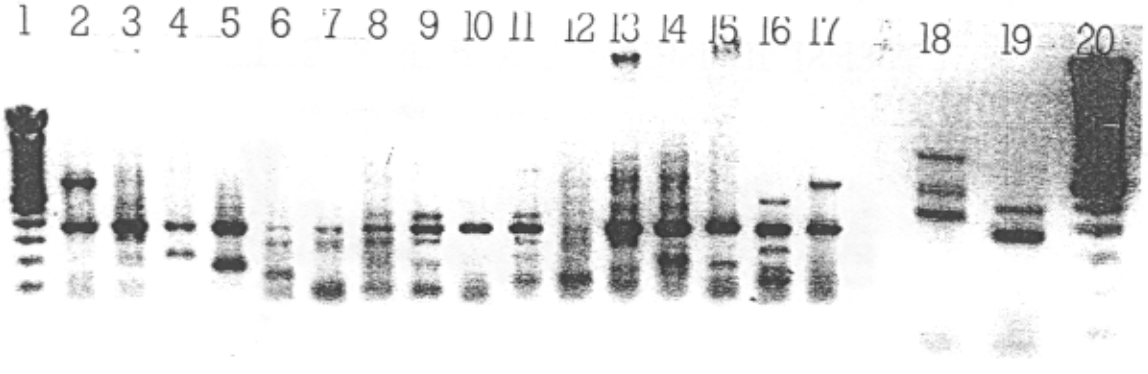


Fig. 3.1B *NifH*-PCR fingerprint patterns of *Nostoc sp.* group (Table 3.1A) with genomic DNA as the template. Lane 1-20; 100 bp DNA ladder marker, I NEA 1-2/2, III NEA 1-1, III NEA 2-11, III NEA 2-5, III CA 1-33, I NB 2-3, I NB 1-20, III NB 1-3, I NEB 1-1, I NEB 2-20, II CB 2-27, III NC* 1-4, II NEC* 1-20, I ND 1-3, I CD 2-2, I CD 1-14, I CD 1-1, III CD 1-17, and 100 bp DNA ladder marker, respectively.

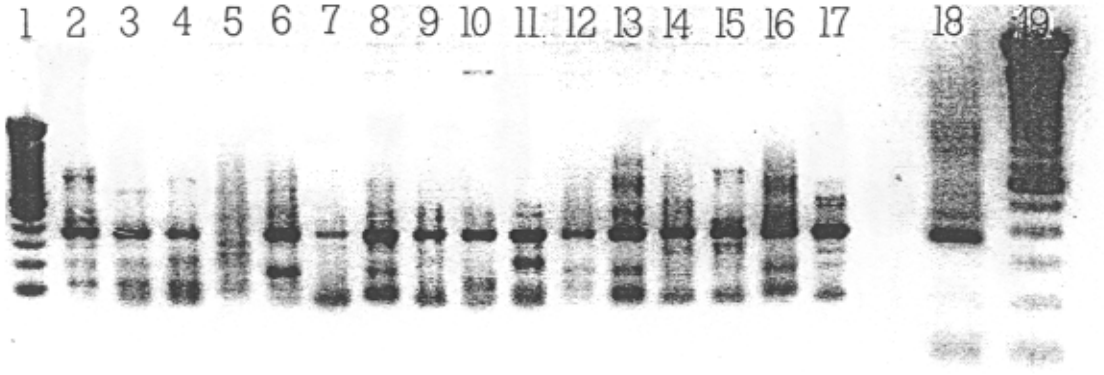


Fig. 3.1C *NifH*-PCR fingerprint patterns of *Nostoc sp.* group (Table 3.1A) with genomic DNA as the template. Lane 1-19, 100 bp DNA ladder marker, INF, INEF 4-2, III NEF 3-4, ICF 3-22, ICF 2-2, II NEC 4-12, III NEC* 1-3, III NEC 3-29, III NEC 2-13, ICC 2-2, I NECR 3-9, I NECR 3-4, I CCR 3-22, II NR 4-8, III NR 2-2, I CR 1-1, III CR 2-1 and 100 bp DNA ladder marker, respectively.

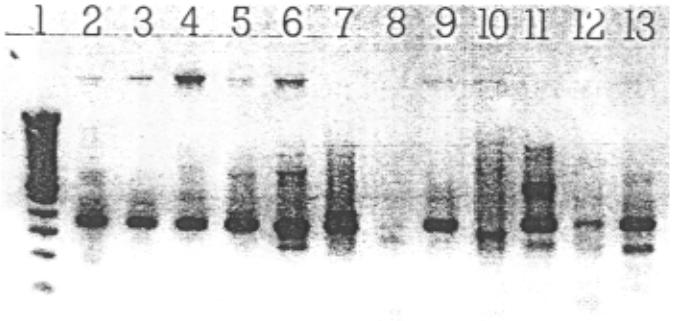


Fig. 3.1D *NifH*-PCR fingerprint patterns of *Anabaena sp.* group (Table 3.1A) with genomic DNA as the template. Lane 1-13; 100 bp DNA ladder marker, *Nostoc sp.*, *Anabaena cylindrica*, *Hapalosiphon sp.* DASH 05101, *Calothrix sp.* DASH 02101, *Scytonema sp.*, I NM 1-1-3, II CM 1-3-40, I NM 2-4-1, I NM 3-1-3, II NM 3-3-13, I NEM 3-3-1 and I CM 3-1-1, respectively.

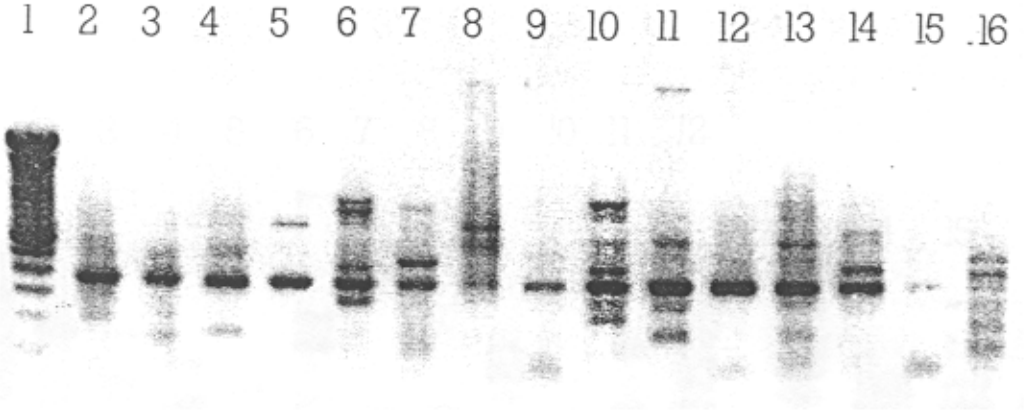


Fig. 3.1E *NifH*-PCR fingerprint patterns of *Anabaena sp.* group (Table 3.1A) with genomic DNA as the template. Lane 1-16; 100 bp DNA ladder marker, I NA 2-1, II NA 2-2, III NEA 2-6 1/2, I CA 2-1, III NEB 2-2, III CB 2-1, III CB 2-2, I NC* 2-6, III CC* 2-10, I ND2, I ND 1-14, I ND 2-3, III ND 2-1, I NED 1-2 and III NED 2-9, respectively.

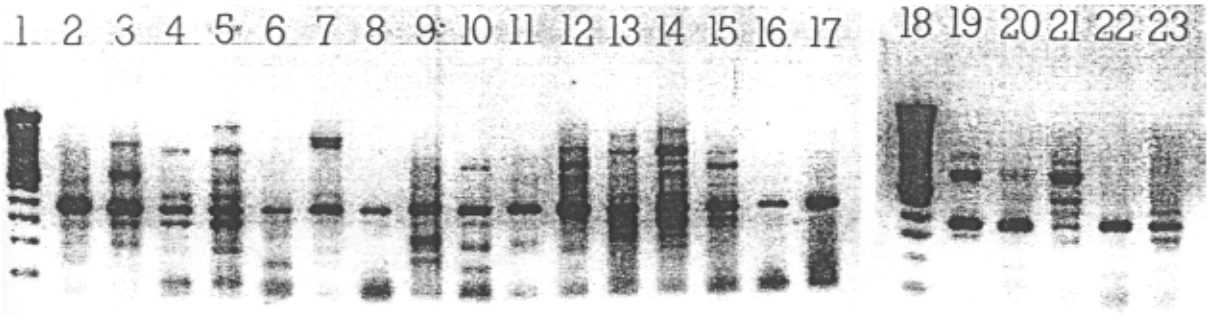


Fig. 3.1F *NifH*-PCR fingerprint patterns *of Anabaena sp.* group (Table 3.1A) with genomic DNA as the template. Lane 1-23; 100 bp DNA ladder marker, III NF 1-4, III CF 3-20, I NC 4-21, I NC 2-1, I NC 3-20, I NEC 4-1, I CC 3-1, III CC 1-22, I NCR 2-3, I NCR 2-3.1, I NCR 4-2, I NCR 3-1, III NCR 1-11, IIINECR 4-22, I CCR 3-22, III CCR 4-24, 100 bp DNA ladder marker, I NR 1-6, I NR 2-2, IV NR 3-9, III NER 2-7 and II CR 1-2, respectively.

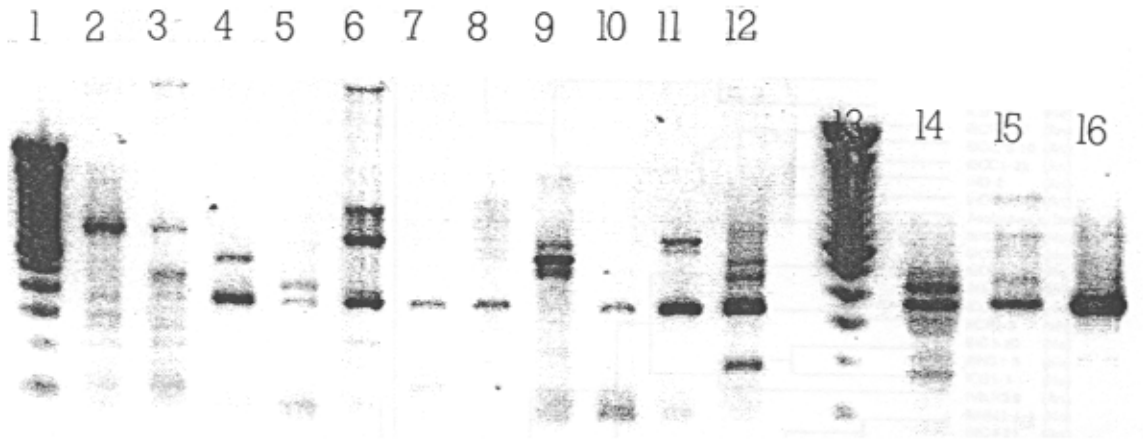
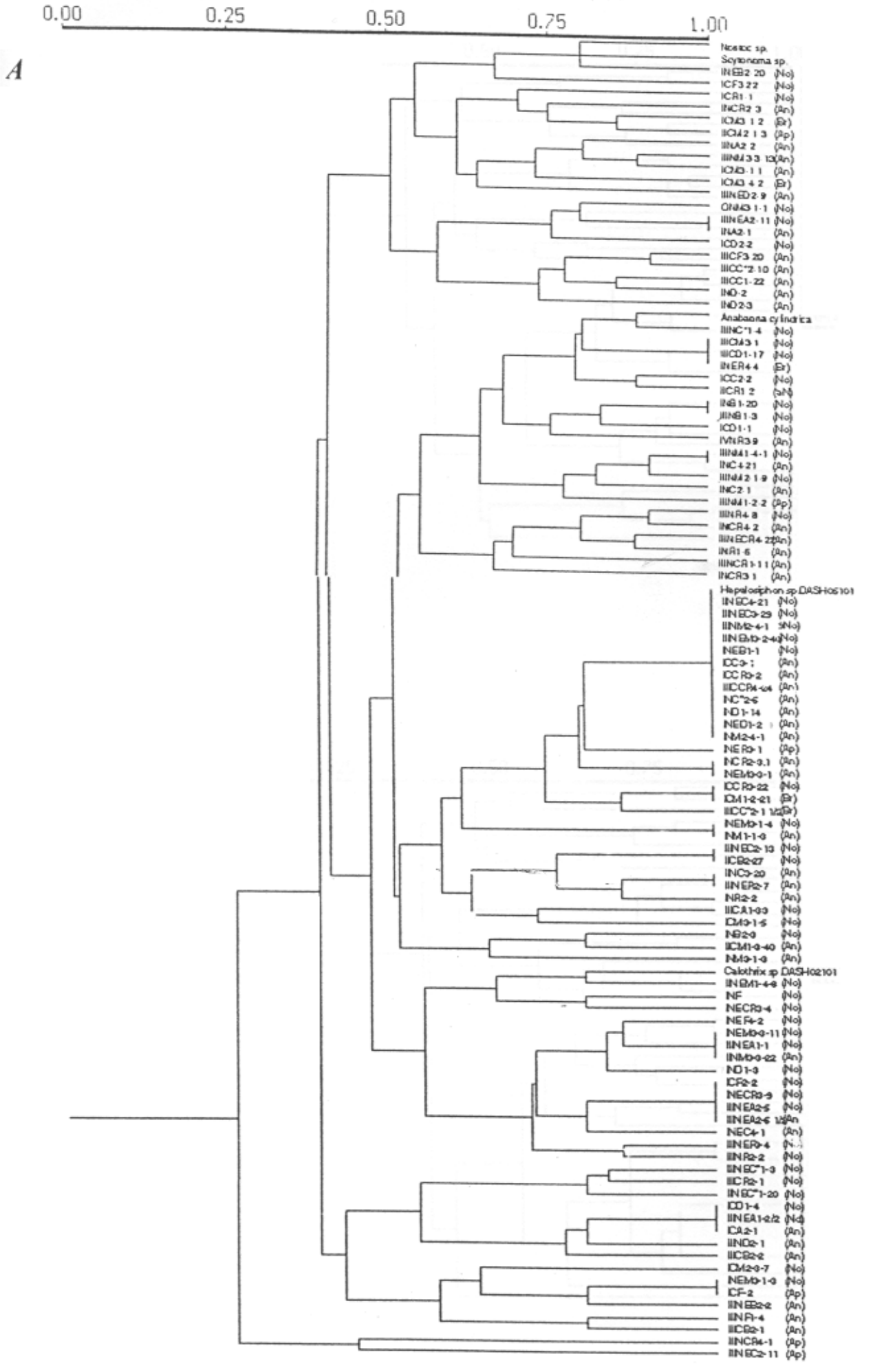
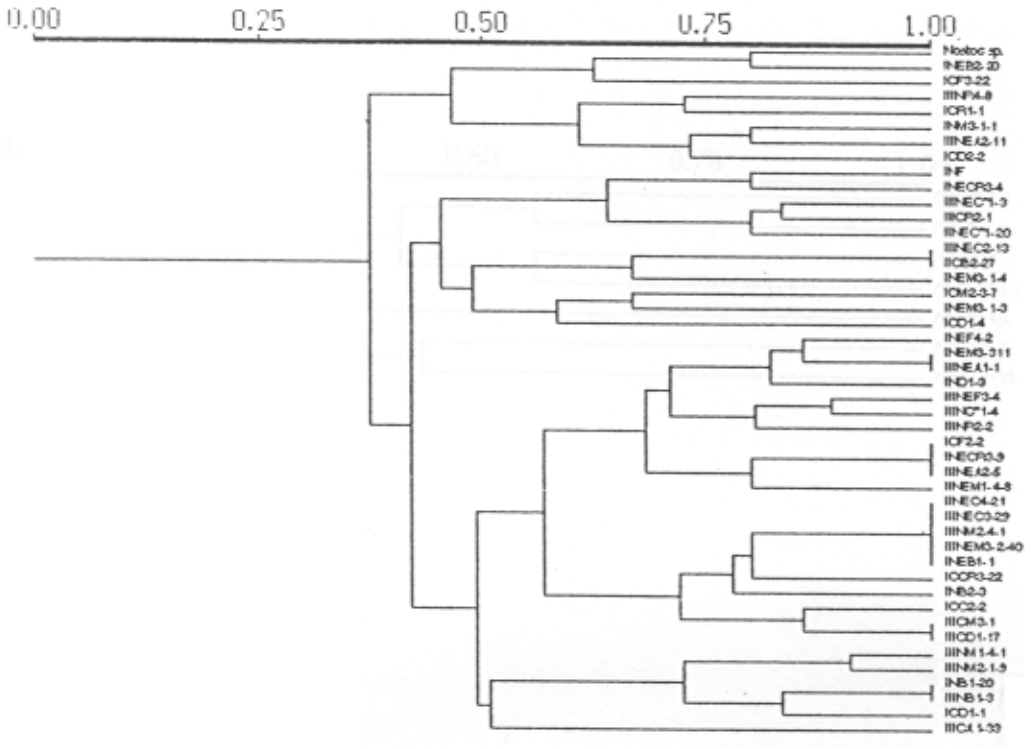


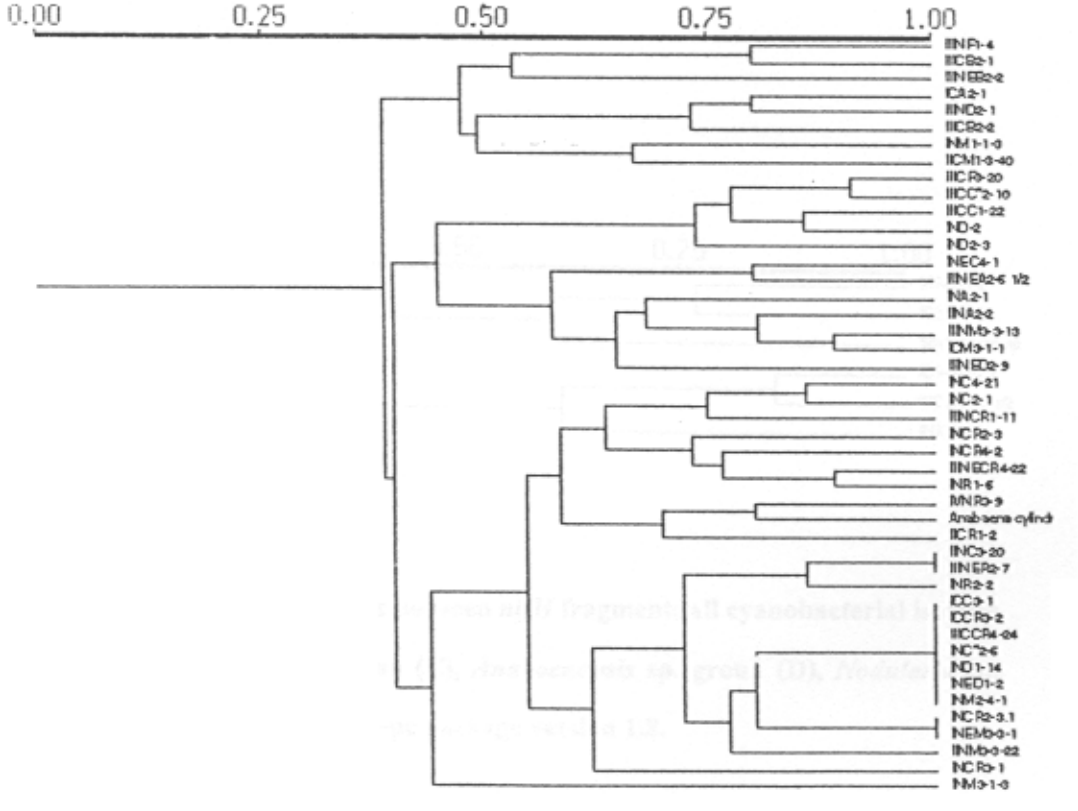
Fig. 3.1G *NifH*-PCR fingerprint patterns of other group (Table 3.1C, 3.1D and 3.1E) with genomic DNA as the template. Lane 1; 100 bp DNA ladder marker, lane 2-5 (branching group); I CM3-1-2, I CM 3-4-2, I CM1-2-21, I NER 4-4, lane 6 and 7 (*Nodularia* sp.); I CM 3-1-5, I CA 1-10, lane 8-12 (*Anabaenopsis* sp.); I CF -2, III NCR 4-1, III NM 1-2-2, I NER 3-1, III NEC 2-11, lane 13; 100 bp DNA ladder marker, lane 14 (*Nodularia* sp.); II CM 2-1-3, lane 15 (Branching group) III CC* 2-1 ½ and lane 16 (*Anabaena* sp.) II NM3-3-22, respectively.


A
0.00
0.25
0.50
0.75
1.00
Nostoc sp.
Scytonema sp.
Anabaena cylindrica
Hapalosiphon sp. DASH05101
Calothrix sp. DASH02101

B



C



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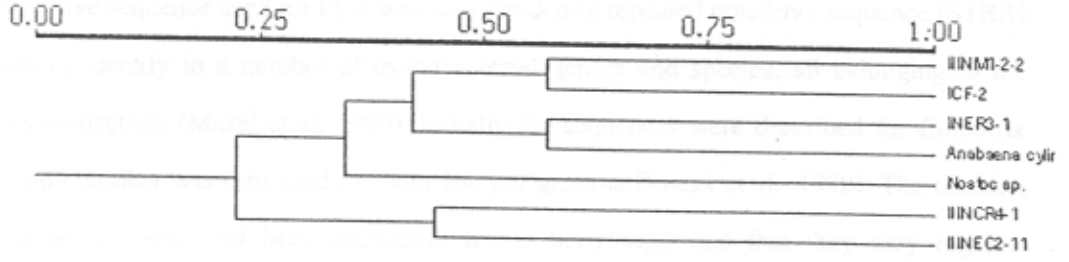

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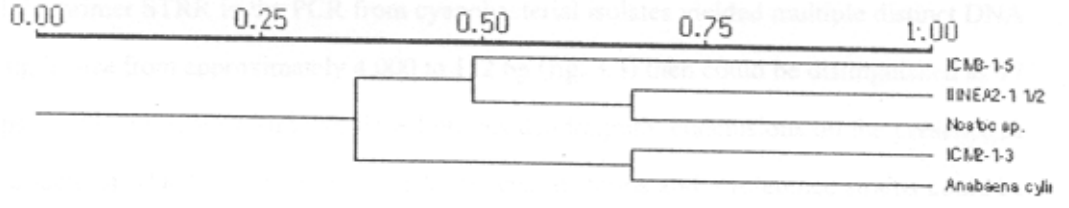

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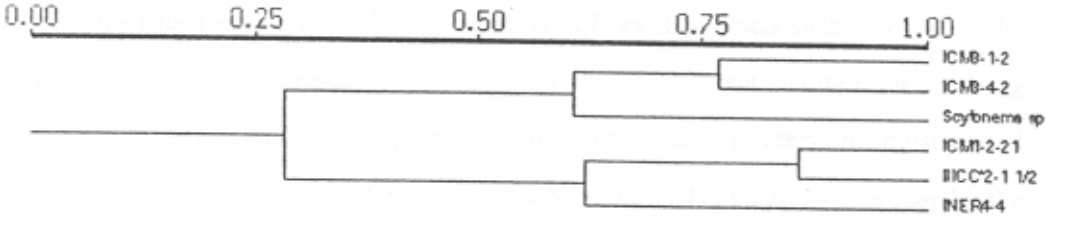


Fig. 3.2 Dendrograms (UPGMA) of similarities between *nifH* fragment; all cyanobacterial isolates (A), *Nostoc* sp. group (B), *Anabaena* sp. group (C), *Anabaenopsis* sp. group (D), *Nodularia* sp. group (E) and branching group (F) by NTSYS-pc package version 1.8.

4. Amplification of cyanobacterial genomic DNA with PCR primer derived from repetitive sequences

The repetitive sequence used for PCR was short tandemly repeated repetitive sequence (STRR) and was applied to identify in a number of cyanobacterial genera and species, all belonging to the heterocystous cyanobacteria (Mazel et al, 1990). Initially the sequences were described for *Calothrix* sp., where the copy number was estimated to about 100 per genome (Mazel et al., 1990). The function of repetitive sequences have still been uncleared. It has been suggested that they may regulate transcription termination (Haselkorn and Buikema, 1992) or be the target of DNA-binding proteins responsible for chromosomal maintenance in the cell (Lupski and Weinstock, 1992 and Mazel et al.). However, the conserved status of these repetitive sequences made them methodologically important tools for diversification studies among related prokaryotes and for identification (fingerprinting) of microorganisms in general.

This study, total genomic DNA was extracted from all isolates as templates for PCR analysis. Application of the primer STRR in the PCR from cyanobacterial isolates yielded multiple distinct DNA products ranging in size from approximately 4,000 to 132 bp (fig. 3.3) then could be distinguished as 97 different groups (include reference strains). Based on this dendrogram, conclusions on the presence or absent band productd of STRR primer from 102 cyanobacterial strains and 5 reference strains could be classified into 2 main clusters (fig. 3.4A). However, some strains have still been undistinguished. When cyanobacterial isolates were analyzed relationship within each genus (fig. 3.4B, 3.4C, 3.4D, 3.4E and 3.4F), Somme strains could not be distinguished such as I NC*2-6 (pale-green colour) and I CC3-1 (brownish colour) in *Anabaena* sp. group or I NEM3-3-11 (filamentous on BG11 solid medium) and III NC*1-4 (erose and curled on BG11 solid medium) in *Nostoc* sp. group. However, STRR primer could distinguish some isolates which unable to be distinguished by *nifH*-PCR products such as I CD1-4, III NEA1-2/2 and I CA2-1. From this results, only applying STRR was not enough for studying diversification of cyanobacteria, thus next steps also used other random primers. Rasmussen and Svenning (1998) compared the clustering of the some cyanobacterial isolates into four groups by the STRR and LTRR (long tandemly repeated repetitive sequences). They revealed the same clustering, however, with the LTRR primers, some differences were obtained among individual within a group. Thus, this study did not used LTRR primers because they had a same result with STRR though LTRR sequences were detected in both heterocystous and nonheterocystous cyanobacteria (Masephol, Gorlitz and Bohmen, 1996).

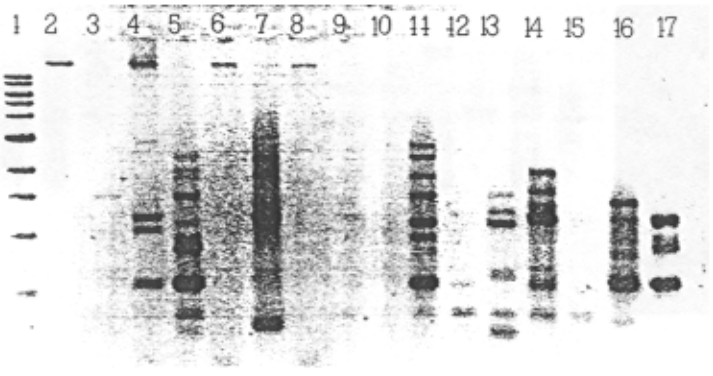


Fig 3.3A STRR-PCR fingerprint patterns of *Nostoc sp.* group (Table 3.1B) with genomic DNA as the template. Lane 1-17; 1kb ladder marker, *Nostoc sp.*, *Anabaena cylindrica*, *Hapalosiphon sp.* DASH 05101, *Calothrix sp.* DASH 02101, *Scytonema sp.*, II NM 1-4-1, II NEM 1-4-8, III NM 2-1-9, III NM 2-4-1, ICM 2-3-7, I NM 3-1-1, I NEM 3-1-3, I NEM 3-1-4 and INEM 3-3-11, respectively.

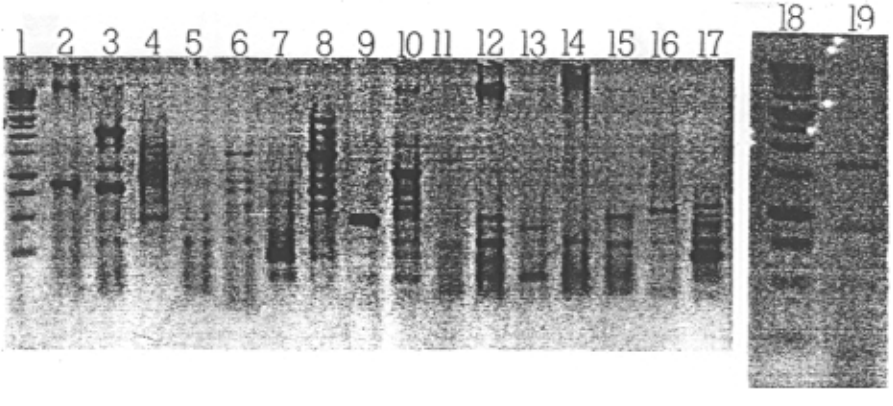


Fig. 3.3B STRR-PCR fingerprint patterns of *Nostoc sp.* group (Table 3.1B) with genomic DNA as the template. Lane 1-19; 1 kb ladder marker, III NEA 1-1, III NEA 2-11, III NEA 2-5, III CA 1-33, I NB 2-3, I NB 1-20, III NB 1-3, I NEB 1-1, I NEB 2-20, II CB 2-27, III NC* 1-4, II NEC* 1-20, I ND 1-3, I CD 2-2, I CD 1-4, I CD 1-1, 1 kb ladder marker and III CD 1-17, respectively.

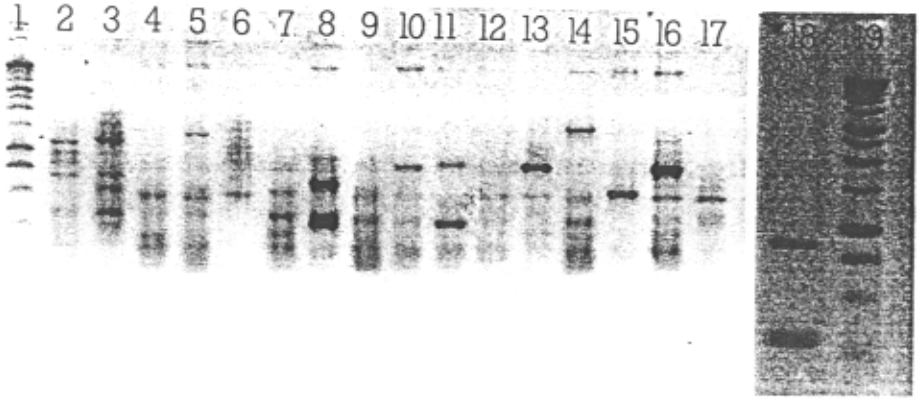


Fig. 3.3C STRR-PCR fingerprint patterns of *Nostoc sp.* group (Table 3.1A) with genomic DNA as the template. Lane 1-19; 1 kb ladder marker, I NF, I NEF 4-2, III NEF 3-4, I CF 3-22, I CF 2-2, II NEC 4-21, III NEC* 1-3, III NEC 3-29, III NEC 2-13, I CC 2-2, I NECR 3-9, I NECR 3-4, II CCR 3-22, III NR 4-8, III NR 2-2 and I CR 1-1, III CR2-1 and 1kb ladder marker, respectively.

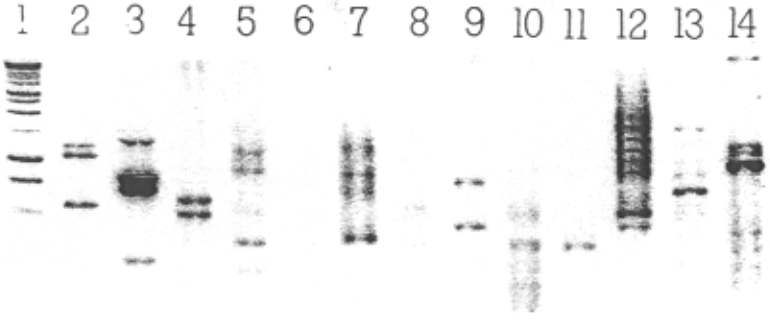


Fig. 3.3D STRR-PCR fingerprint patterns of *Anabaena sp.* group (Table 3.1B) with genomic DNA as the template. Lane 1-14; 1 kb ladder marker, *Nostoc sp.*, *Anabaena cylindrica*, *Hapalosiphon sp.* DASH 05101, *Calothrix sp.* DASH 02101, *Scytonema sp.*, I NM 1-1-3, II CM 1-3-40, I NM 2-4-1, I NM 3-1-3, II NM 3-3-13, I NEM 3-3-1, I CM 3-1-1, and II NM 3-3-22, respectively.

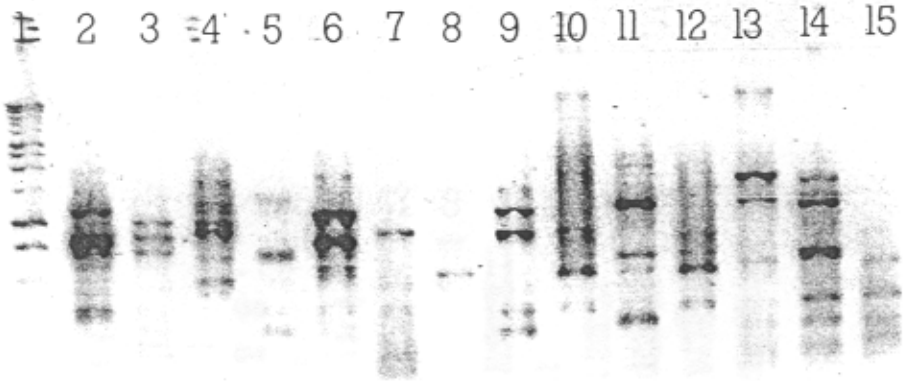


Fig. 3.3E STRR-PCR fingerprint patterns of *Anabaena sp.* group (Table 3.1B) with genomic DNA as the template. Lane 1-15; 1 kb ladder marker, I NA 2-1, II NA 2-2, III NEA 2-6½, I CA 2-1, III NEB 2-2, III CB 2-1, I NC* 2-6, III CC* 2-10, I ND -2, I ND 1-14, I ND 2-3, III ND 2-1, I NED 1-2 and III CB2 -2, respectively.

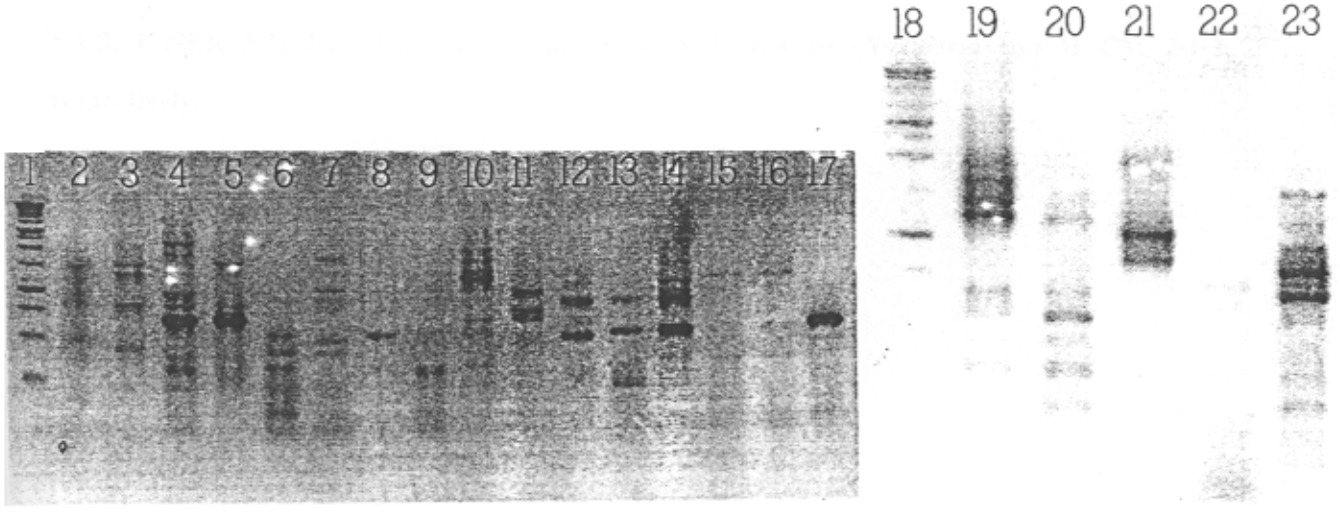


Fig. 3.3F STRR-PCR fingerprint patterns of *Anabaena sp.* group (Table 3.1B) with genomic DNA as the template. Lane 1-23; 1 kb ladder marker, III NF 1-4, III CF 3-20, I NC 4-21, I NC 2-1, II NC 3-20, I NEC 4-1, I CC 3-1, III CC 1-22, I NCR 2-3, I NCR 2-3.1, I NCR 4-2, I NCR 3-1, III NCR 1-11, III NECR 4-22, I CCR 3-2, III CCR 4-22, 1 kb ladder marker, I NR 1-6, I NR 2-2, IV NR 3-9, III NER 2-7 and II CR 1-2, respectively.

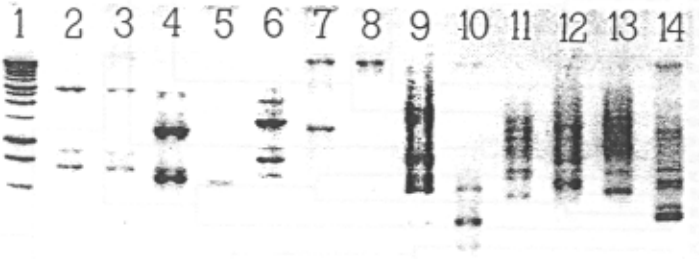
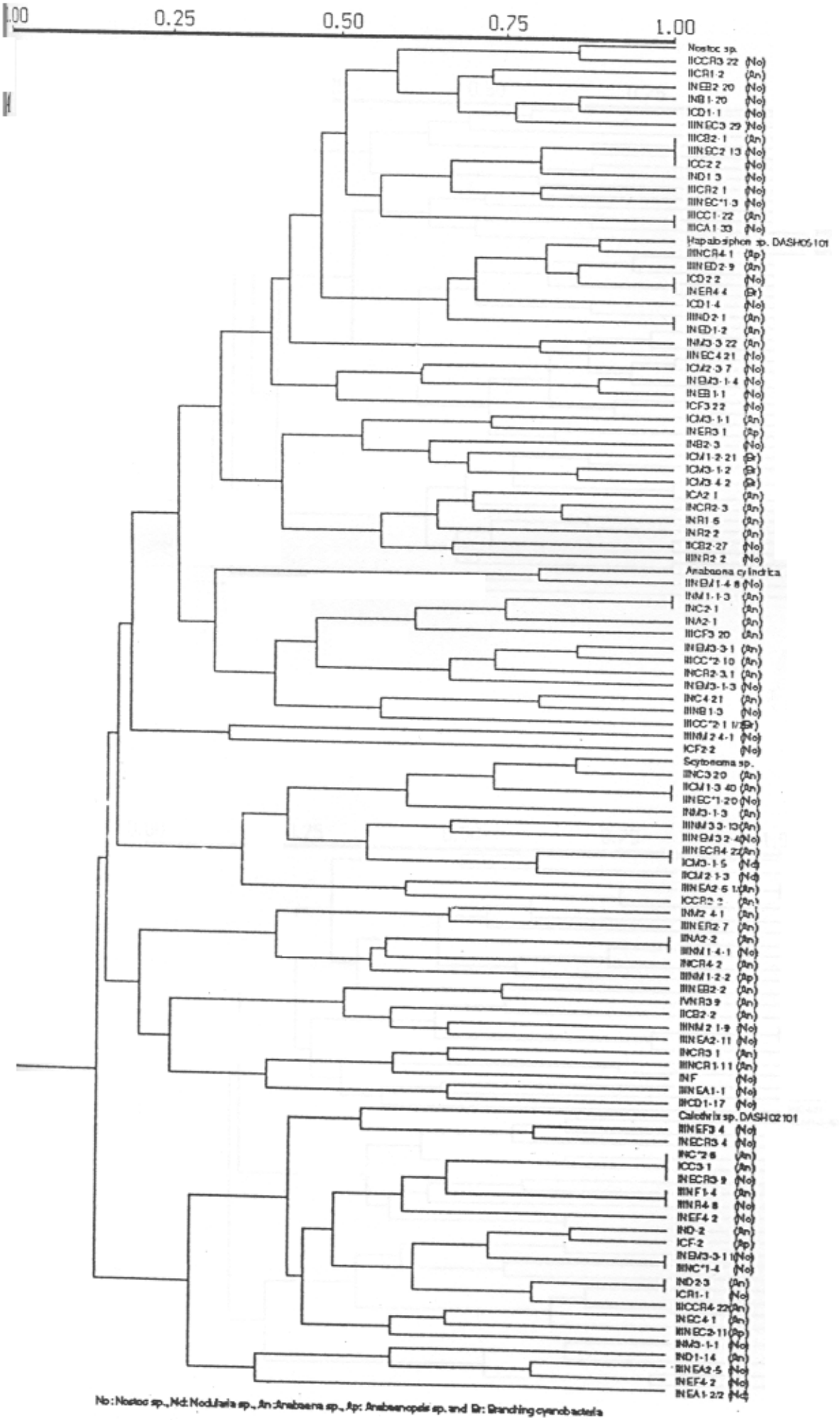
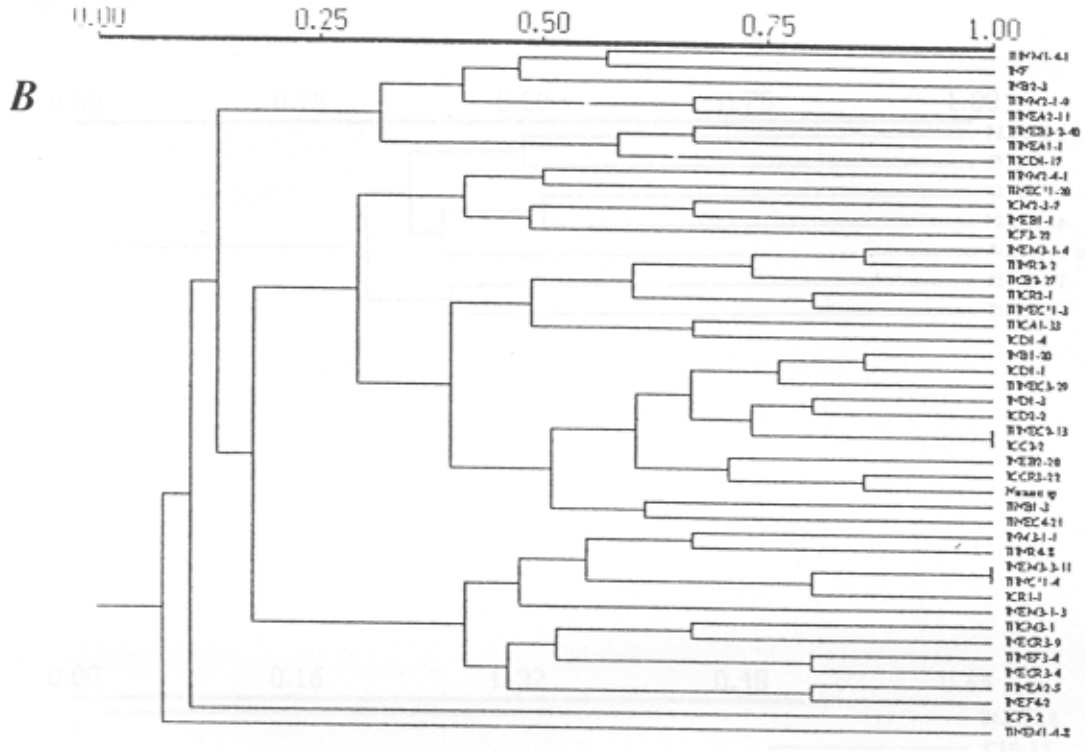


Fig. 3.3G STRR-PCR fingerprint patterns of other group (Table 3.1C, 3.1D and 3.1E) with genomic DNA as the template. Lane 1; 1 kb ladder marker, lane 2-5 (Branching group); I CM 3-12, ICM 1-2-21, I CM 3-4.2, I NER 4-4, lane 6 (*Anabaenopsis* sp.); III CC* 2-1 1/2, lane 7-8 (*Nodularia* sp.) I CM 3-1-5, I CA 1-10, lane 9-13 (*Anabaenopsis* sp.); I CF -2, III NCR 4-1, III NM 1-2-2, I NER 3-1, III NEC 2-11, respectively, and lane 14 (*Nodularia* sp.) II CM 2-1-3, respectively.



No: Nostoc sp., Nd: Nodularia sp., An: Anabaena sp., Ap: Anabaenopsis sp. and Br: Branching cyanobacteria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0.25
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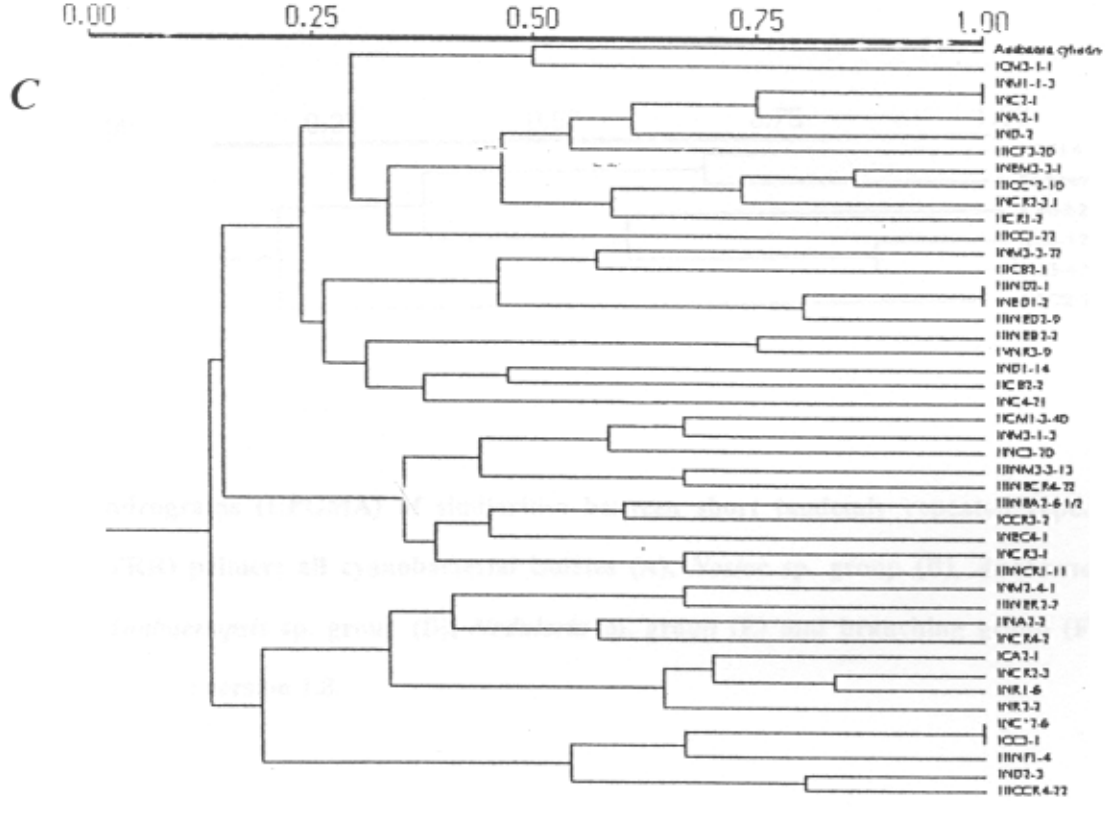
0.75
1.00


C
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0.50
0.75
1.00

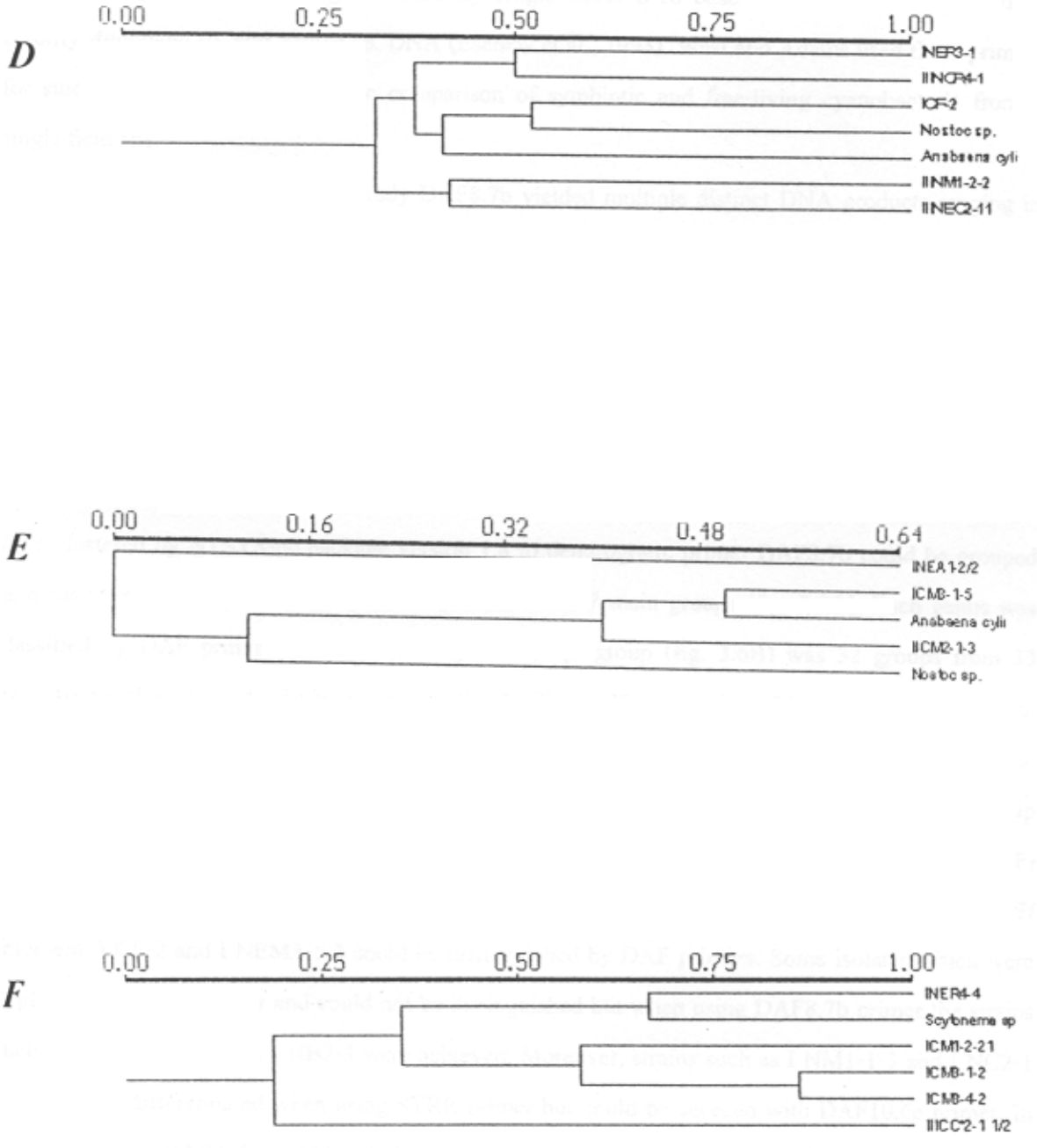


Fig. 3.4 Dendrograms (UPGMA) of similarities between short tandemly repeated repetitive sequence (STRR) primer; all cyanobacterial isolates (A), *Nostoc* sp. group (B), *Anabaena* sp. group (C), *Anabaenopsis* sp. group (D), *Nodularia* sp. group (E) and branching group (F) by NTSYS-pc package version 1.8.

5. DAF analysis

The primers were designed randomly length about 8-10 base and were originally used to amplify *Anabaena* sp. and *Azalea* sp. DNA (Eschew et al., 1993). West and Adams used these primers for study phenotypic and genotypic comparison of symbiotic and free-living cyanobacteria from a single field site.

The use of primer in this study DAF8.7b yielded multiple distinct DNA products ranging in size from approximately 3,000 to 178 bp (fig. 3.5) and using DAF10.6e primer yielded about 4,000 to 178 bp (fig. 3.7). Only DNAs from 78 cyanobacterial isolates were able to be amplified by DAF8.7b primer (include 3 reference strains; *Nostoc sp.*, *Anabaena cylindrica* and *Hapalosiphon sp*. DASH 05101) and 94 cyanobacterial isolates by DAF10.6e primer (include 5 reference strains). From band products obtained from DAF8.7b primer, they were distinguished into 71 different groups from 78 cyanobacterial isolate and 75 groups from 94 cyanobacterial isolates (DAF10.6e primer). When they were clustered by NTSYS-pc package version 1.8 to dendrogram, primer DAF8.7b could be grouped as 6 main groups (fig. 3.6A) and primer DAF10.6e was 7 main groups (fig. 3.8A). Each genus was classified by DAF primer; DAF8.7b primer, *Nostoc* sp. group (fig. 3.6B) was 32 groups from 33 cyanobacterial isolates, *Anabaena* sp. group (fig. 3.6C) was 32 groups from 36 cyanobacterial isolates; for DAF10.6e primer, *Nostoc* sp. group (fig. 3.8B) was 35 groups from 42 cyanobacterial isolates and *Anabaena* sp. group (fig. 3.8C) was 34 groups in 39 cyanobacterial isolates. *Anabaenopsis* sp. group (fig. 3.6D and 3.8D), *Nodularia* sp. group (fig. 3.6E and 3.8E) and branching group (fig. 3.6F and 3.8F) were clearly differentiated by the both of primers. When both of DAF primers were compared with *nifH* fragment, I CF-2 and I NEM3-1-3 could be distinguished by DAF primers. Some isolates which were applied with STRR primer and could not be distinguished but when using DAF8.7b primer the strains such as I ND2-3, I CR1-1, I NB2-3 were achieved. Moreover, strains such as I NM1-1-3 and I NC2-1 could not be differentiated when using STRR primer but could be succeed with DAF10.6e primer. In addition, primer DAF8.7b could be distinguish isolates III NECR4-22 and I NER3-1 more clearly than primer DAF10.6e. Thus, PCR products obtained from these primers (STRR, DAF8.7b and DAF10.96e) were combined to generate for the most clearly distinguished dendrogram.

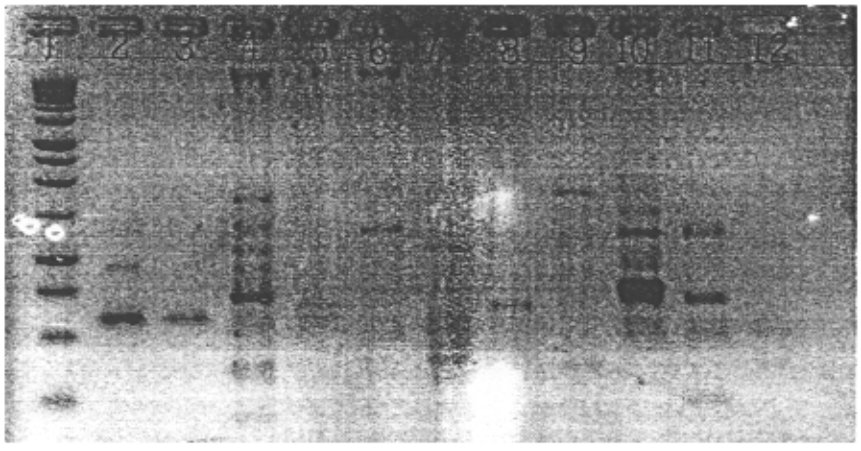


Fig. 3.5A DAF8, 7B-PCR fingerprint patterns of *Nostoc* sp. group (Table 3.1A) with genomic DNA as the template. Lane 1-12; 1 kb ladder marker, *Nostoc* sp., *Anabaena cylindrica*, *Hapalosiphon* sp. DASH 05101, III NM 1-4-1, III NM 2-1-9, III NM 2-4-1, I CM 2-3-7, I NM 3-1-1, I NEM 3-1-3, I NEM 3-1-4 and III CM 3-1, respectively.

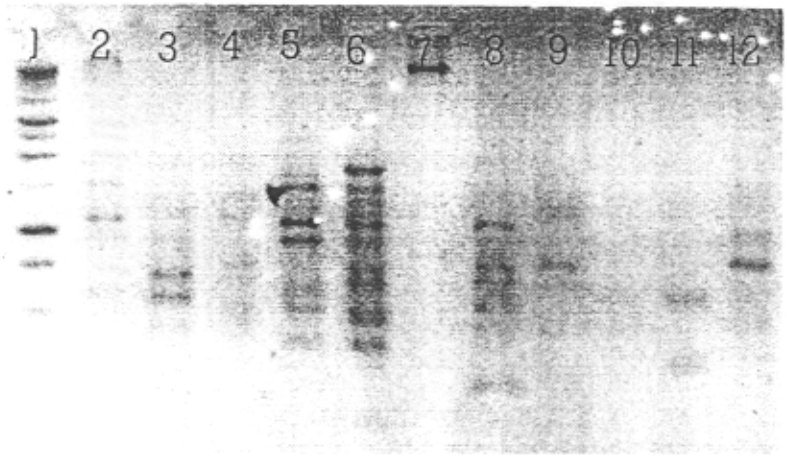


Fig. 3.5B DAF8, 7b-PCR fingerprint patterns of *Nostoc* sp. group (Table 3.1A) with genomic DNA as the template. Lane 1-12; 1 kb ladder marker, III NEA 2-11, III NEA 2-5, III CA 1-33, I NB 2-3, INB 1-20, III NC*1-4, II NEC*1-20, I CD 2-2, I CD 1-4, I CD 1-1* and III CD 1-17, respectively.

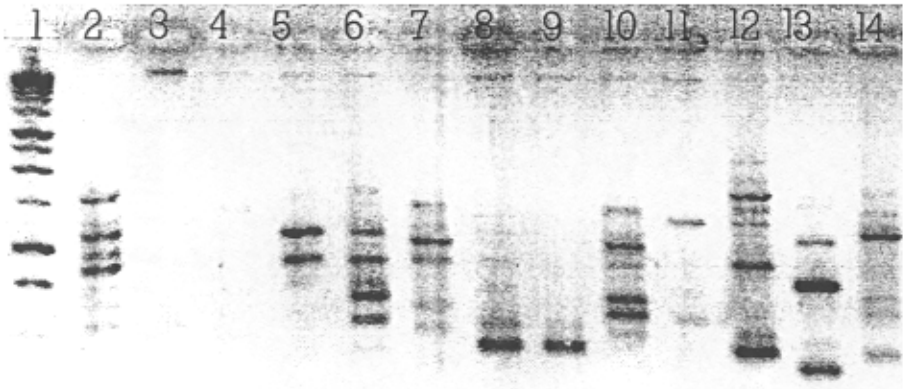


Fig. 3.5C DAF 8.7b-PCR fingerprint patterns of *Nostoc* sp. group (Table 3.1A) with genomic DNA as the template. Lane 1-14; 1 kb ladder marker, I NF, I NEF 4-2, III NEF 3-4, I CF 3-22, I CF 2-2, III NEC 3-20, I NECR 3-9, I NECR 3-4, I CCR 3-22, III NR 4-8, III NR 2-2, I CR 1-1 and III CR 2-1, respectively.

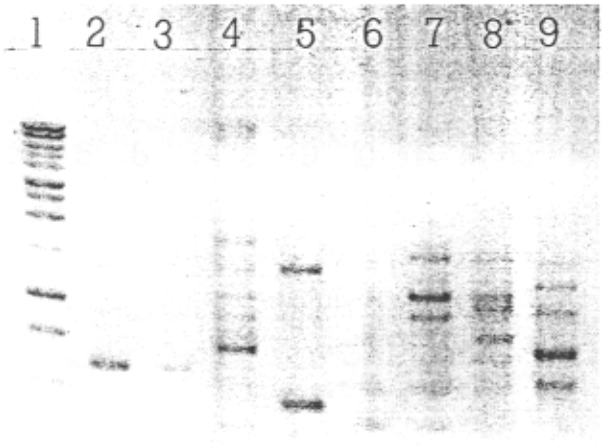


Fig. 3.5D DAF 8.7b-PCR fingerprint patterns of *Anabaena* sp. group (Table 3.1B) with genomic DNA as the template. Lane 1-9; 1 kb ladder marker, *Nostoc* sp., *A. cylindrica, Hapalopsiphon* sp. DASH 05101, I NM 1-1-3, I NM 2-4-1, I NM 3-1-3, II NM 3-3-22 and I NEM 3-3-1, respectively.

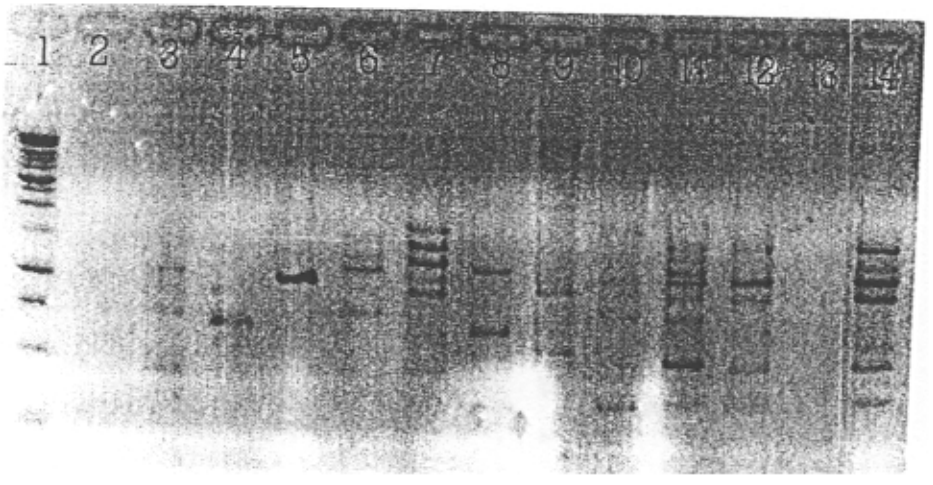


Fig. 3.5E DAF 8.7b-PCR fingerprint patterns of *Anabaena* sp. group (Table 2B) with genomic DNA as the template. Lane 1-14; 1 kb ladder marker, I NA 2-1, III NEA 2-6 1/2, I CA 2-1, III NEB 2-2, III CB 2-1, III CB 2-2, III CC*2-10, I ND-2, I ND 1-14, I ND 2-3, III ND 2-1, I NED 1-2 and III NED 2-9, respectively

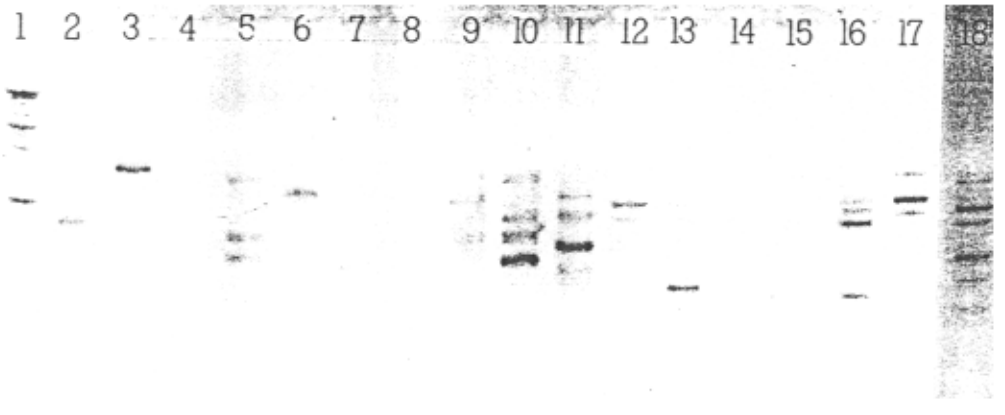


Fig. 3.5F DAF 8.7b-PCR fingerprint patterns of *Anabaena* sp. group (Table 2B) with genomic DNA as the template. Lane 1-18; 1 kb ladder marker, III NF 1-4, III CF 3-20, I NC 4-21, I NC 2-1, I NEC 4-1, I CC 3-1, I NCR 2-3, I NCR 4-2, I NCR 3-1, III NCR 1-11, III NCR 4-22, III CCR 4-24, I NR 1-6, I NR 2-2, IV NR 3-9, III NER 2-7 and II CR 1-2, respectively.

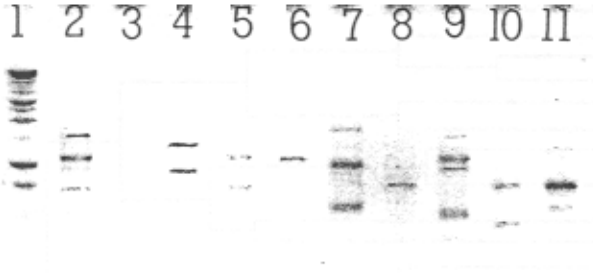
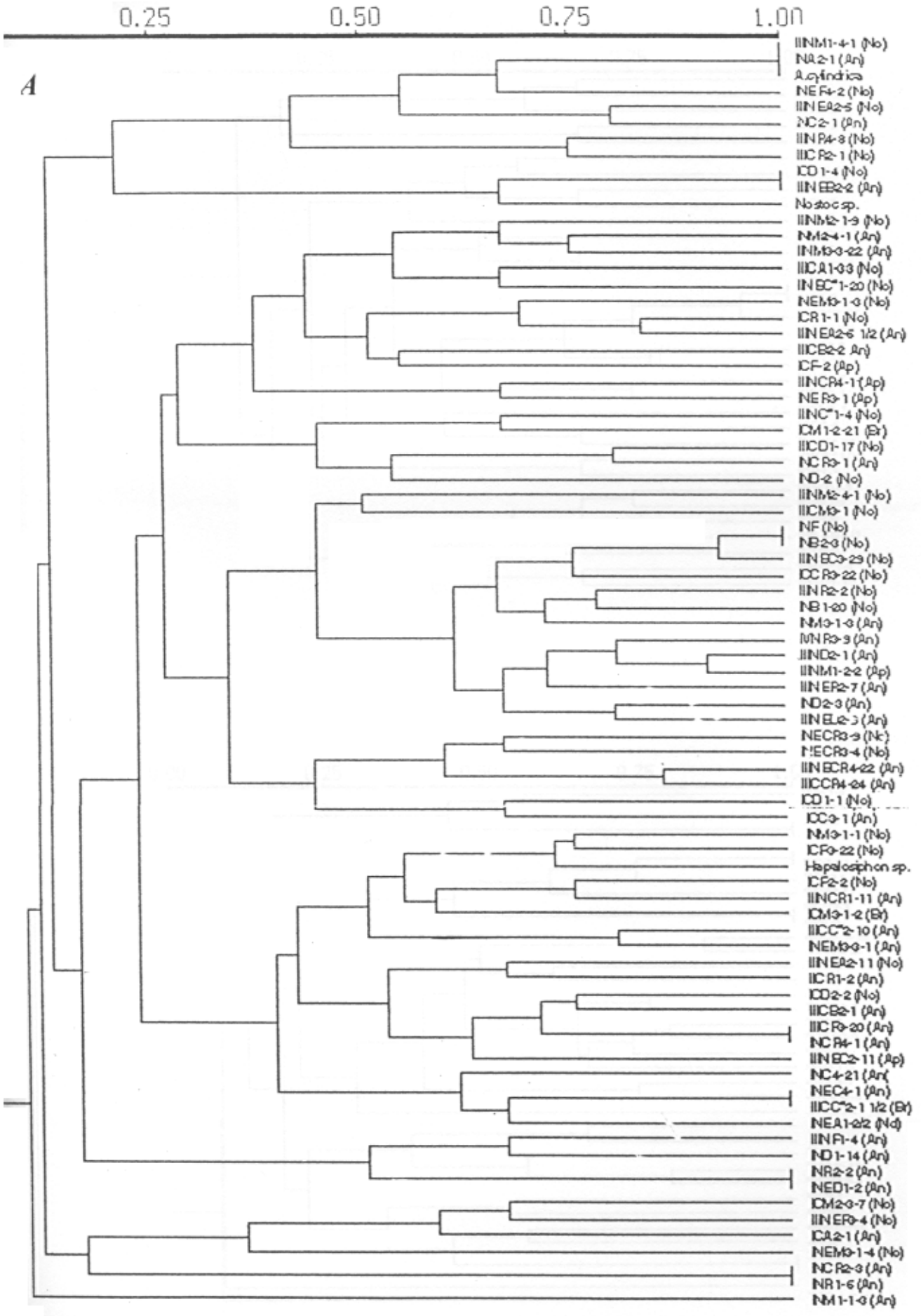
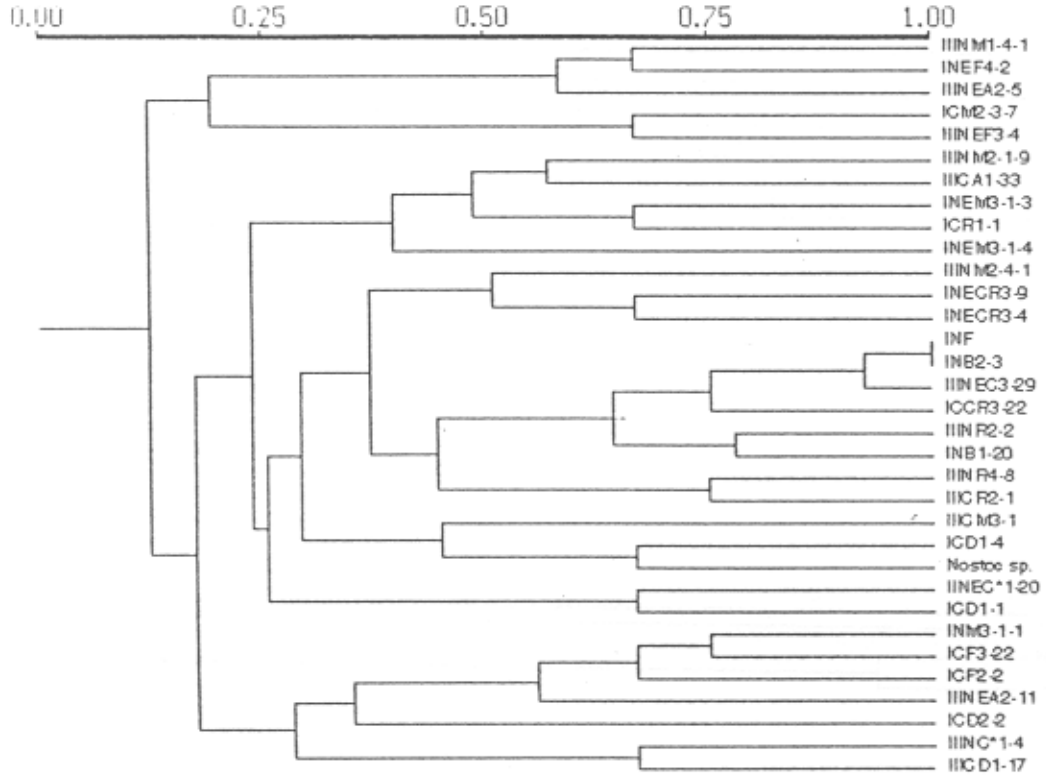


Fig. 3.5G DAF 8.7b-PCR fingerprint patterns of other group (Table 3.1C, 3.1D and 3.1E) with genomic DNA as the template. Lane 1; 1 kb ladder marker, lane 2-4 (branching group); I CM 3-1-2, I CM 3-4-2, I CM 1-2-21, III CC* 2-1 1/2, lane 5 (*Nodularia* sp.); I NEA 1-2/2, lane 6-11 (*Anabaenopsis* sp.); I CF-2, III NCR 4-1, III NM 1-2-2, I NER 3-1 and III NEC 2-11, respectively.



No Nostoc sp., Nd; Nodularie sp., An; Anabaena sp., Ap; Anabaenopsis sp. and Br; Branching cyanobacteria

B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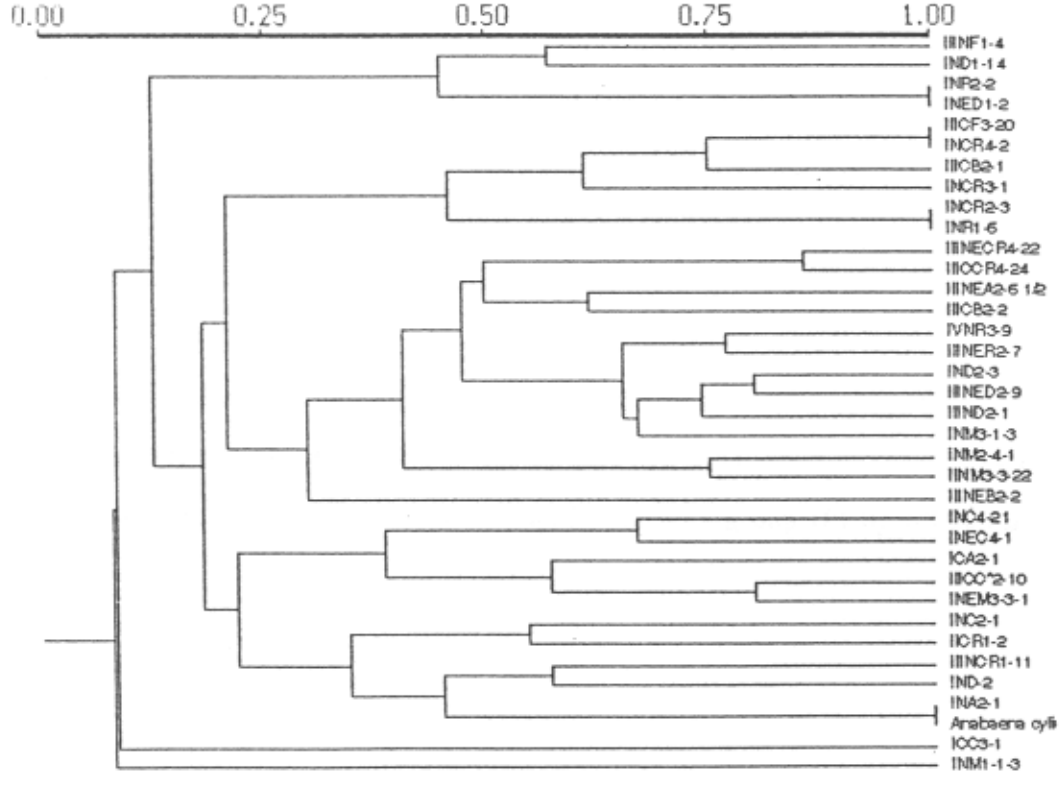
IIINM2-1-9
IIICA1-33
INEM3-1-3
ICR1-1
INEM3-1-4
IIINM2-4-1
INECR3-9
INECR3-4
INF
INB2-3
IIINEC3-29
ICCR3-22
IIINR2-2
INB1-20
IIINR4-8
IIICR2-1
IIICM3-1
ICD1-4
Nostoc sp.
IIINEC*1-20
ICD1-1
INM3-1-1
ICF3-22
ICF2-2
IIINEA2-11
ICD2-2
IIINC*1-4
IIICD1-17

C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IIICB2-1
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INCR2-3
INR1-6
IIINECR4-22
IIICCR4-24
IIINEA2-6 1-2
IIICB2-2
IVNR3-9
IIINER2-7
IND2-3
IIINED2-9
IIIND2-1
INM3-1-3
INM2-4-1
IINM3-3-22
IIINEB2-2
INC4-21
INEC4-1
ICA2-1
IIICC*2-10
INEM3-3-1
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ICC3-1
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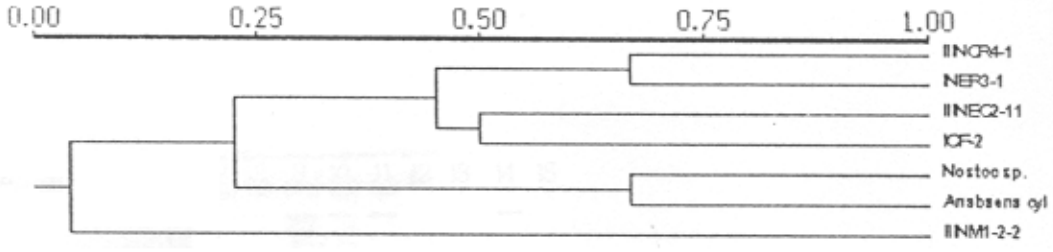
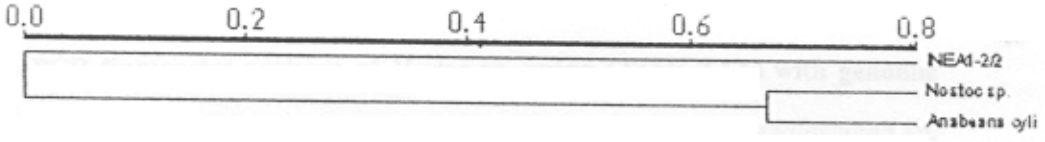
D



E



F

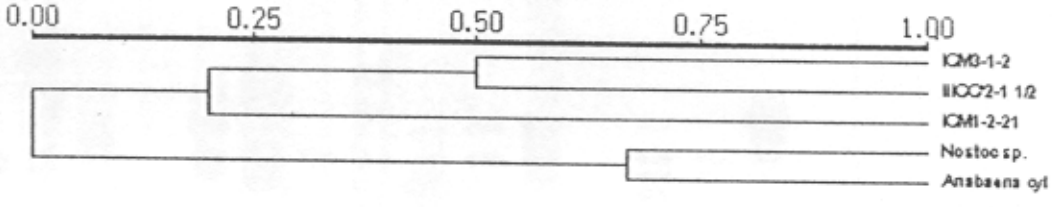


Fig. 3.6 Dendrograms (UPGMA) of similarities between DNA amplification fingerprint (DAF8.7b) primer; **all cyanobacterial isolates (A), *Nostoc* sp. group (B), *Anabaena* sp. group (C), *Anabaenopsis* sp. group (D), *Nodularia* sp. group (E) and branching group (F) by NTSYS-pc package version 1.8.**

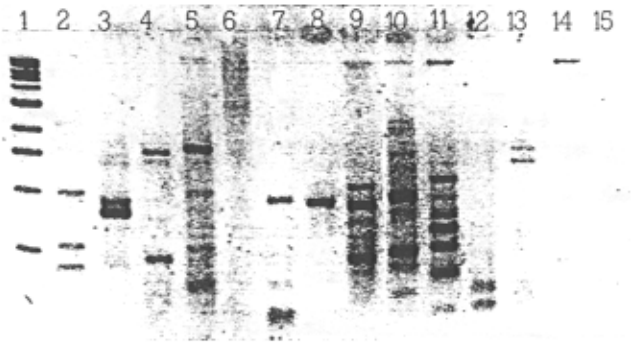


Fig. 3.7A DAF 10.6e-PCR fingerprint patterns of *Nostoc* sp. group (Table 3.1A) with genomic DNA as the template. Lane 1-15; 1 kb ladder marker, *Nostoc* sp., *A. cylindrica, Hapalosiphon* sp. DASH 05101, *Calothrix* sp. DASH 02101, *Scytonema* sp., III NM 1-4-1, III NM 2-1-9, III NM 2-4-1, I CM 2-3-7, I NM 3-1-1, I NEM 3-1-3, I NEM 3-1-4, I NEM 3-3-11 and III NEM 3-2-40, respectively.

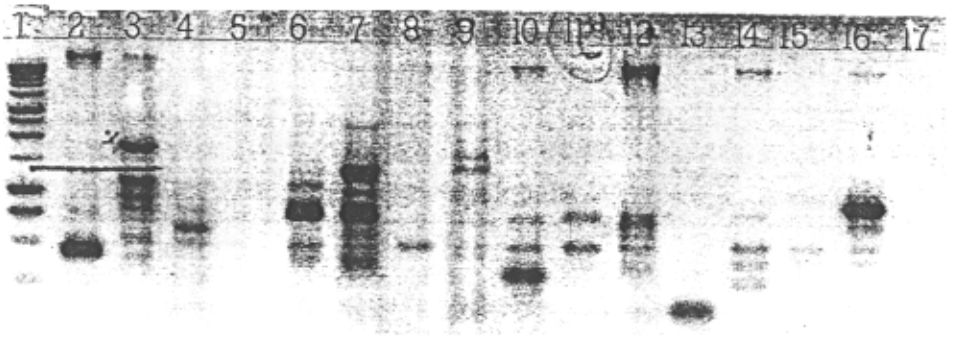


Fig. 3.7B DAF 10.6e-PCR fingerprint patterns of *Nostoc* sp. group (Table 3.1A) with genomic DNA as the template. Lane 1-17; 1 kb ladder marker, III NEA 1-1, III NEA 2-11, III NEA 2-5, III CA 1-33, I NB 2-3, I NB 1-20, III NB 1-3, I NEB 1-1, I NEB 2-20, II CB 2-27, III NC* 1-4, II NEC* 1-20, III NEC* 1-3, I CD 2-2, I CD 1-1 and III CD 1-17, respectively.

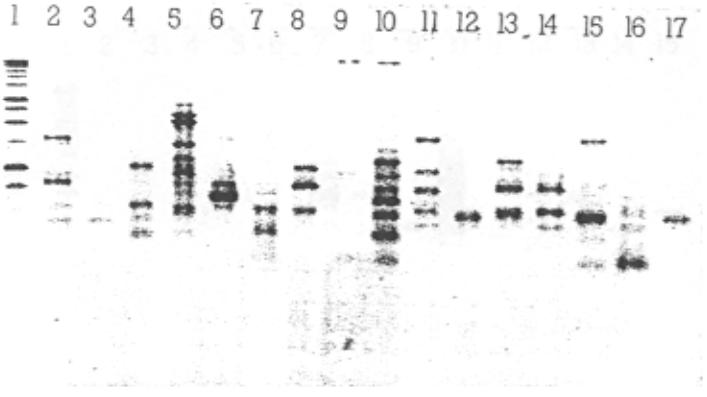


Fig. 3.7C DAF 10.6e-PCR fingerprint patterns of *Nostoc* sp. group (Table 3.1A) with genomic DNA as the template. Lane 1-17; 1 kb ladder marker, I NF, I NEF 4-2, III NEF 3-4, I CF 3-22, I CF 2-2, II NEC 4-21, III NEC 3-29, III NEC 2-13, I CC 2-2, I NECR 3-9, I NECR 3-4, I CCR 3-22, III NR 4-8, III NR 2-2, I CR 1-1 and III CR 2-1, respectively.

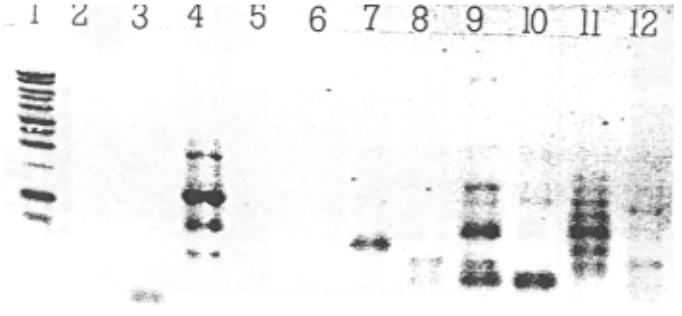


Fig. 3.7D DAF 10.6e-PCR fingerprint patterns of *Anabaena* sp. group (Table 3.1B) with genomic DNA as the template. Lane 1-12; 1 kb ladder marker, *Nostoc* sp., *A. cylindrica, Hapalosiphon* sp. DASH 05101, *Calothrix* sp. DASH 02101, *Scytonema* sp., I NM 1-1-3, II CM 1-3-40, I NM 3-1-3, III NM 3-3-13, II NM 3-3-22 and I CM 3-1-1, respectively.

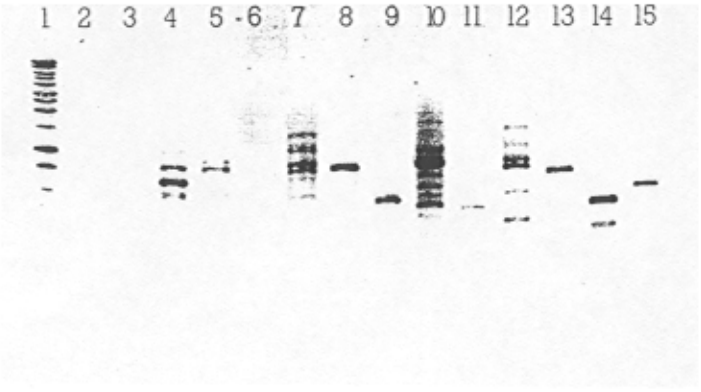


Fig. 3.7E DAF 10.6e-PCR fingerprint patterns of *Anabaena* sp. group (Table 3.1B) with genomic DNA as the template. Lane 1-15; 1 kb ladder marker, I NA 2-1, II NA 2-2, III NEA 2-6½, I CA 2-1, III NEB 2-2, III CB 2-2, I NC* 2-6, III CC* 2-10, I ND-2, I ND 1-14, I ND 2-3, III ND 2-1, I NED 1-6 and III NED 2-9, respectively.

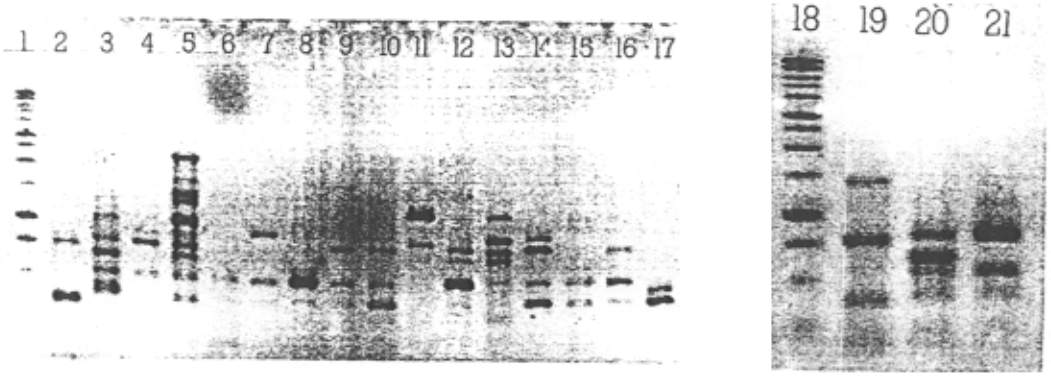


Fig. 3.7F DAF 10.6e-PCR fingerprint patterns of *Anabaena* sp. group (Table 3.1B) with genomic DNA as the template. Lane 1-21; 1 kb ladder marker, III NF 1-4, III CF 3-20, I NC 4-21, I NC 2-1, II NC 3-20, I NEC 4-1, I CC 3-1, III CC 1-22, I NCR 2-3, I NCR 4-2, I NCR 3-1, III NCR 1-11, III NECR 4-22, I CCR 3-2, III CCR 4-24, I NR 1-6, 1 kb ladder marker, IV NR 3-9, III NER 2-7 and II CR 1-2, respectively.

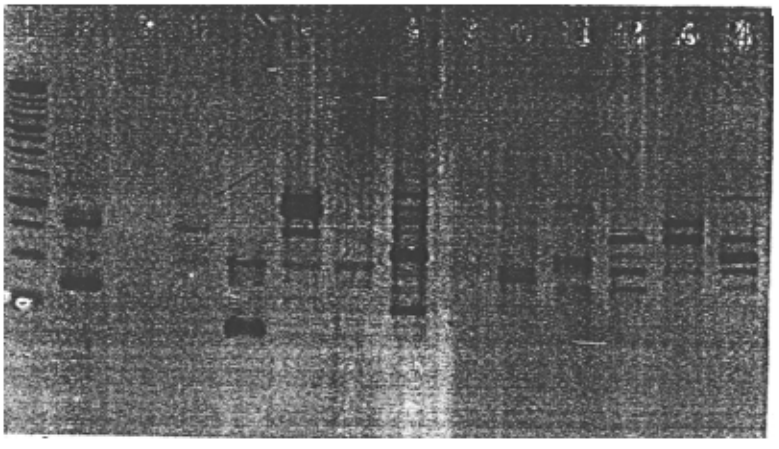
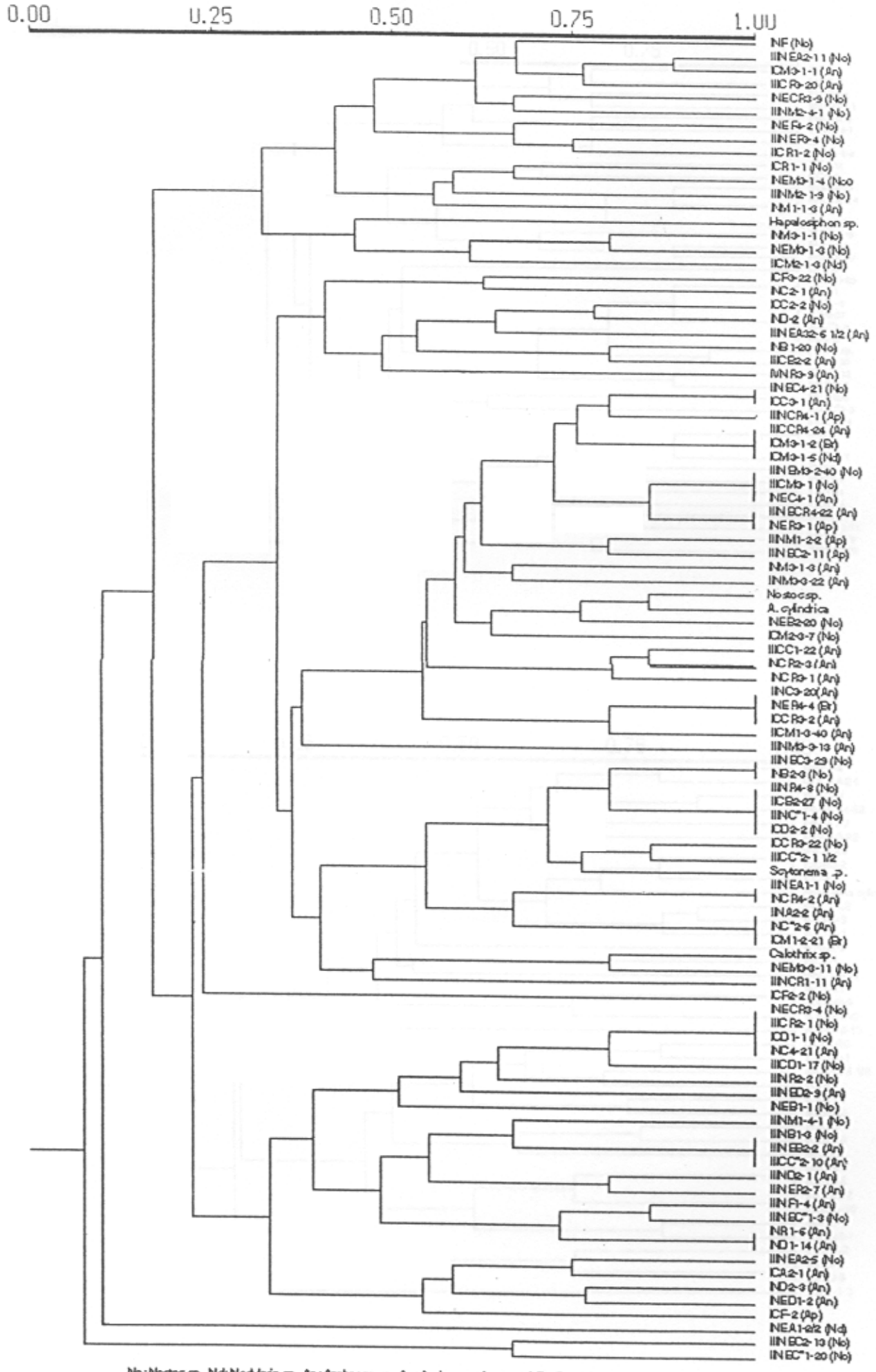
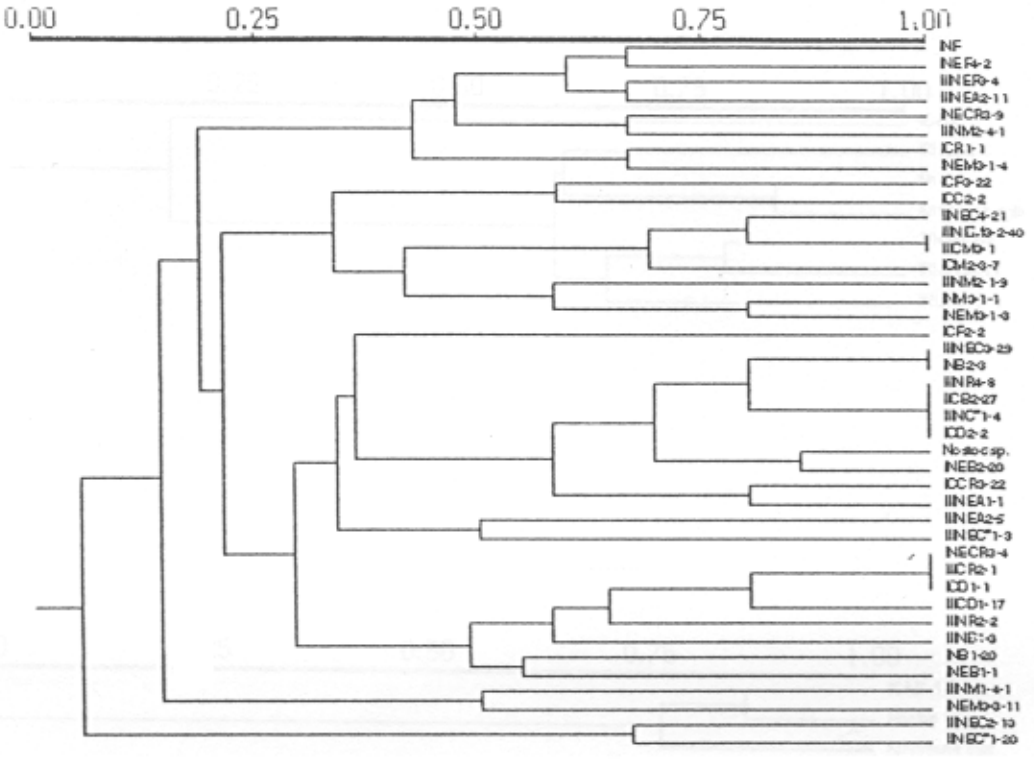


Fig. 3.7G DAF 10.6e-PCR fingerprint patterns of other sp. group (Table 3.1C, 3.1D and 3.1E) with genomic DNA as the template. Lane 1; 1 kb ladder marker, lane 2-6 (branching group); I CM 3-1-2, I CM 3-4-2, I CM 1-2-21, I NER 4-4, III CC* 2-1 1/2, lane 7 (*Nodularia* sp.); I CM 3-1-5, lane 8 (*Anabaenopsis* sp.); II CM 2-1-3, lane 9 (*Nodularia* sp.); I NEA 1-2/2, lane 10-14 (*Anabaenopsis* sp.); I CF-2, III NCR 4-1, III NM 1-2-2, I NER 3-1 and III NEC 2-11, respectively.



No : Nostoc sp., Nd: Nodularia sp., An : Anabaena sp., Ap: Anabaenopsis sp. and Br: Branching sp.

B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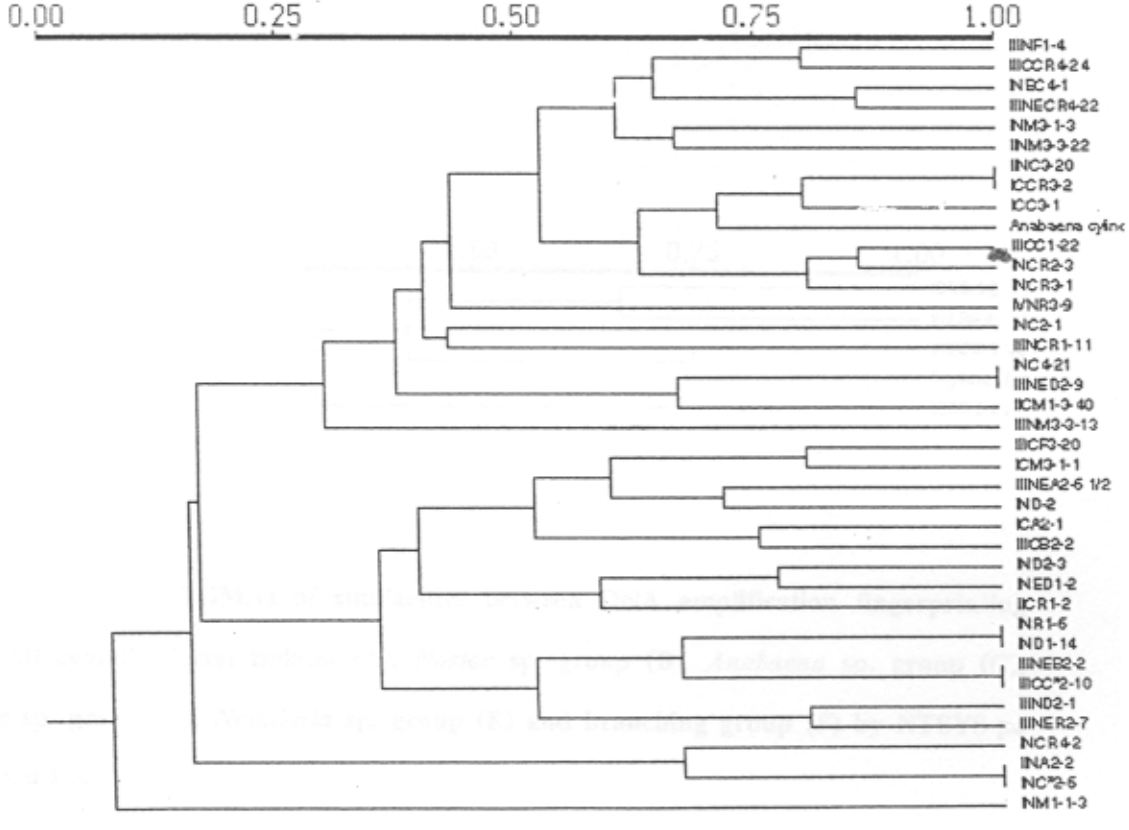
IINEC*1-20

C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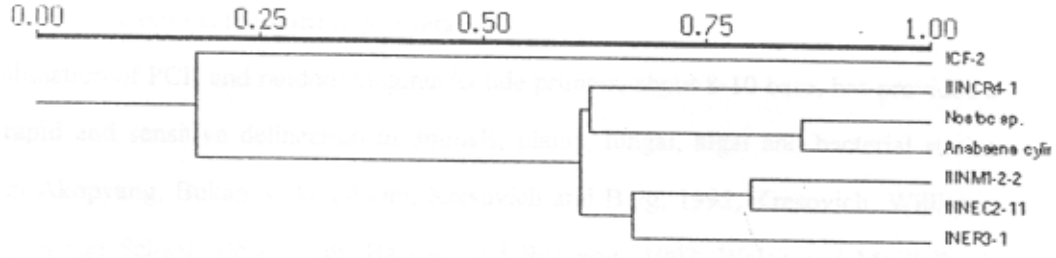
D



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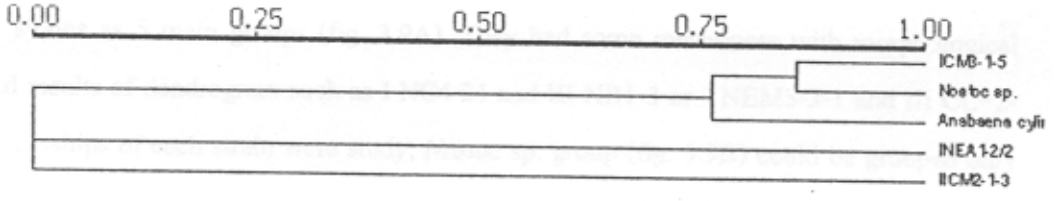


F

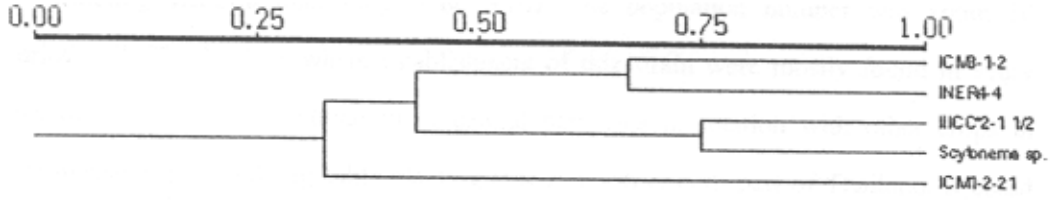
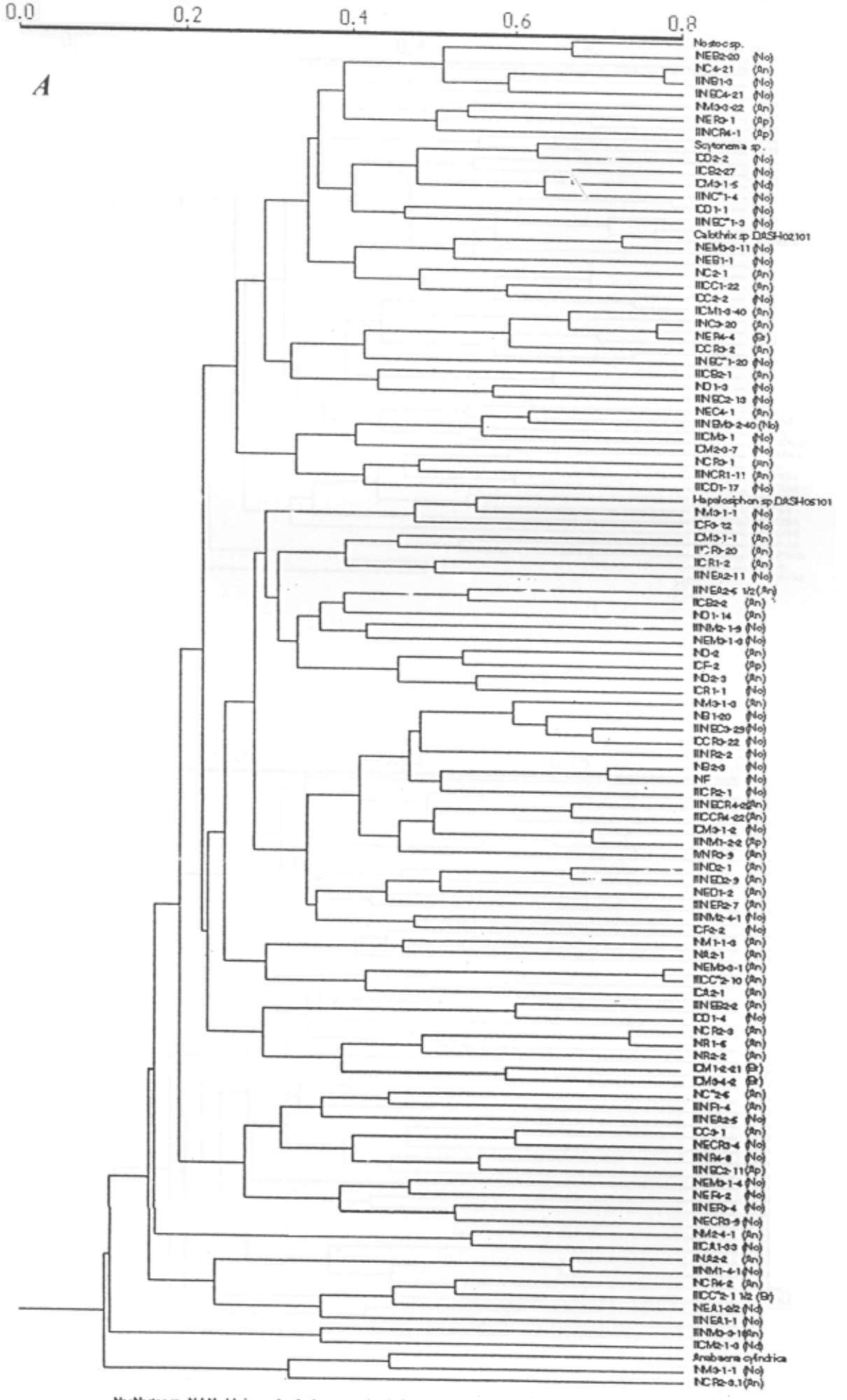


Fig. 3.8 Dendrograms (UPGMA) of similarities between DNA amplification fingerprinting (DAF10.6e); all cyanobacterial isolates (A), *Nostoc* sp. group (B), *Anabaena* sp. group (C), *Anabaenopsis* sp. group (D), *Nodularia* sp. group (E) and branching group (F) by NTSYS-pc package version 1.8.

6. Combination of PCR products from three primers

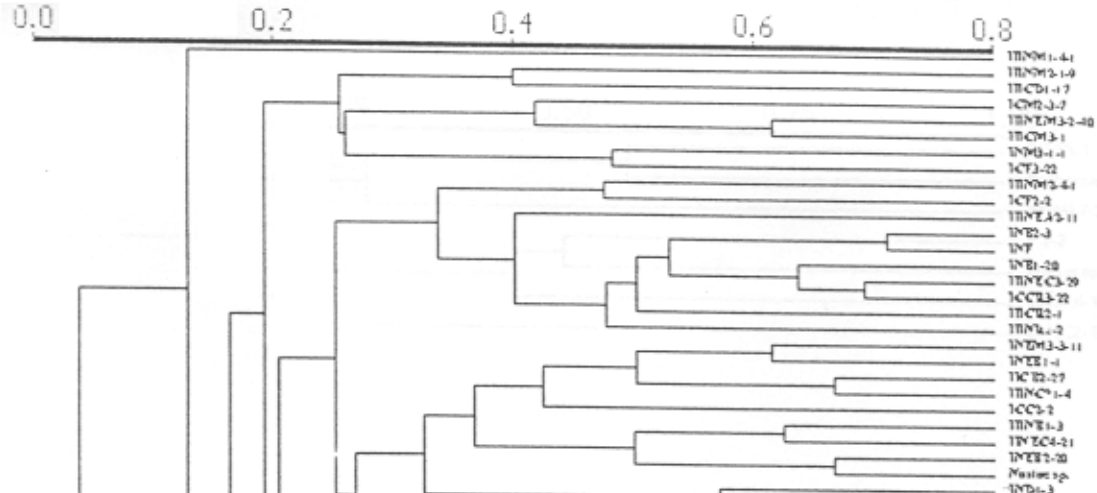
The combination of PCR and random oligonucleotide primers, about 8-10 base, has provided a method for the rapid and sensitive delineation of animals, plants, fungal, algal and bacterial strains (Neilan, quoted in Akopyang, Bukanov, Westblom, Kresovich and Berg, 1992, Kresovich, Williams, McFerson, Routman and Schaal, 1992, Scott, Haymes and Williams, 1992, Welsh and McClelland, 1990 and Williams, Kubelik, Livak, Rafalski and Tingey, 1990). RAPD profiling of these organism's genomes has been widely accepted as a valid taxonomic and phylogenetic tool (Neilan, 1995). This study, PCR products from using STRR, DAF8.7b and DAF10.6e were combined for clustering based on the presence or absence of band products by NYSYS-pc package version 1.8. Reproducibility of dendrogram was achieved from combined PCR products which could be to clearly distinguished all cyanobacterial strains as 5 main groups (fig. 3.9A). They had some relatedness with morphological when compared results of dendrogram such as I NC4-21 and III NB1-3 or I NEM3-3-1 and III CC*2-10. Then, Relationships of each strain were study; *Nostoc* sp. group (fig. 3.9B) could be grouped only into 3 groups but clearly distinguished among strains as well as *Anabaena* sp. group (fig. 3.9C), *Anabaenopsis* sp. group (fig. 3.9D), *Nodularia* sp. group (fig. 3.9E) and branching group (fig. 3.9F), 5, 2, 2 and 2 main clusters, respectively. From this results, only one primer was not enough to differentiate or clarified the diversity of cyanobacteria.

Moreover, we found unidentified cyanobacteria isolates (IV NR3-9) (fig. A12) which had reddish- purple colour filamentous, consisted of vegetative cell (1-2 μm), spherical form of heterocyst cell (0-1 μm) and abundant of akinete cell with the size larger than5 μm. Furthermore, this isolate was figured under scanning electron microscope (fig. 3.10). The population number was about 10 cells/gram of dried soil. The habitats where establishment of this strain were mostly found in every cultivation areas of Thailand, crop cultivation of central part, rice in rotation with other crops in northern and north-eastern part and vegetable planting area in north-eastern part of Thailand. It could fix N_2 about 0.462 and 0.291 μmolC_2H_4/mg of chrolophyll a/h in light and dark condition, respectively. Chromosomal DNA of this strain was amplified with primer STRR, DAF8.7b and DAF10.6e. Results suggested that this isolate was not clearly related with any reference strains whereas data analysis from each primer indicated that it was rather closely related to genus *Anabaena* than other.

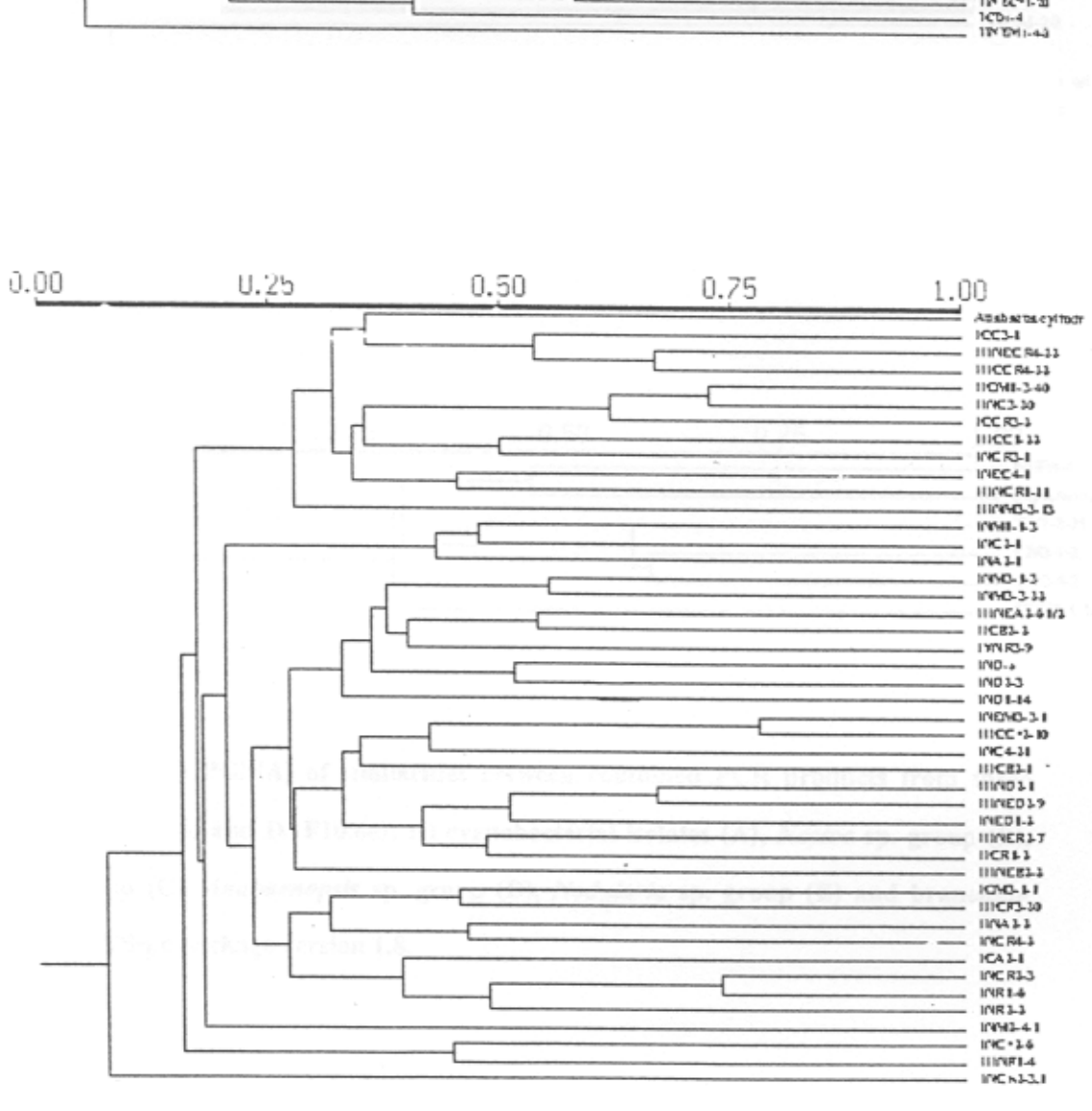


No : Nostoc sp., Nd: Nodularia sp., An : Anabaena sp., Ap: Anabaenopsis sp. and Br: Branching cyanobacteria

B



C



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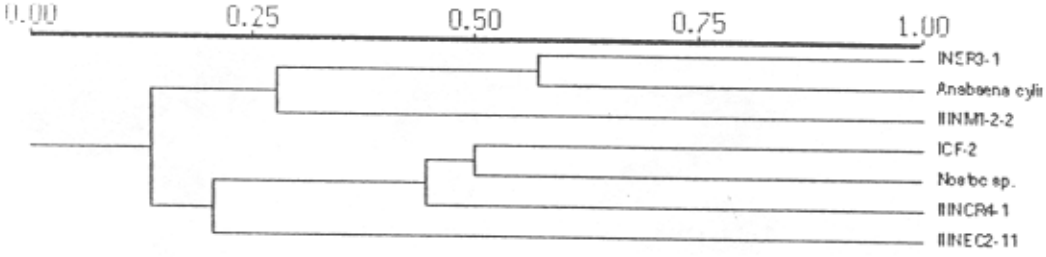

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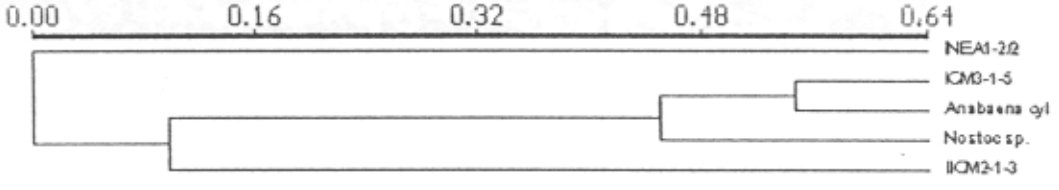

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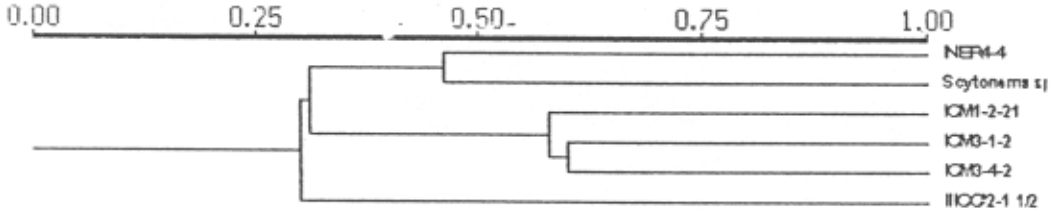

Fig. 3.9 Dendrograms (UPGMA) of similarities between combined PCR products from three primers (STRR, DAF8.7b and DAF10.6e); all cyanobacterial isolates (A), *Nostoc* sp. group (B), *Anabaena* sp. group (C), *Anabaenopsis* sp. group (D), *Nodularia* sp. group (E) and branching group (F) by NTSYS-pc package version 1.8.

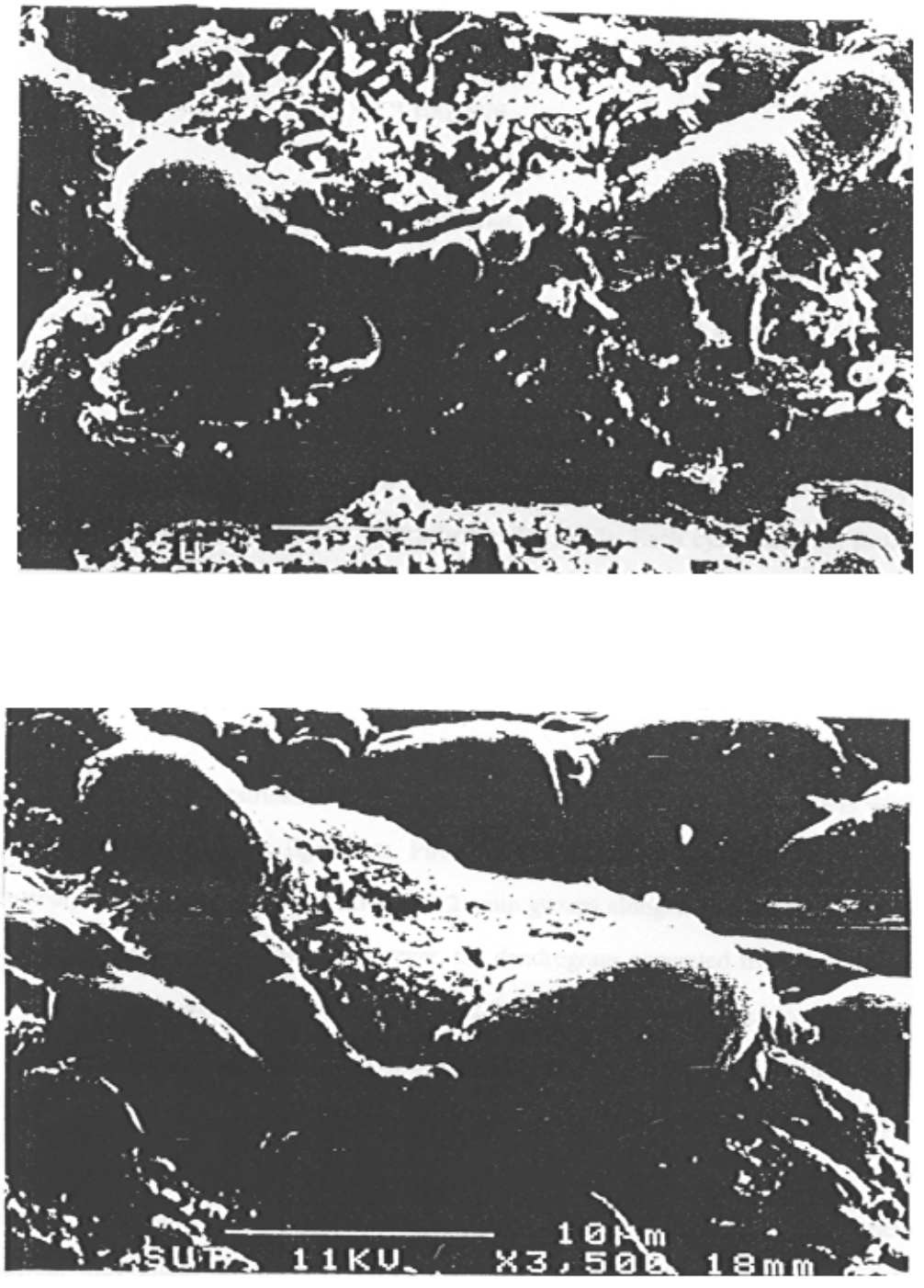

Fig. 3.10 Cell morphology of cyanobacterial isolate IV NR3-9 from 30000X and 35000X magnification under Scanning Electron Microscope.

CHAPTER IV

CONCLUSIONS

One-hundred and two N_2-fixing cyanobacterial strains were isolated from soils in different ecosystems of Northern, North-eastern and Central parts of Thailand. The great diversities in cell morphology, colony forming and clump formation in BG11 liquid medium were observed. Most ot the strains were heterocystous form. In each area of specific site (such as the top mountain area (M1)) was found high diversity of cyanobacteria when compared with agricultural practice area. From morphological study of cyanobacteria showed that *Nostoc spp.* and *Anabaena spp.* were the major genera established in Thai soils. Each cyanobacterial isolate could fix nitrogen under both light and dark conditions when detected by acetylene reduction assay (ARA). However most of cyanobacteria were able to fix higher nitrogen under light than under dark condition, except some strains such as I NM3-1-3 could fix nitrogen under dark more than light condition.

To investigate the diversification among strains in DNA level, some PCR primers were conducted for generating the DNA figerprints. Firstly, *NifH* -PCR products obtained from all strains were analyzed. They could be classified into 2 main groups along with PCR patterns and some of them still remain undifferntiated. However, the dendrogram generated from this primer indicated the group of strains in one separated cluster containing high efficiency in N_2 fixing ability. Attempt to analyse more diversification among strains, group of random primers were employed. STRR, DAF 8.7 b and DAF10.6e were choosen for strains differentiation. Each primer did not show the efficiency to discriminate all strains. They could be grouped into 2,6 and 7 different main groups, respectively, but when combaination of PCR products from these primers were conducted, they could be grouped in to 5 main groups and all strains were clearly differntiated. This was able to clearly distinguished all cyanobacterial strains even in intraspecies level. This addressed the great divergent of N_2 fixing cyanobacteria in Thai soil. Database from these results would be very fruitful since it has never been investigated before in Thailand. Thus agricultural management with appropriate strategies to conserve the microbial diversity should be concerned. Another approach for agricultural application such as utilizing N_2 fixing cyanobacterial inoculum as biofertilizer in sustainable agriculture in stead of chemical fertilizer is an intreguing way to promote the sense of environmental friendly.

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Appendix

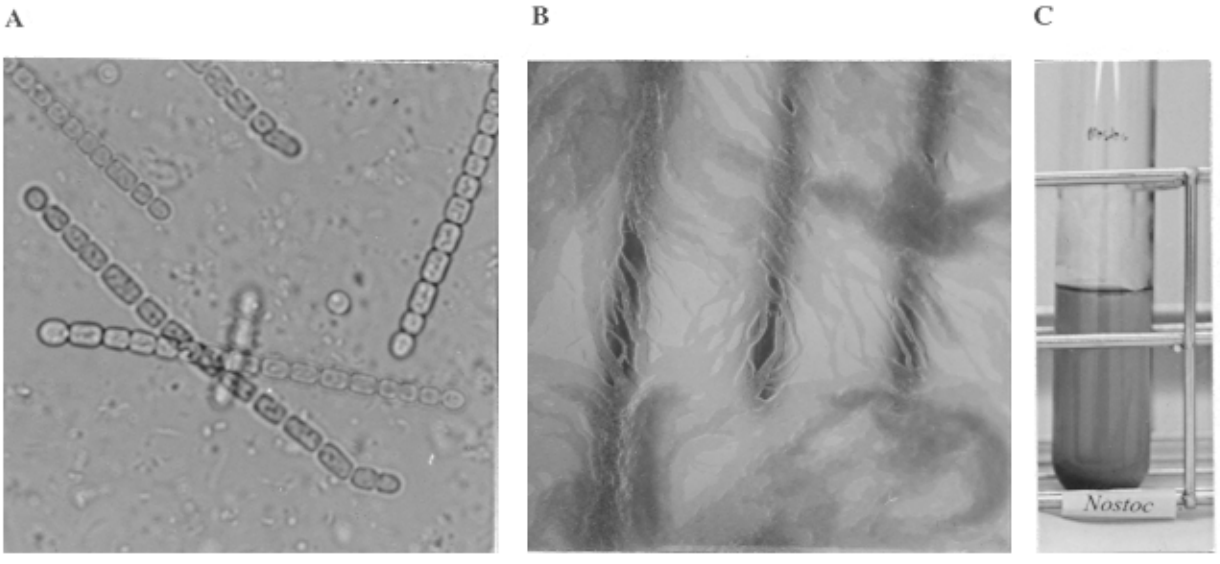

Fig. A1 Cell morphology under microscope with 400X magnification (A), colony forming on BG11 solid (B) and clump forming in BG11 liquid medium (C) of reference strain *Nostoc sp.*

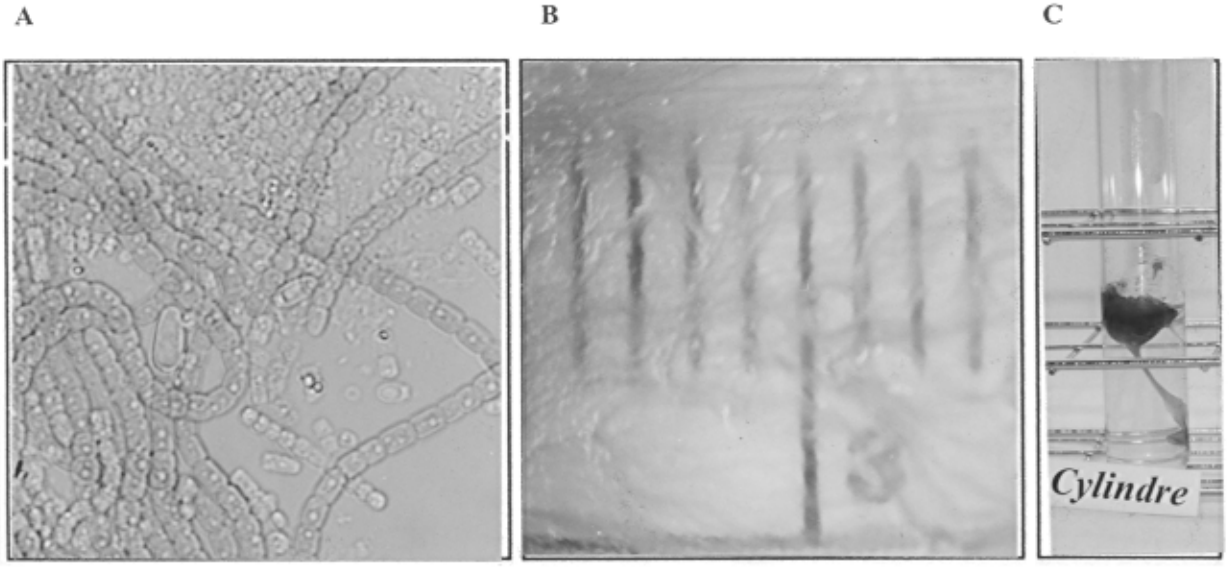

Fig. A2 Cell morphology under microscope with 400X magnification (A), colony forming on BG11 solid (B) and clump forming in BG11 liquid medium (C) of reference strain *Anabaena cylindriga*

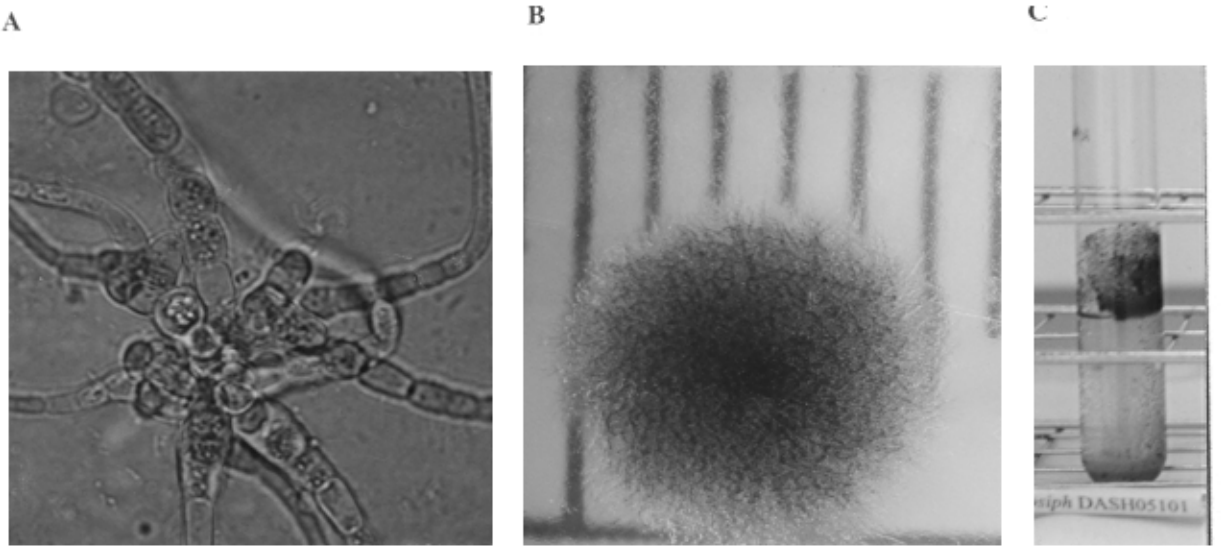

Fig. A3 Cell morphology under microscope with 400X magnification (A), colony forming on BG11 solid (B) and clump forming in BG11 liquid medium (C) of reference strain *Hapalosiphon sp.* DASH 05101

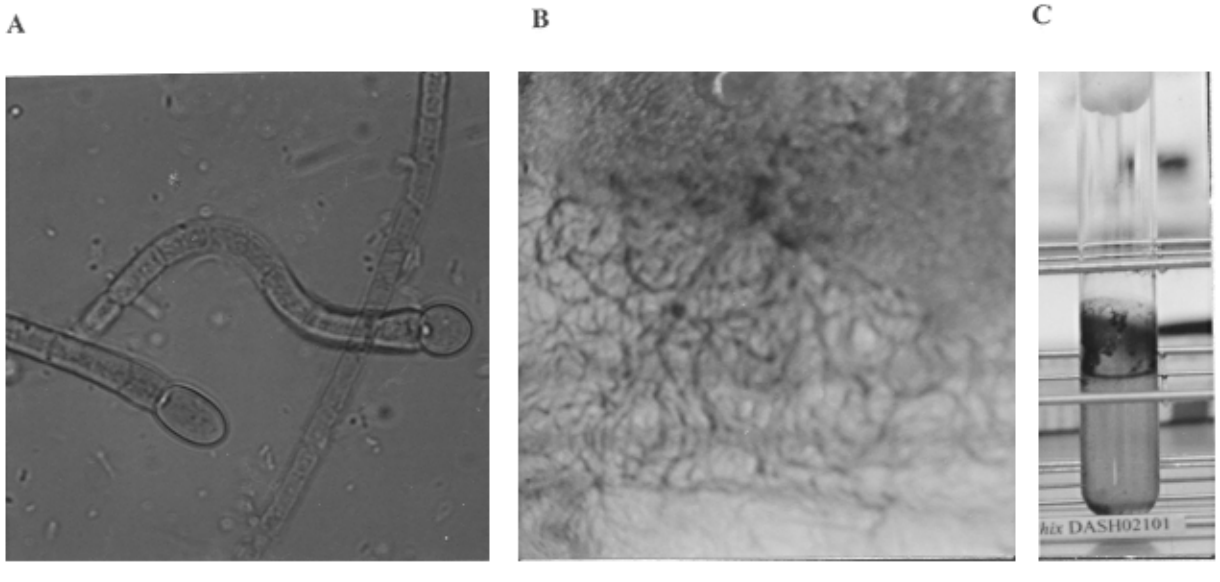

Fig. A4 Cell morphology under microscope with 400X magnification (A), colony forming on BG11 solid (B) and clump forming in BG11 liquid medium (C) of reference strain *Calothrix sp.* DASH 02101

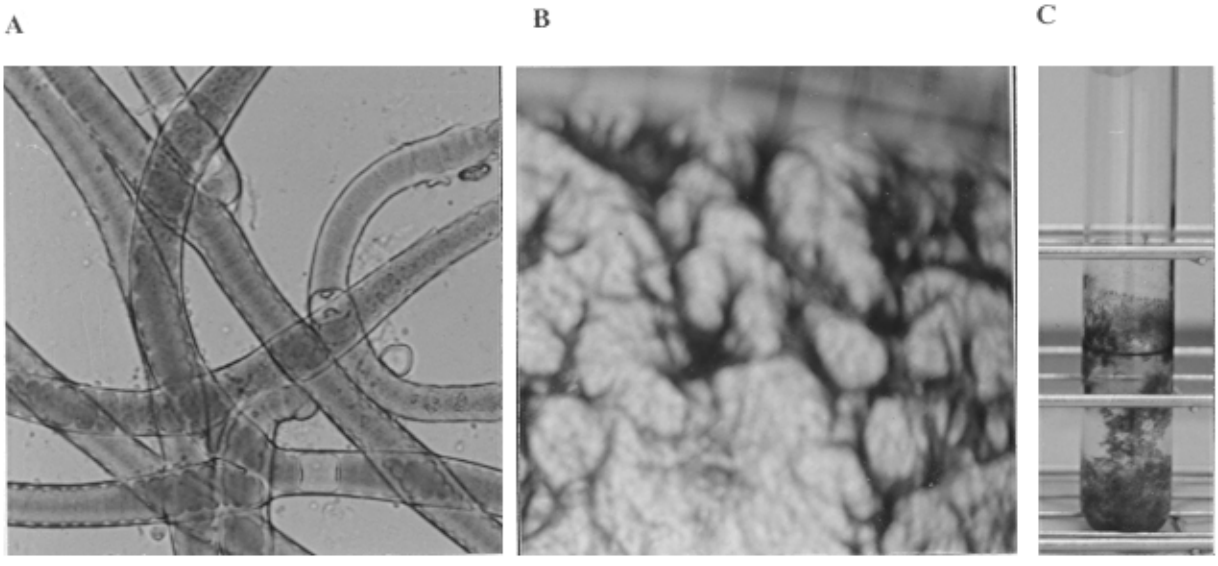

Fig. A5 Cell morphology under microscope with 400X magnification (A), colony forming on BG11 solid (B) and clump forming in BG11 liquid medium (C) of reference strain *Scytonema sp.*

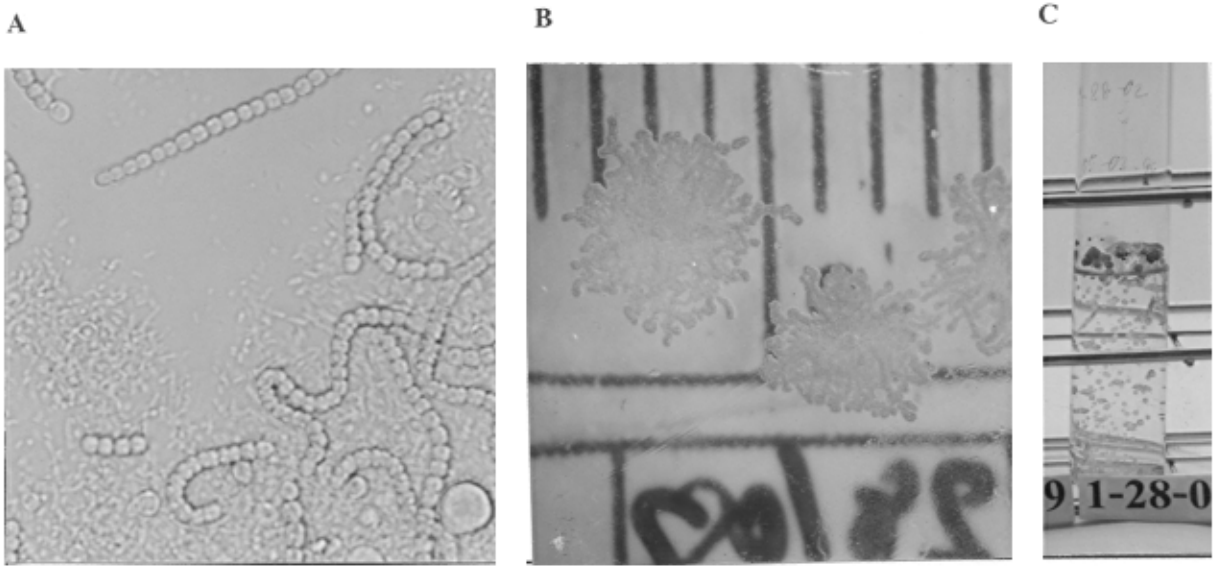

Fig. A6 Cell morphology under microscope with 400X magnification (A), colony forming on BG11 solid (B) and clump forming in BG11 liquid medium (C) of cyanobacterial isolate I NF

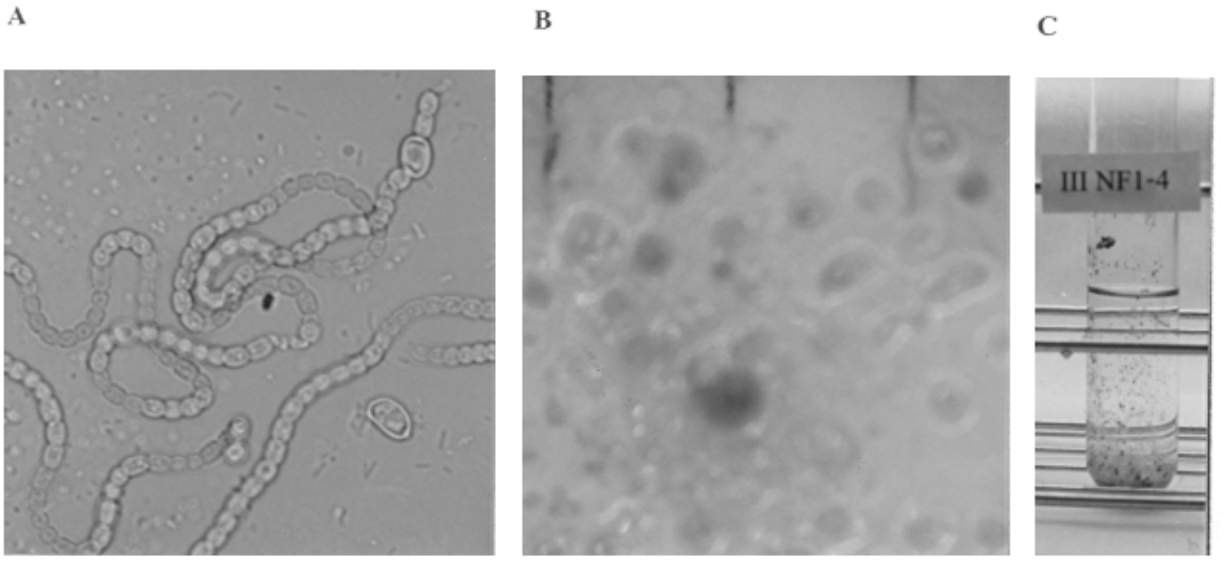

Fig. A7 Cell morphology under microscope with 400X magnification (A), colony forming on BG11 solid (B) and clump forming in BG11 liquid medium (C) of cyanobacterial isolate III NF1-4

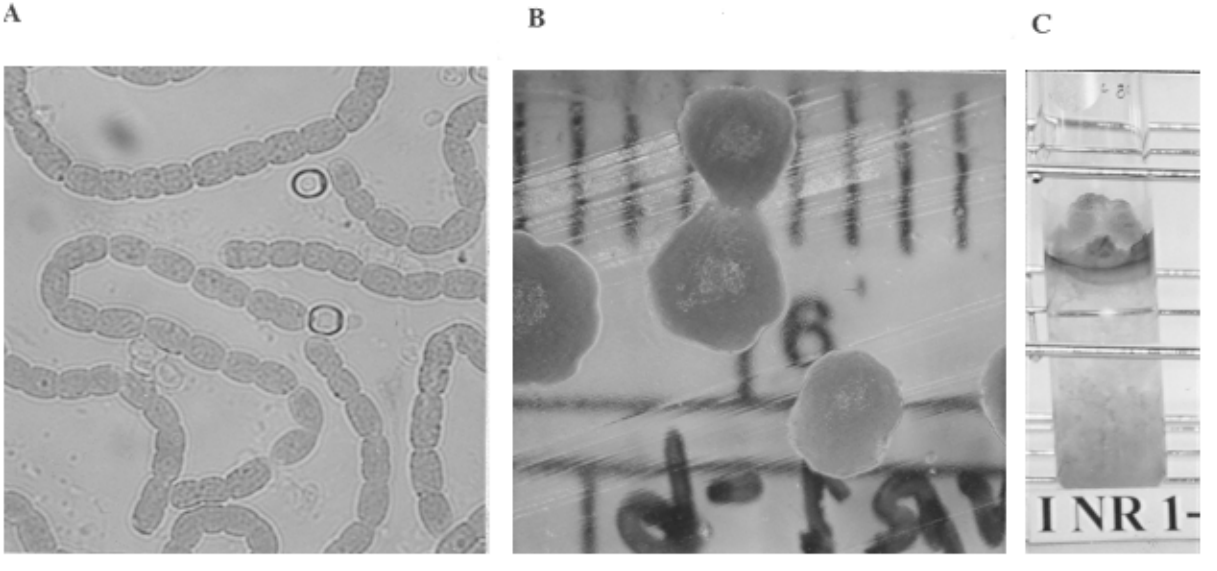

Fig. A8 Cell morphology under microscope with 400X magnification (A), colony forming on BG11 solid (B) and clump forming in BG11 liquid medium (C) of cyanobacterial isolate I NR1-6

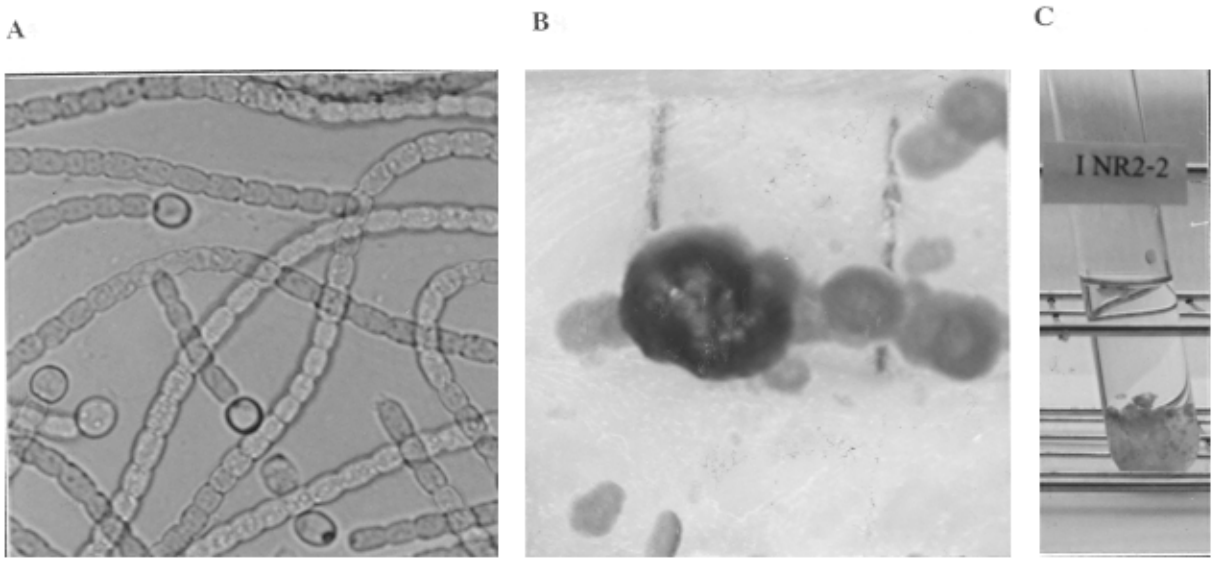

Fig. A9 Cell morphology under microscope with 400X magnification (A), colony forming on BG11 solid (B) and clump forming in BG11 liquid medium (C) of cyanobacterial isolate I NR2-2

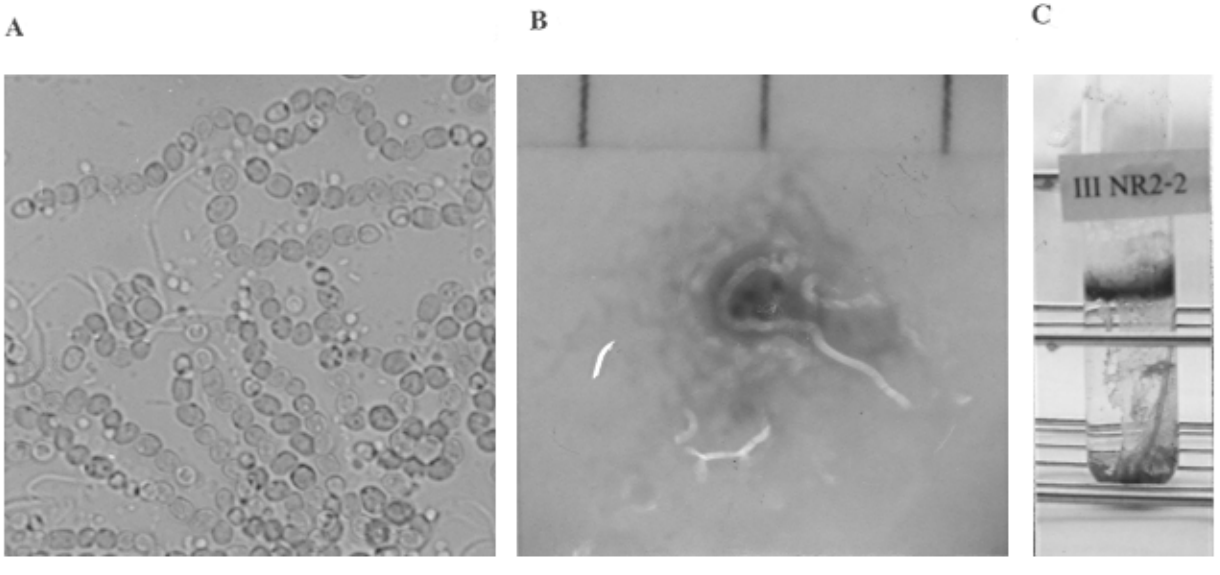

Fig. A10 Cell morphology under microscope with 400X magnification (A), colony forming on BG11 solid (B) and clump forming in BG11 liquid medium (C) of cyanobacterial isolate III NR2-2

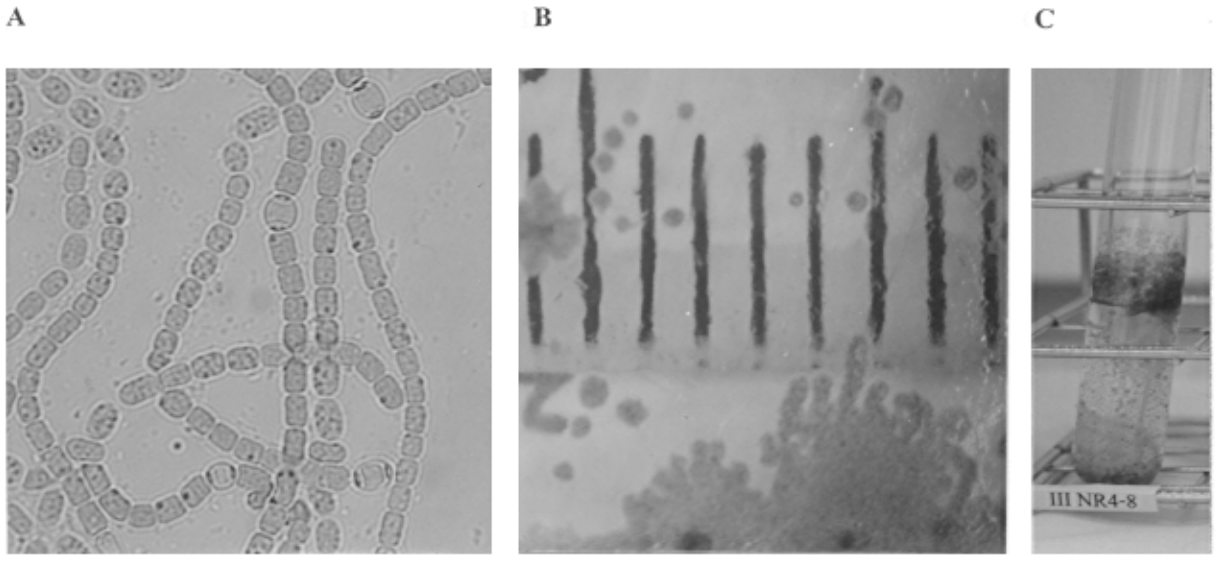

Fig. A11 Cell morphology under microscope with 400X magnification (A), colony forming on BG11 solid (B) and clump forming in BG11 liquid medium (C) of cyanobacterial isolate III NR4-8

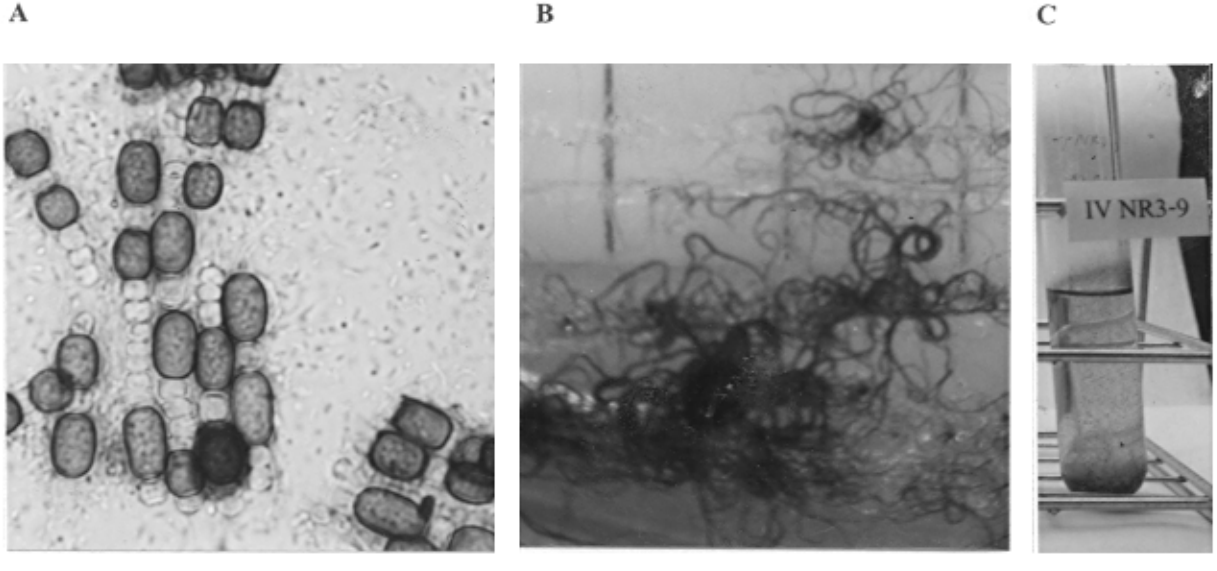

Fig. A12 Cell morphology under microscope with 400X magnification (A), colony forming on BG11 solid (B) and clump forming in BG11 liquid medium (C) of cyanobacterial isolate IV NR3-9

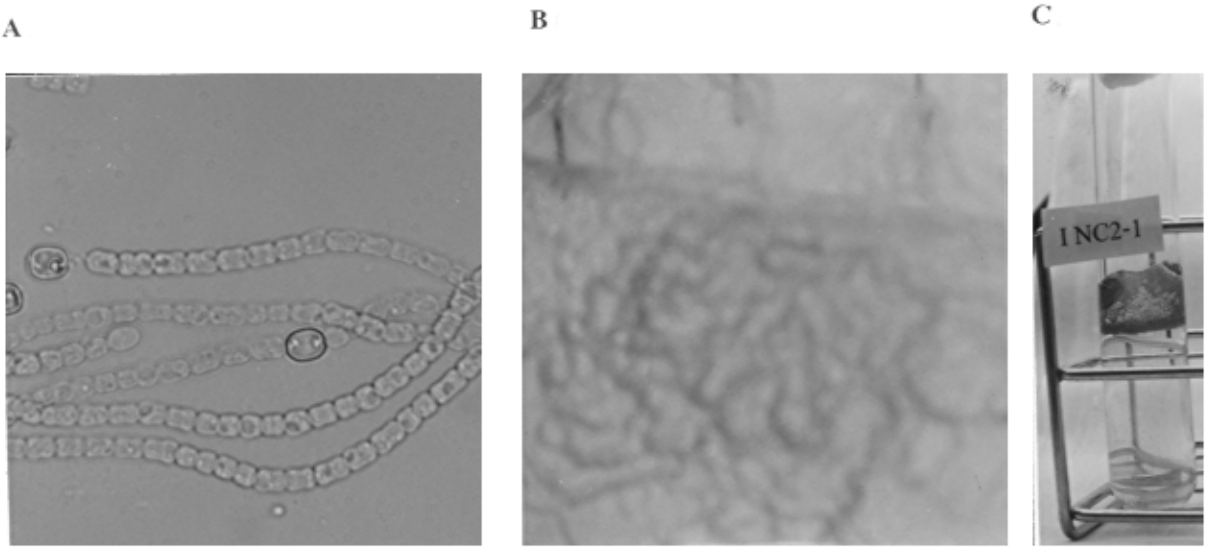

Fig. A13 Cell morphology under microscope with 400X magnification (A), colony forming on BG11 solid (B) and clump forming in BG11 liquid medium (C) of cyanobacterial isolate I NC2-1

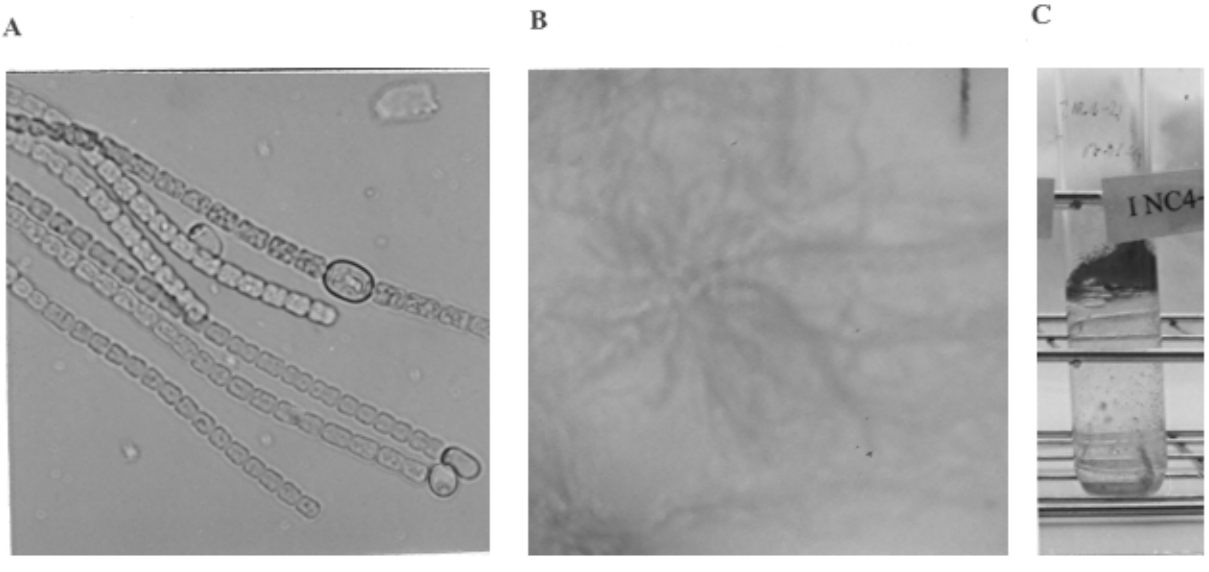

Fig. A14 Cell morphology under microscope with 400X magnification (A), colony forming on BG11 solid (B) and clump forming in BG11 liquid medium (C) of cyanobacterial isolate I NC4-21

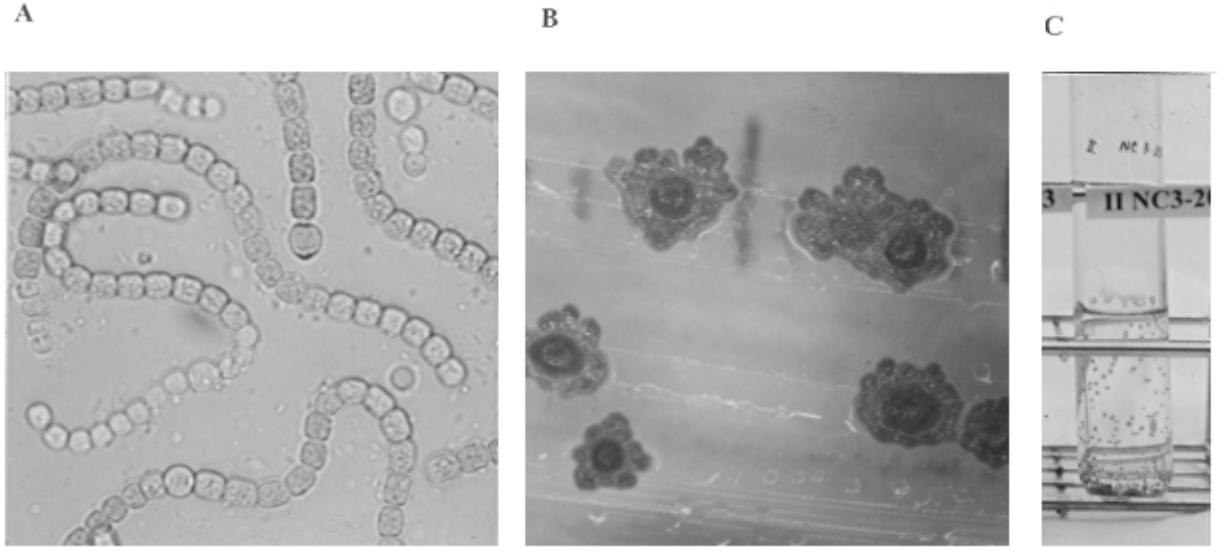

Fig. A15 Cell morphology under microscope with 400X magnification (A), colony forming on BG11 solid (B) and clump forming in BG11 liquid medium (C) of cyanobacterial isolate II NC3-20

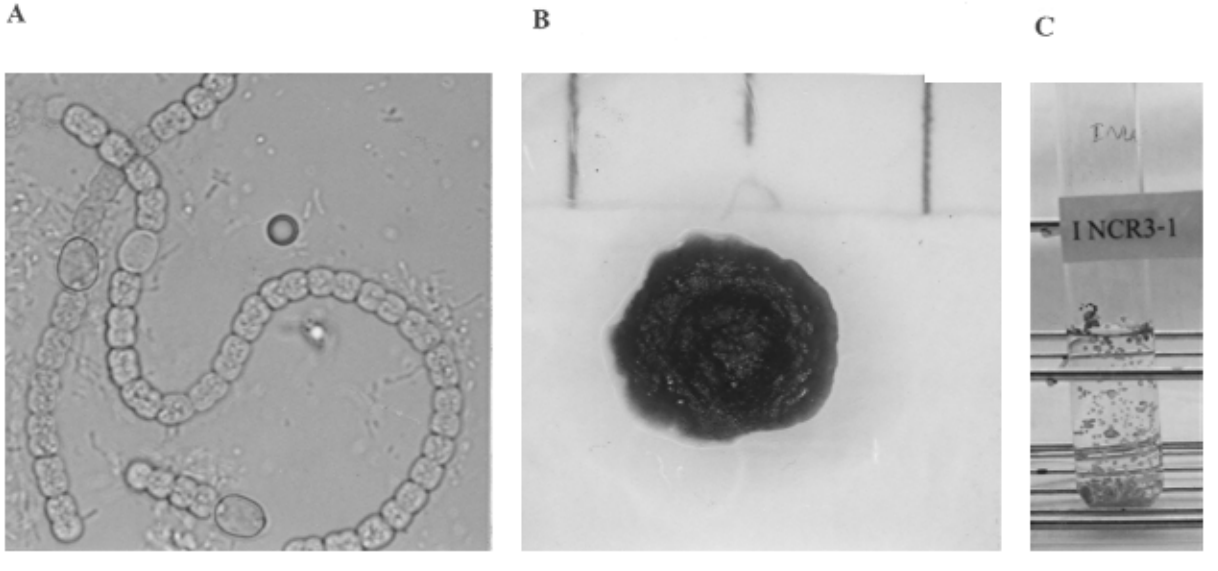

Fig. A16 Cell morphology under microscope with 400X magnification (A), colony forming on BG11 solid (B) and clump forming in BG11 liquid medium (C) of cyanobacterial isolate I NCR3-1

A B C

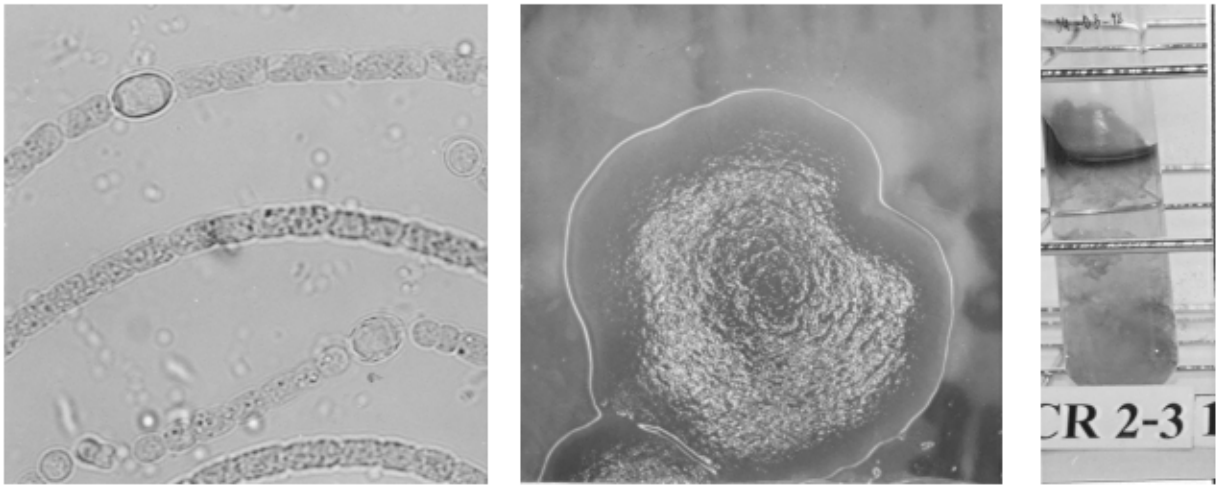

Fig. A17 Cell morphology under microscope with 400X magnification (A), colony forming on BG11 solid (B) and clump forming in BG11 liquid medium (C) of cyanobacterial isolate I NCR2-3

A B C

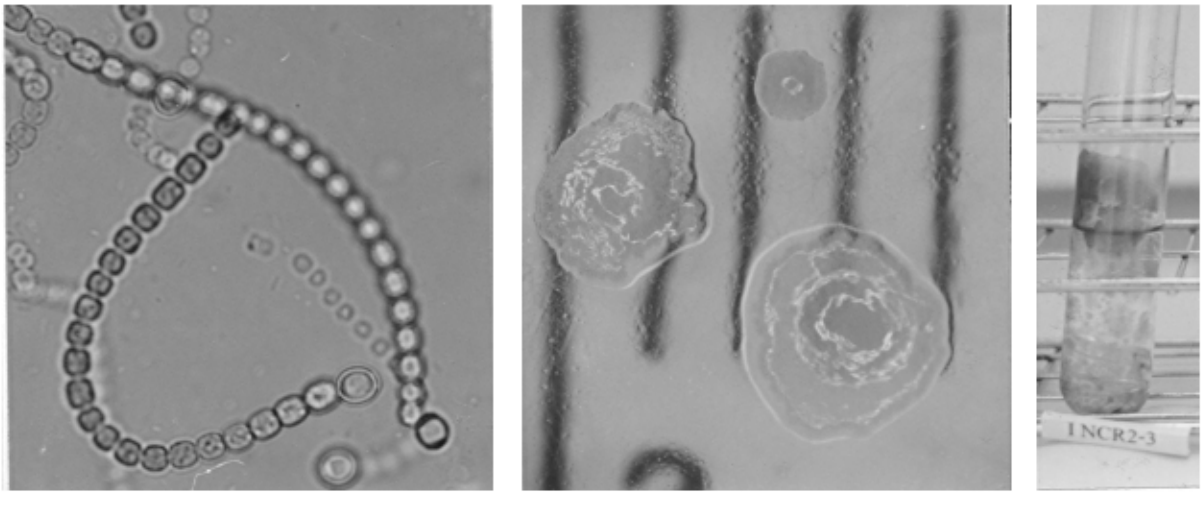

Fig. A18 Cell morphology under microscope with 400X magnification (A), colony forming on BG11 solid (B) and clump forming in BG11 liquid medium (C) of cyanobacterial isolate I NCR2-3.1

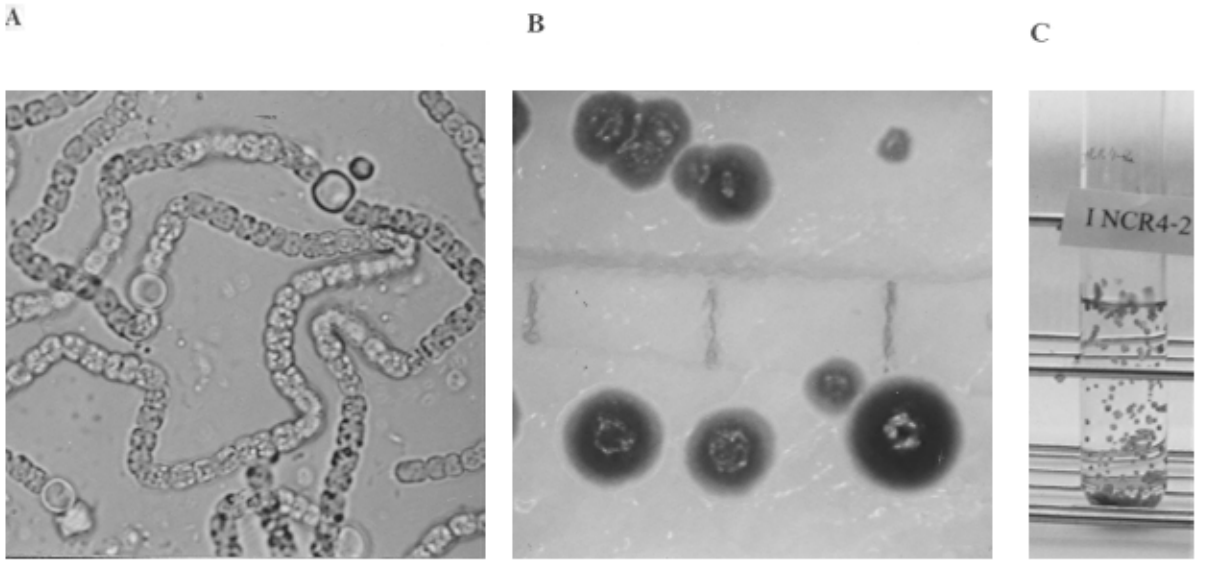

Fig. A19 Cell morphology under microscope with 400X magnification (A), colony forming on BG11 solid (B) and clump forming in BG11 liquid medium (C) of cyanobacterial isolate I NCR4-2

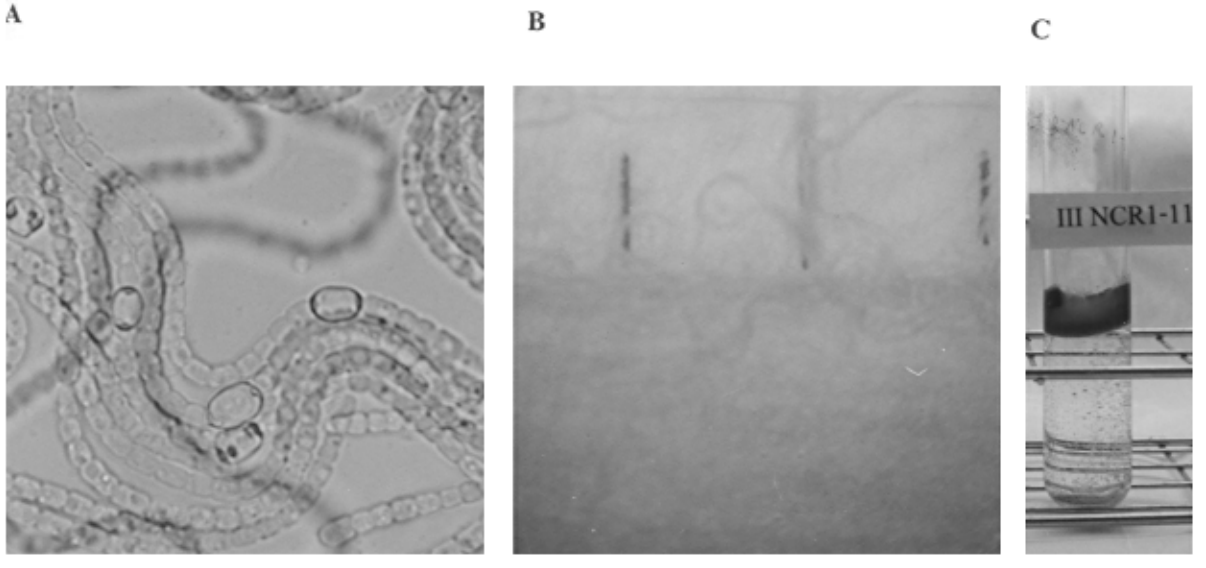

Fig. A20 Cell morphology under microscope with 400X magnification (A), colony forming on BG11 solid (B) and clump forming in BG11 liquid medium (C) of cyanobacterial isolate III NCR1-11

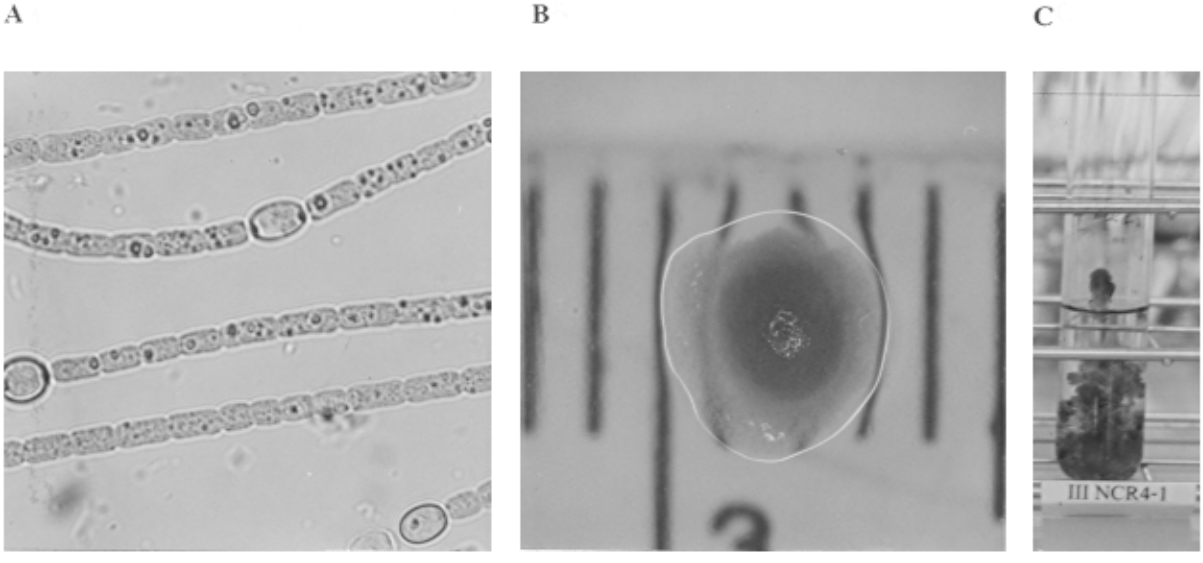

Fig. A21 Cell morphology under microscope with 400X magnification (A), colony forming on BG11 solid (B) and clump forming in BG11 liquid medium (C) of cyanobacterial isolate III NCR4-1

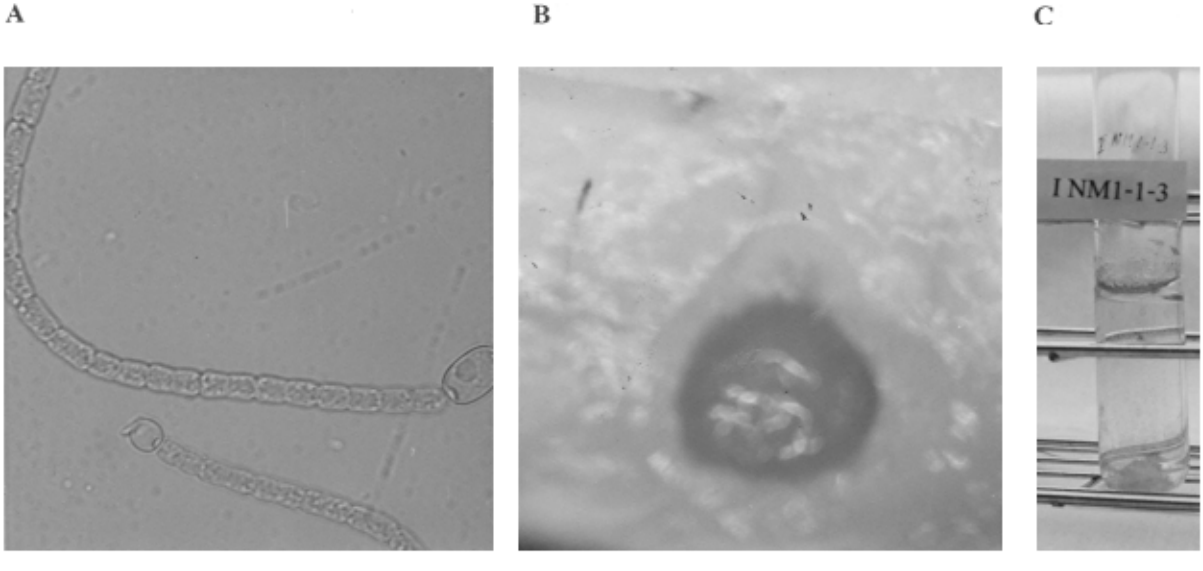

Fig. A22 Cell morphology under microscope with 400X magnification (A), colony forming on BG11 solid (B) and clump forming in BG11 liquid medium (C) of cyanobacterial isolate I NM1-1-3

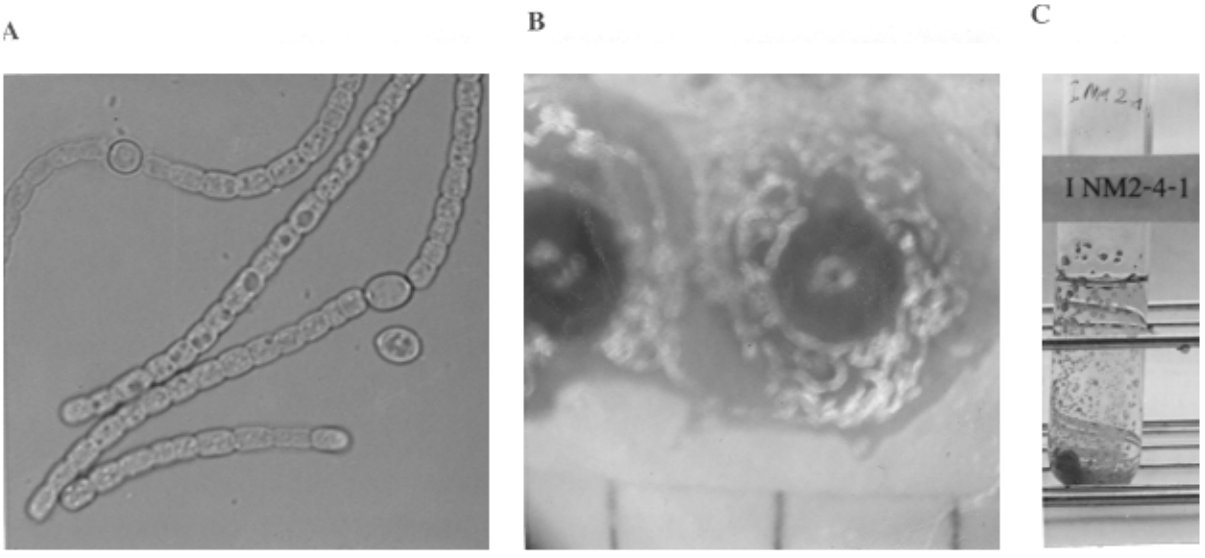

Fig. A23 Cell morphology under microscope with 400X magnification (A), colony forming on BG11 solid (B) and clump forming in BG11 liquid medium (C) of cyanobacterial isolate I NM2-4-1

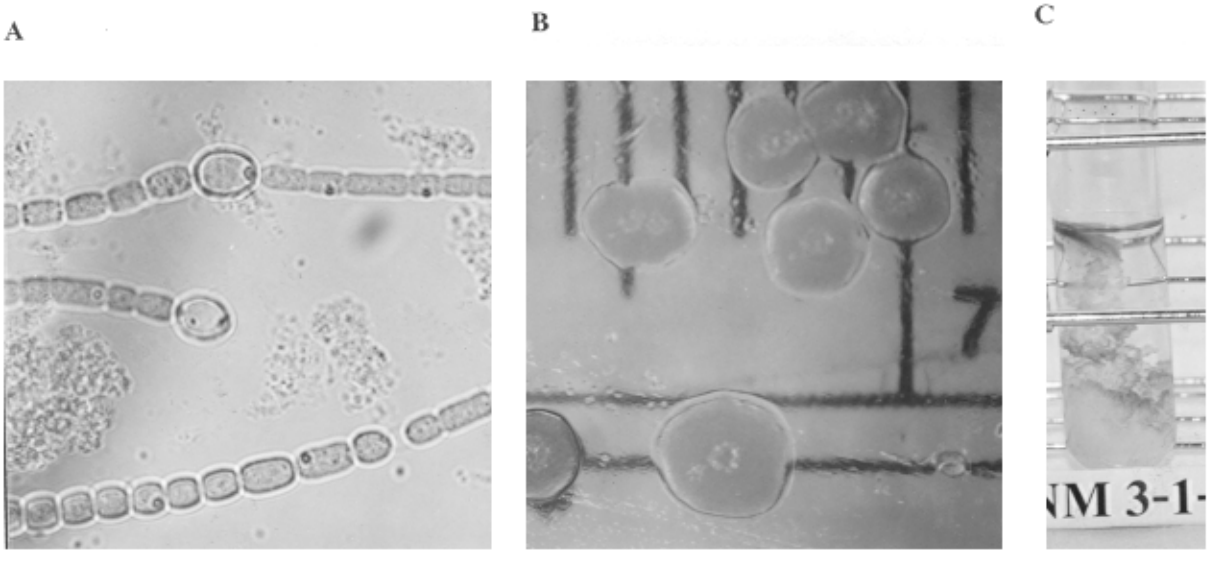

Fig. A24 Cell morphology under microscope with 400X magnification (A), colony forming on BG11 solid (B) and clump forming in BG11 liquid medium (C) of cyanobacterial isolate I NM3-1-1

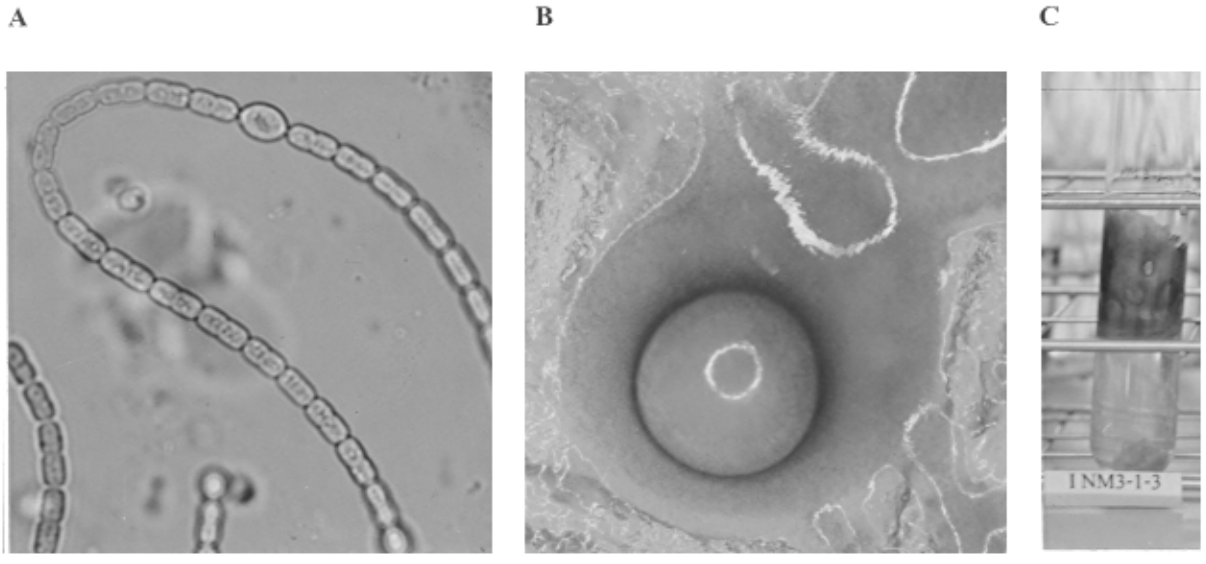

Fig. A25 Cell morphology under microscope with 400X magnification (A), colony forming on BG11 solid (B) and clump forming in BG11 liquid medium (C) of cyanobacterial isolate I NM3-1-3

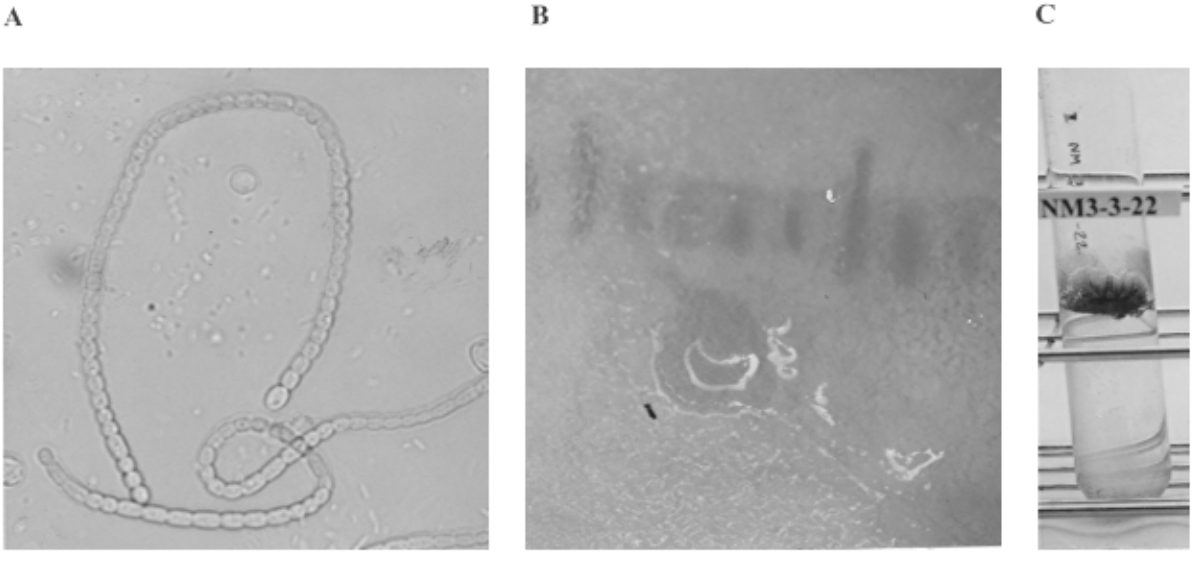

Fig. A26 Cell morphology under microscope with 400X magnification (A), colony forming on BG11 solid (B) and clump forming in BG11 liquid medium (C) of cyanobacterial isolate II NM3-3-22

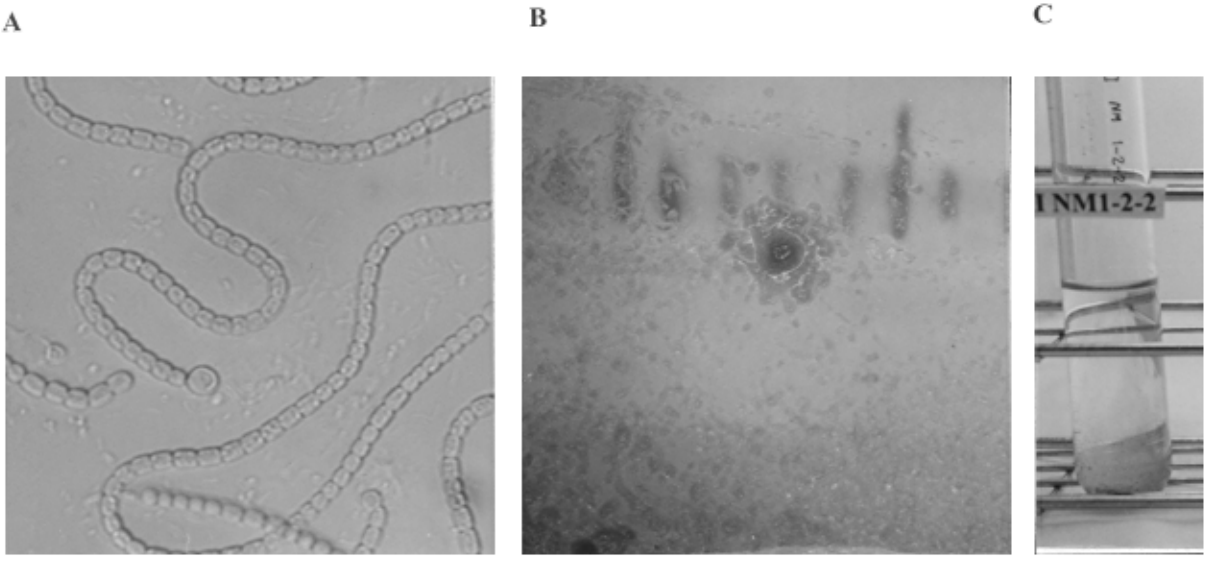

Fig. A27 Cell morphology under microscope with 400X magnification (A), colony forming on BG11 solid (B) and clump forming in BG11 liquid medium (C) of cyanobacterial isolate III NM1-2-2

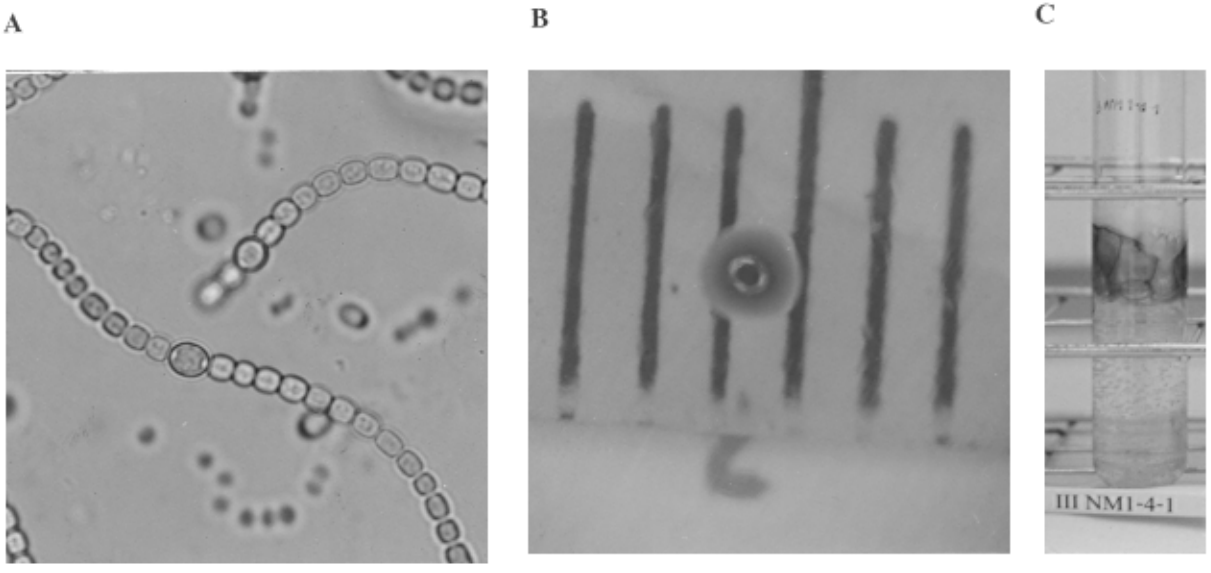

Fig. A28 Cell morphology under microscope with 400X magnification (A), colony forming on BG11 solid (B) and clump forming in BG11 liquid medium (C) of cyanobacterial isolate III NM1-4-1

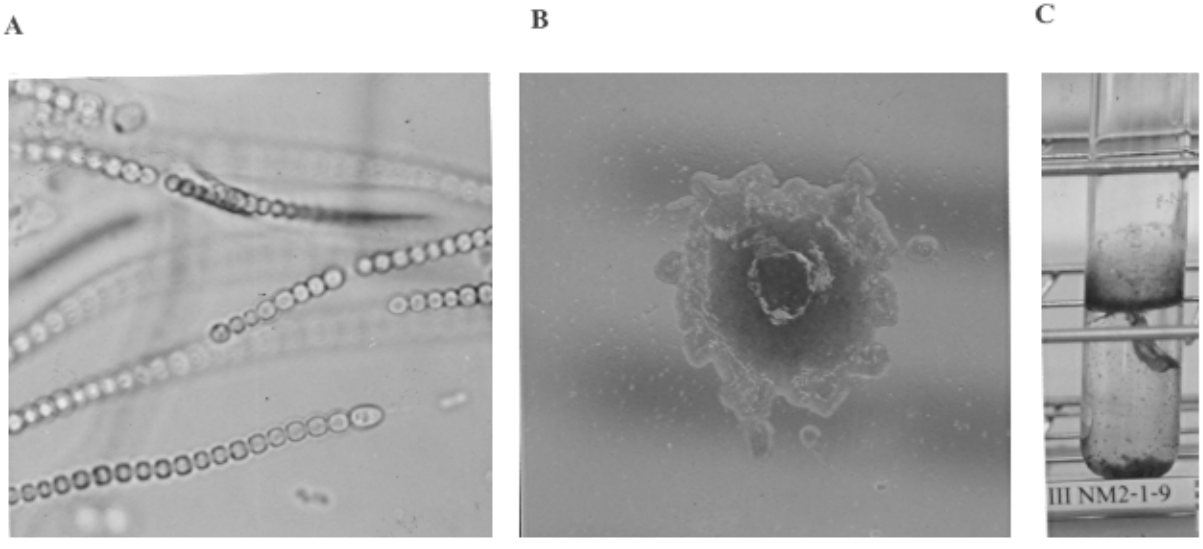

Fig. A29 Cell morphology under microscope with 400X magnification (A), colony forming on BG11 solid (B) and clump forming in BG11 liquid medium (C) of cyanobacterial isolate III NM2-1-9

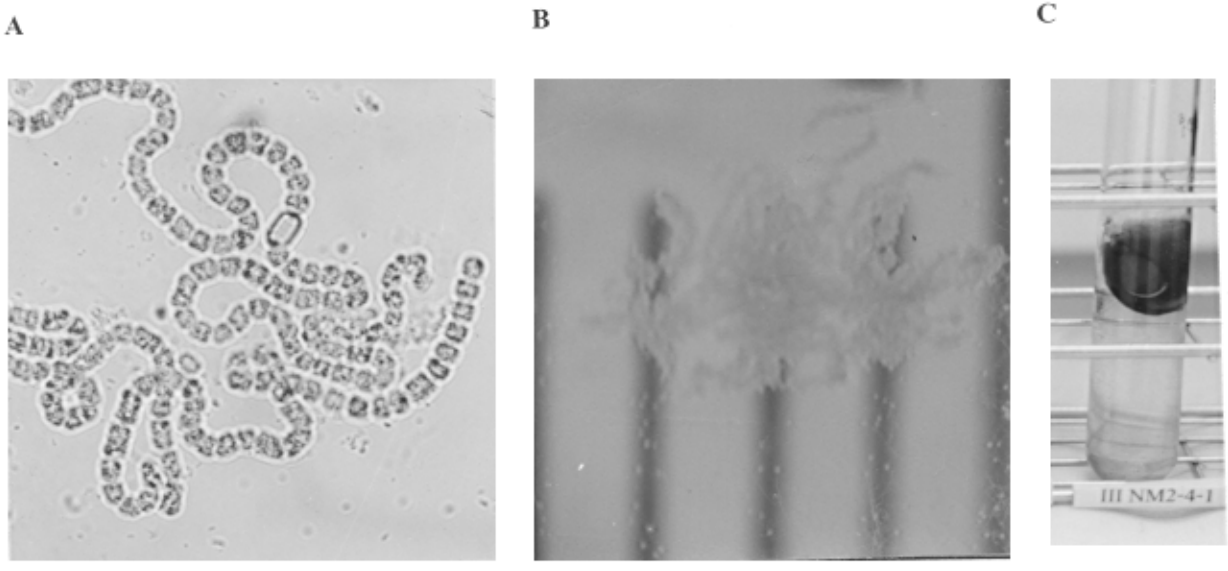

Fig. A30 Cell morphology under microscope with 400X magnification (A), colony forming on BG11 solid (B) and clump forming in BG11 liquid medium (C) of cyanobacterial isolate III NM2-4-1

A B C

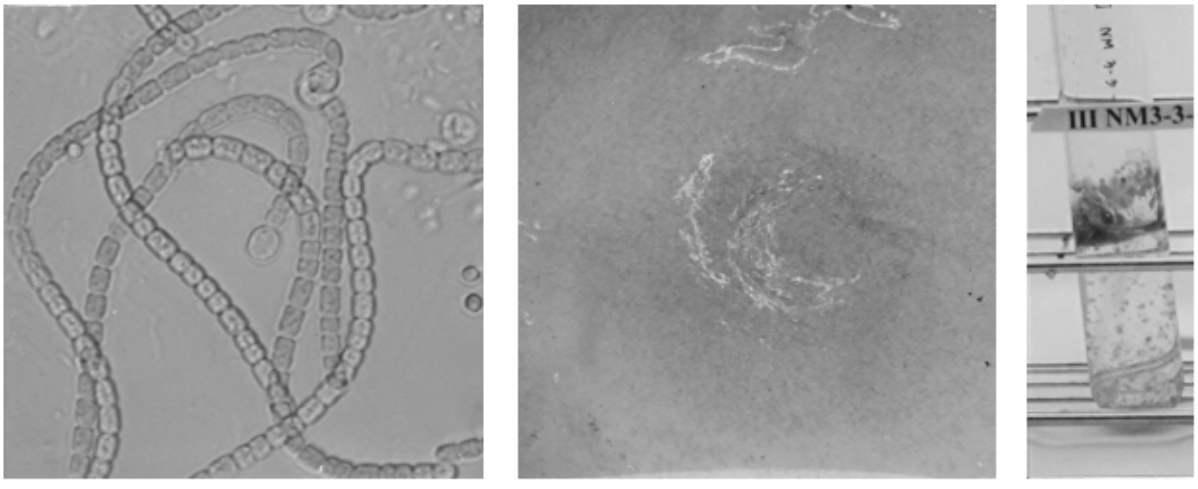

Fig. A31 Cell morphology under microscope with 400X magnification (A), colony forming on BG11 solid (B) and clump forming in BG11 liquid medium (C) of cyanobacterial isolate III NM3-3-13

A B C

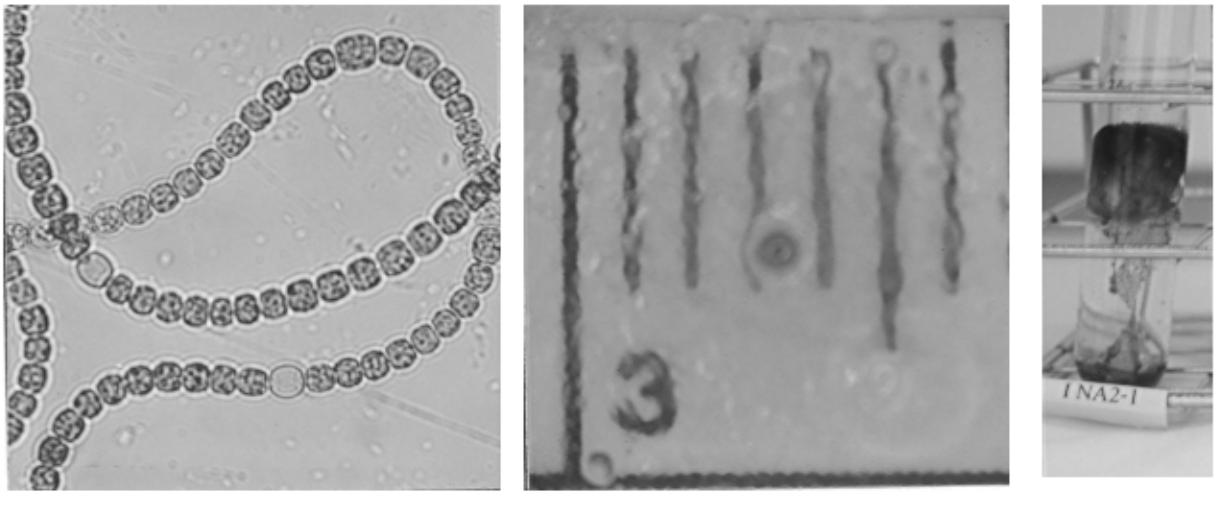

Fig. A32 Cell morphology under microscope with 400X magnification (A), colony forming on BG11 solid (B) and clump forming in BG11 liquid medium (C) of cyanobacterial isolate I NA2-1

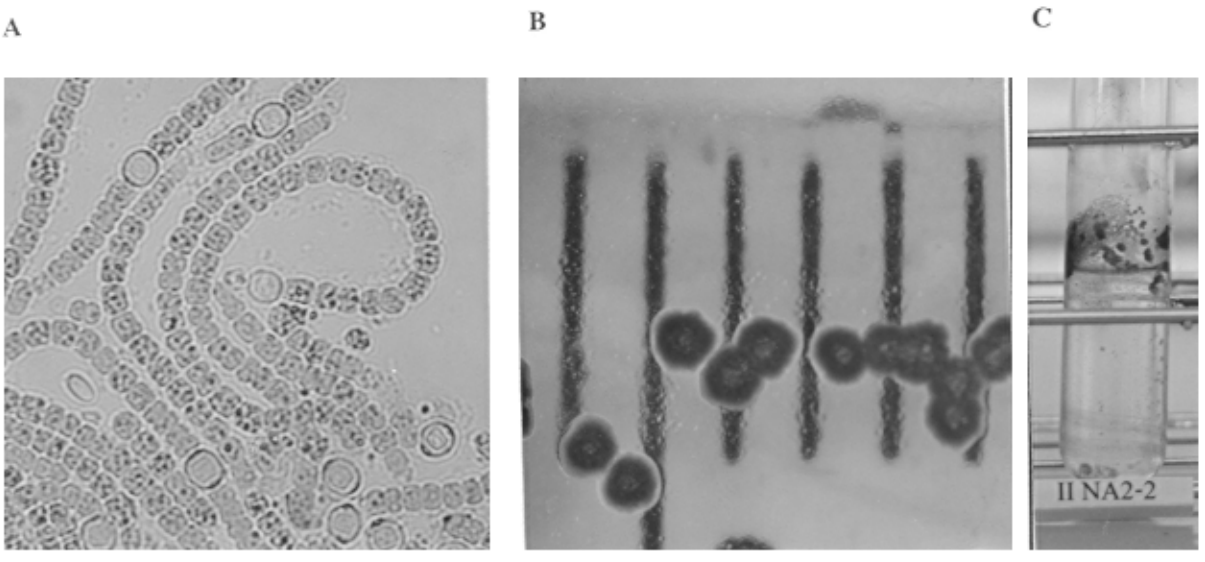

Fig. A33 Cell morphology under microscope with 400X magnification (A), colony forming on BG11 solid (B) and clump forming in BG11 liquid medium (C) of cyanobacterial isolate II NA2-2

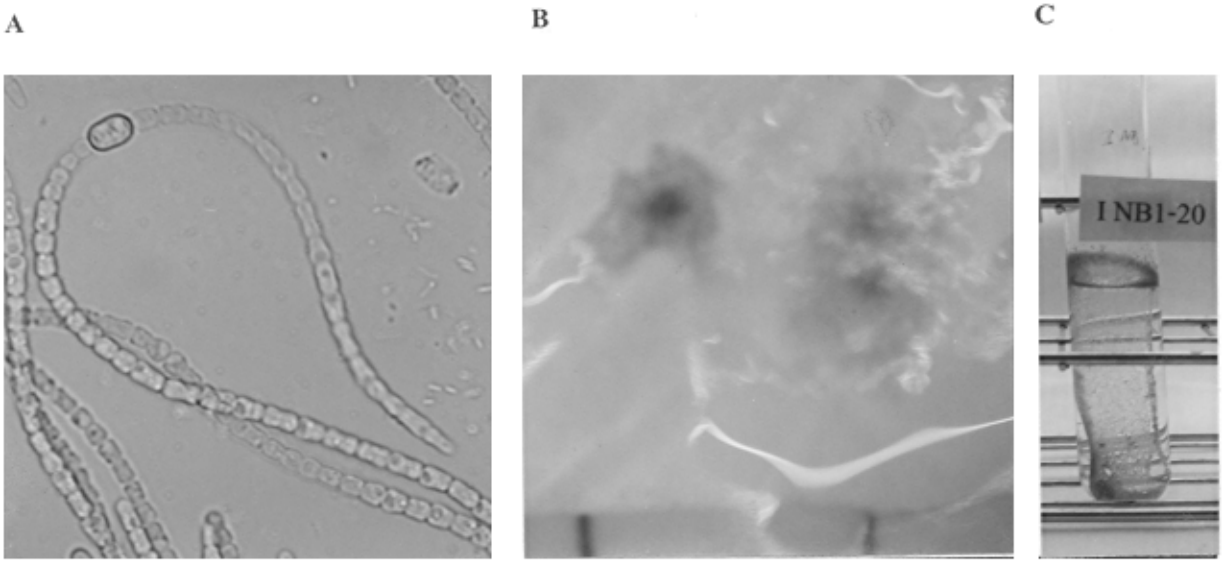

Fig. A34 Cell morphology under microscope with 400X magnification (A), colony forming on BG11 solid (B) and clump forming in BG11 liquid medium (C) of cyanobacterial isolate I NB1-20

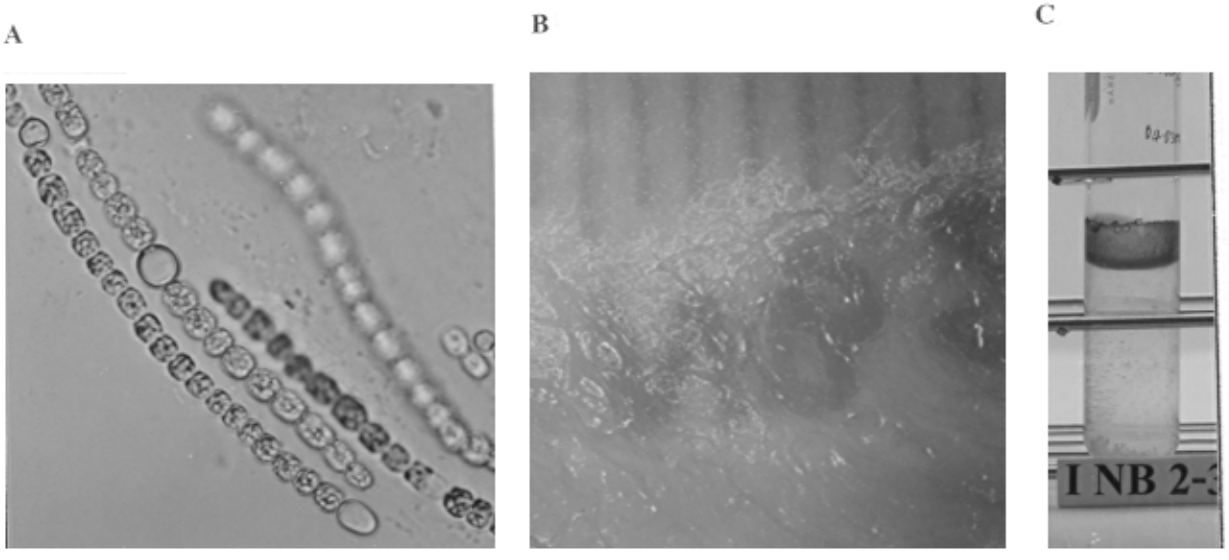

Fig. A35 Cell morphology under microscope with 400X magnification (A), colony forming on BG11 solid (B) and clump forming in BG11 liquid medium (C) of cyanobacterial isolate I NB2-3

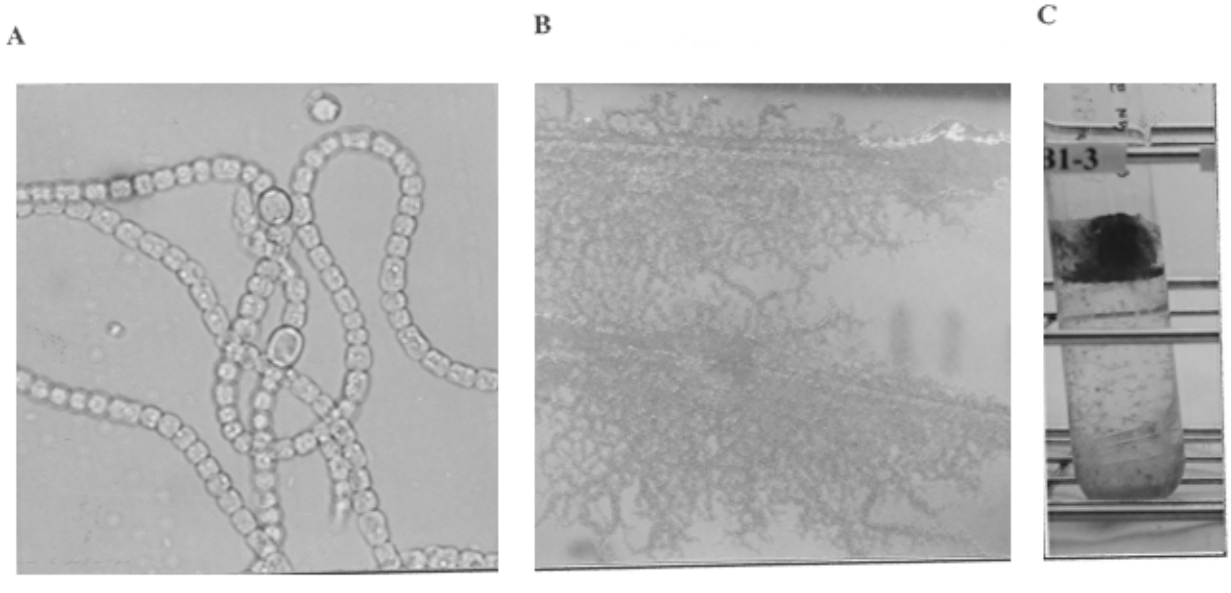

Fig. A36 Cell morphology under microscope with 400X magnification (A), colony forming on BG11 solid (B) and clump forming in BG11 liquid medium (C) of cyanobacterial isolate III NB1-3

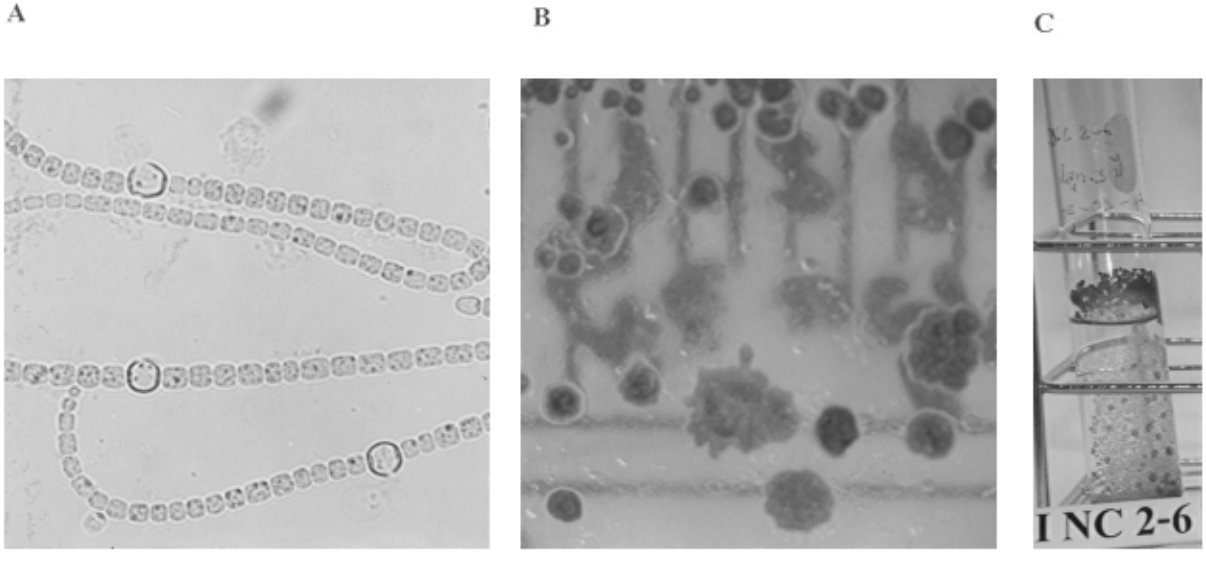

Fig. A37 Cell morphology under microscope with 400X magnification (A), colony forming on BG11 solid (B) and clump forming in BG11 liquid medium (C) of cyanobacterial isolate I NC*2-6

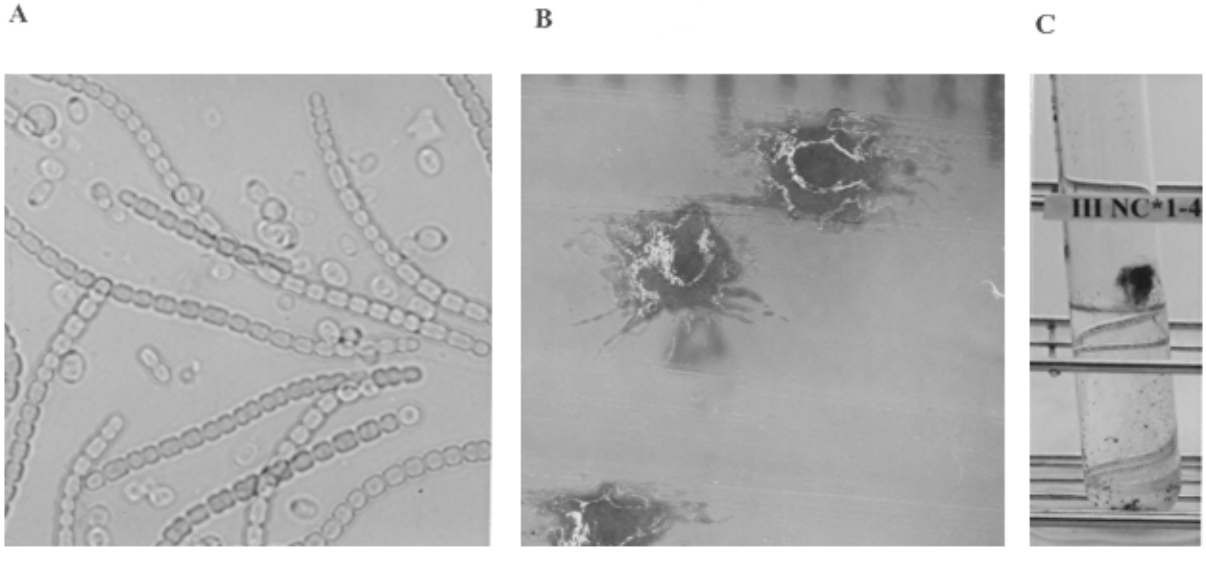

Fig. A38 Cell morphology under microscope with 400X magnification (A), colony forming on BG11 solid (B) and clump forming in BG11 liquid medium (C) of cyanobacterial isolate III NC*1-4

A B C

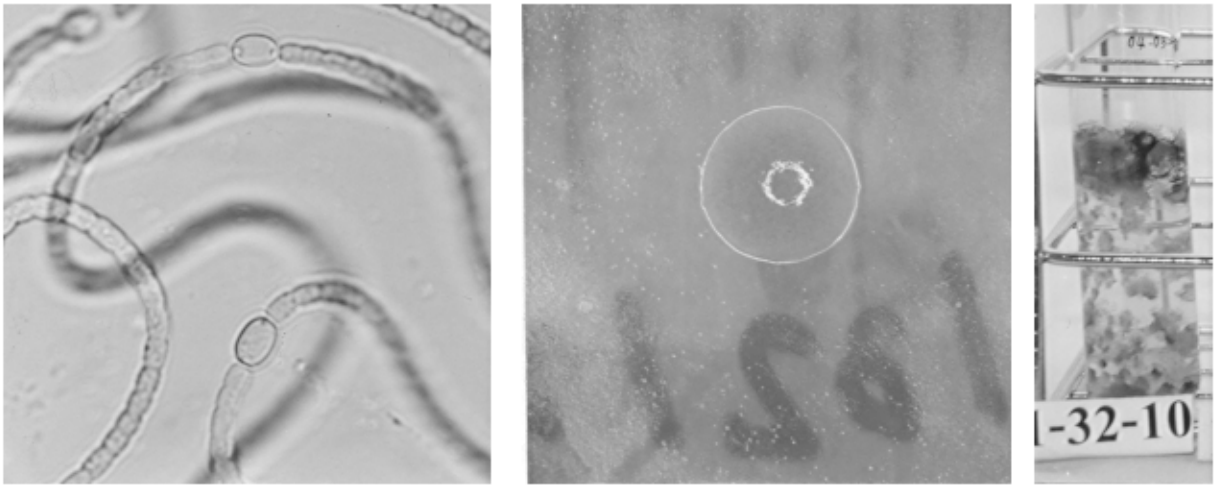

Fig. A39 Cell morphology under microscope with 400X magnification (A), colony forming on BG11 solid (B) and clump forming in BG11 liquid medium (C) of cyanobacterial isolate I ND-2

A B C

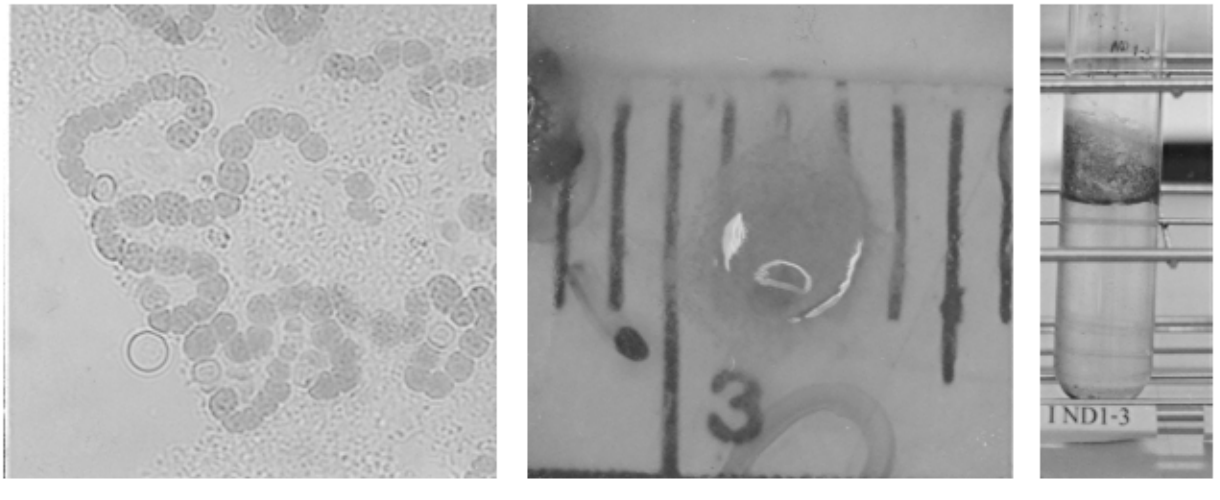

Fig. A40 Cell morphology under microscope with 400X magnification (A), colony forming on BG11 solid (B) and clump forming in BG11 liquid medium (C) of cyanobacterial isolate I ND1-3

A B C

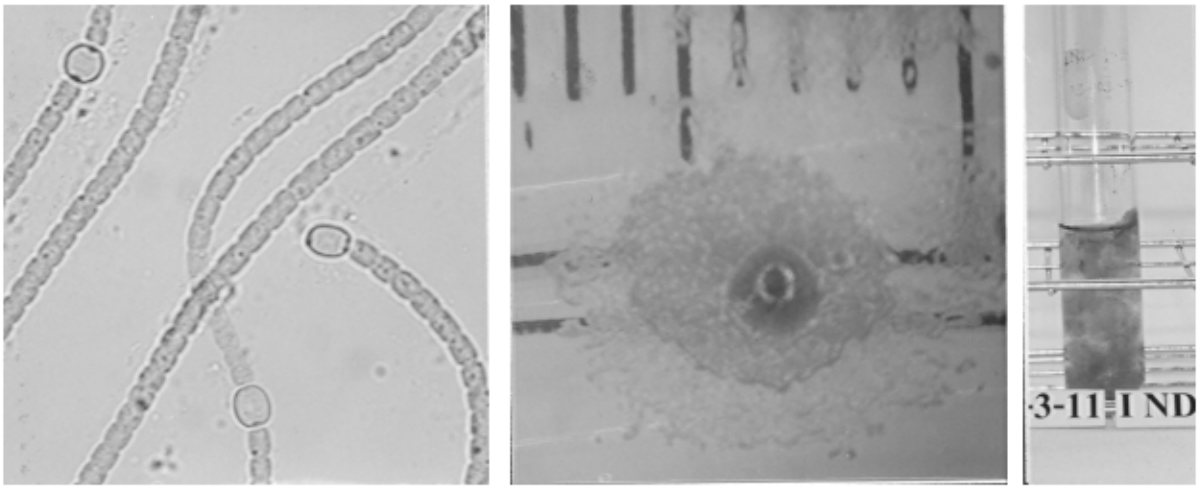

Fig. A41 Cell morphology under microscope with 400X magnification (A), colony forming on BG11 solid (B) and clump forming in BG11 liquid medium (C) of cyanobacterial isolate I ND1-14

A B C

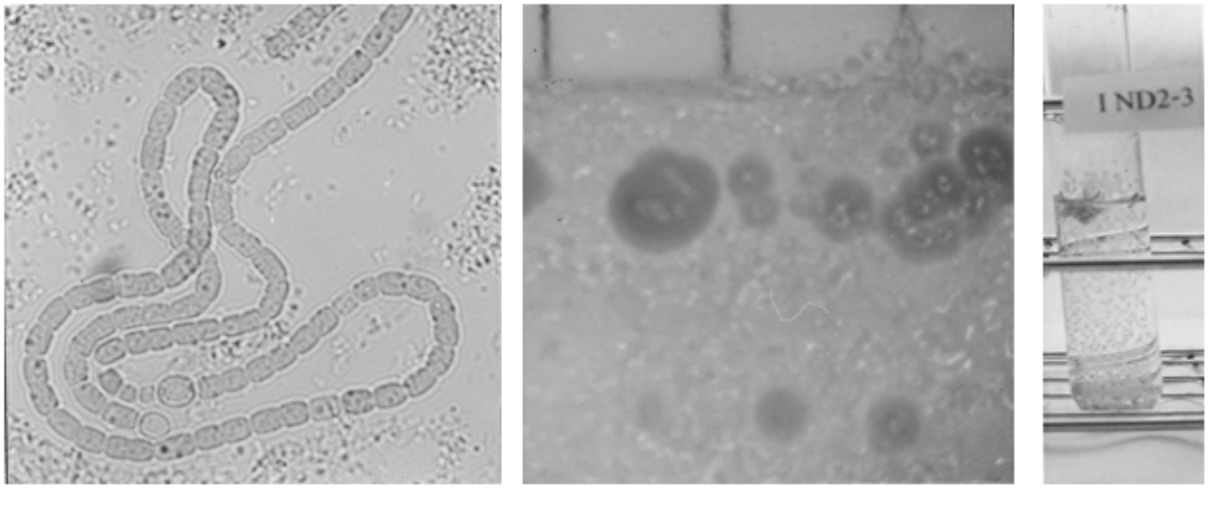

Fig. A42 Cell morphology under microscope with 400X magnification (A), colony forming on BG11 solid (B) and clump forming in BG11 liquid medium (C) of cyanobacterial isolate I ND2-3

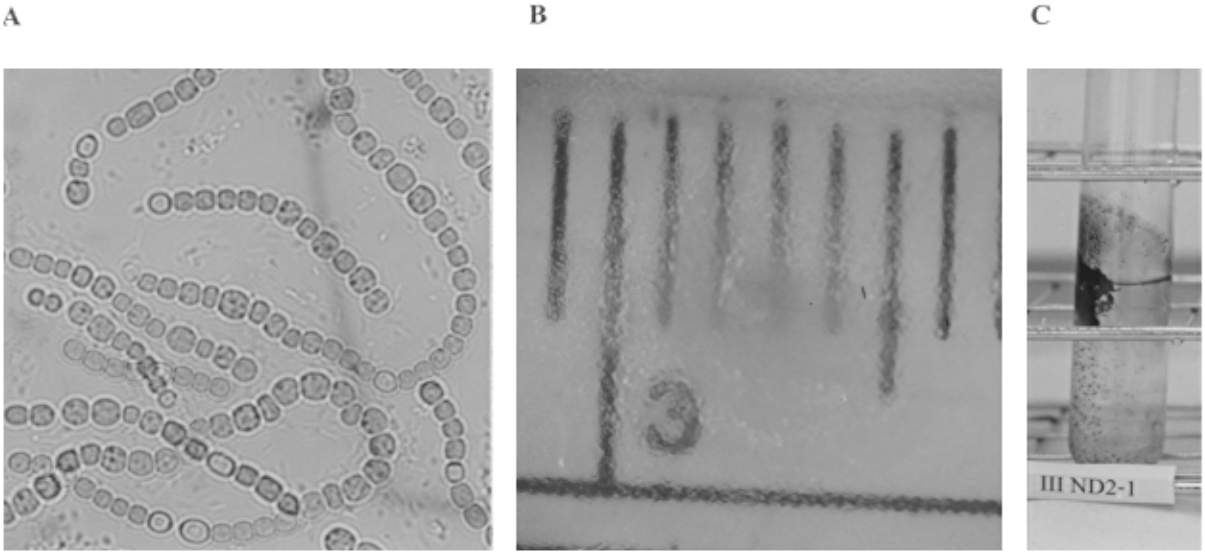

Fig. A43 Cell morphology under microscope with 400X magnification (A), colony forming on BG11 solid (B) and clump forming in BG11 liquid medium (C) of cyanobacterial isolate III ND2-1

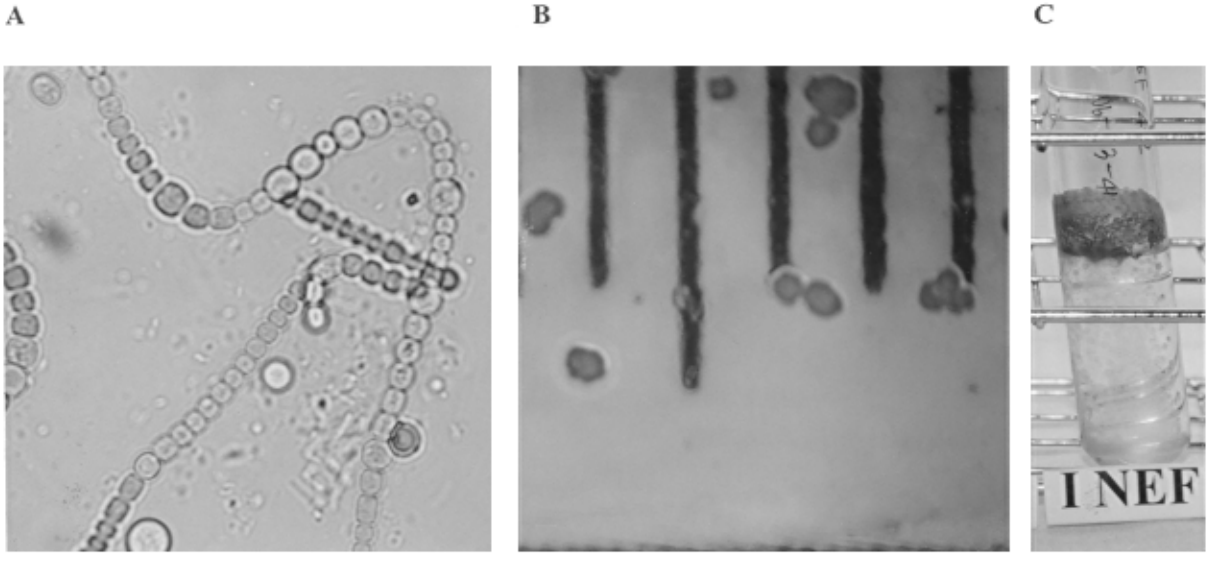

Fig. A44 Cell morphology under microscope with 400X magnification (A), colony forming on BG11 solid (B) and clump forming in BG11 liquid medium (C) of cyanobacterial isolate I NEF4-2

A B C

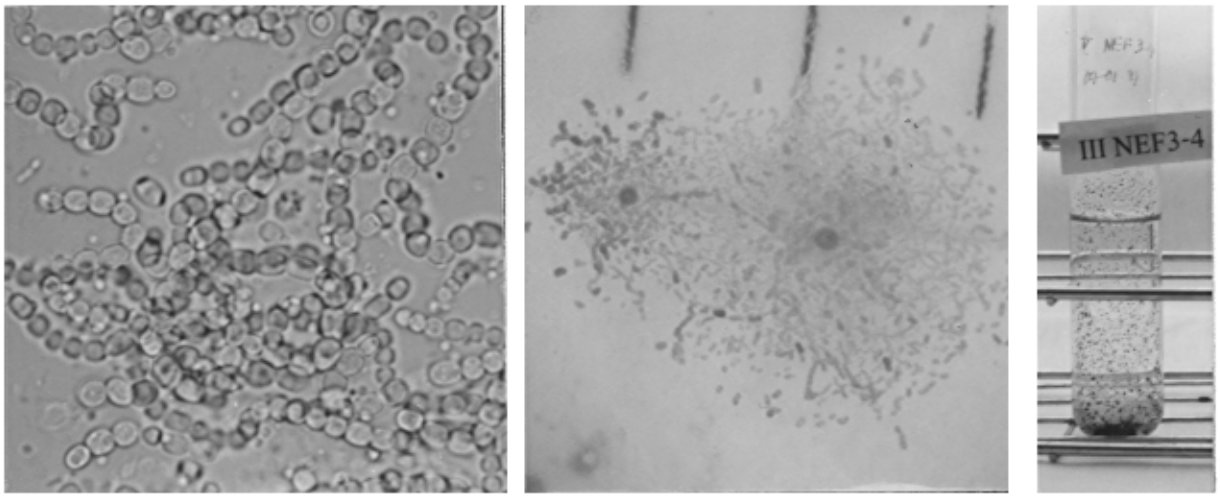

Fig. A45 Cell morphology under microscope with 400X magnification (A), colony forming on BG11 solid (B) and clump forming in BG11 liquid medium (C) of cyanobacterial isolate III NEF3-4

A B C

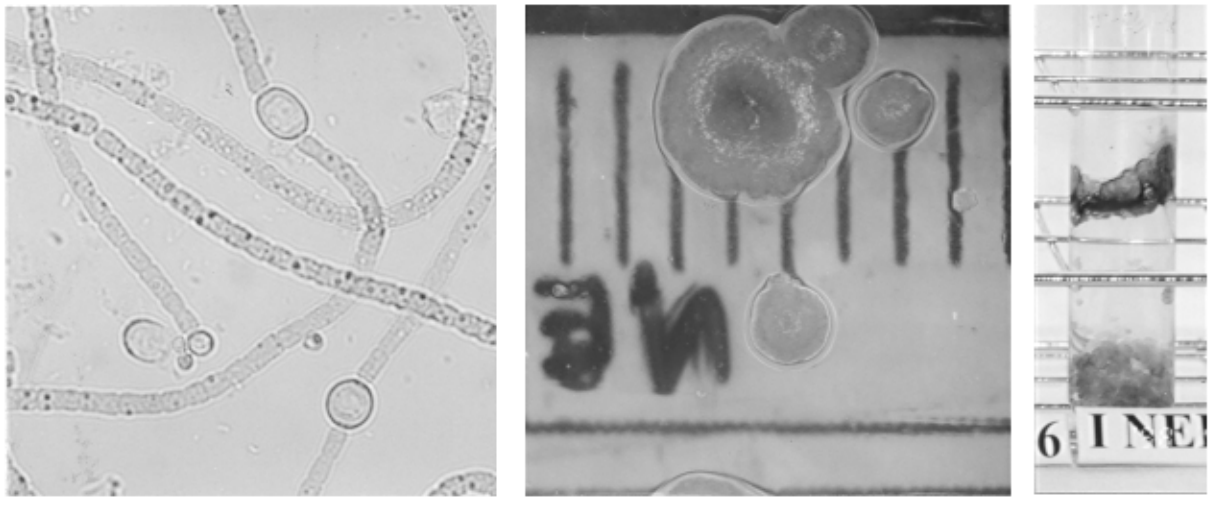

Fig. A46 Cell morphology under microscope with 400X magnification (A), colony forming on BG11 solid (B) and clump forming in BG11 liquid medium (C) of cyanobacterial isolate I NER3-1

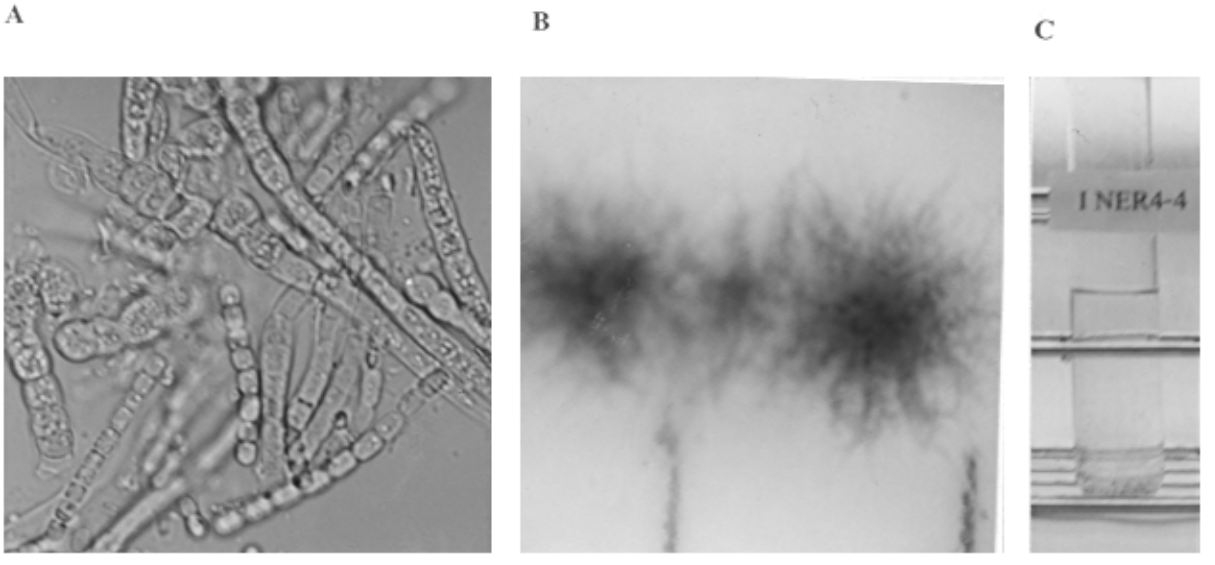

Fig. A47 Cell morphology under microscope with 400X magnification (A), colony forming on BG11 solid (B) and clump forming in BG11 liquid medium (C) of cyanobacterial isolate I NER4-4

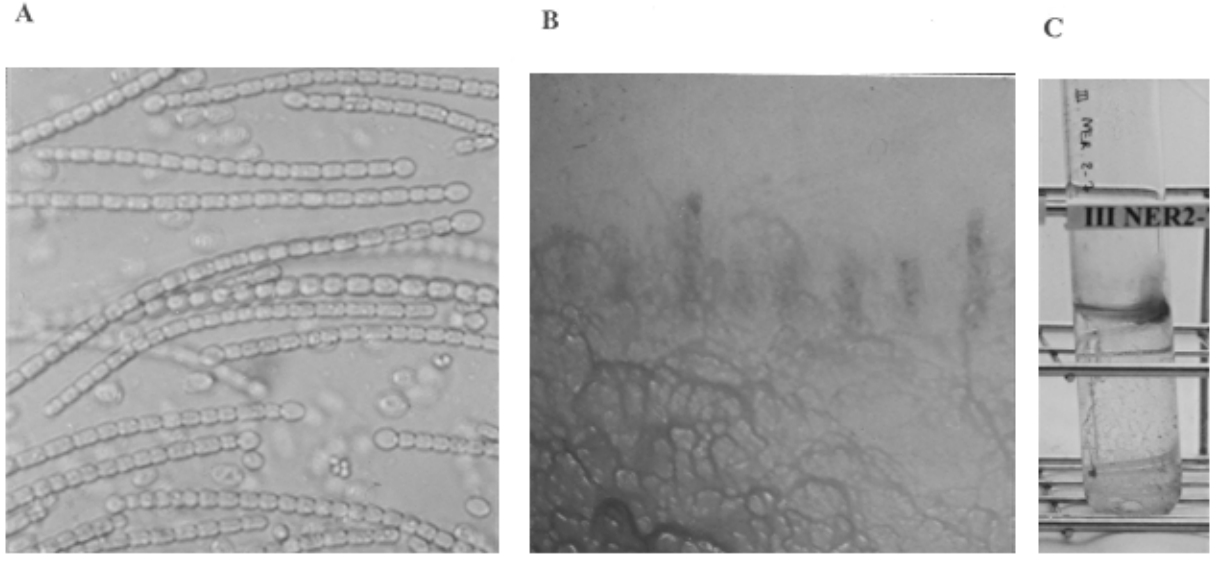

Fig. A48 Cell morphology under microscope with 400X magnification (A), colony forming on BG11 solid (B) and clump forming in BG11 liquid medium (C) of cyanobacterial isolate III NER2-7

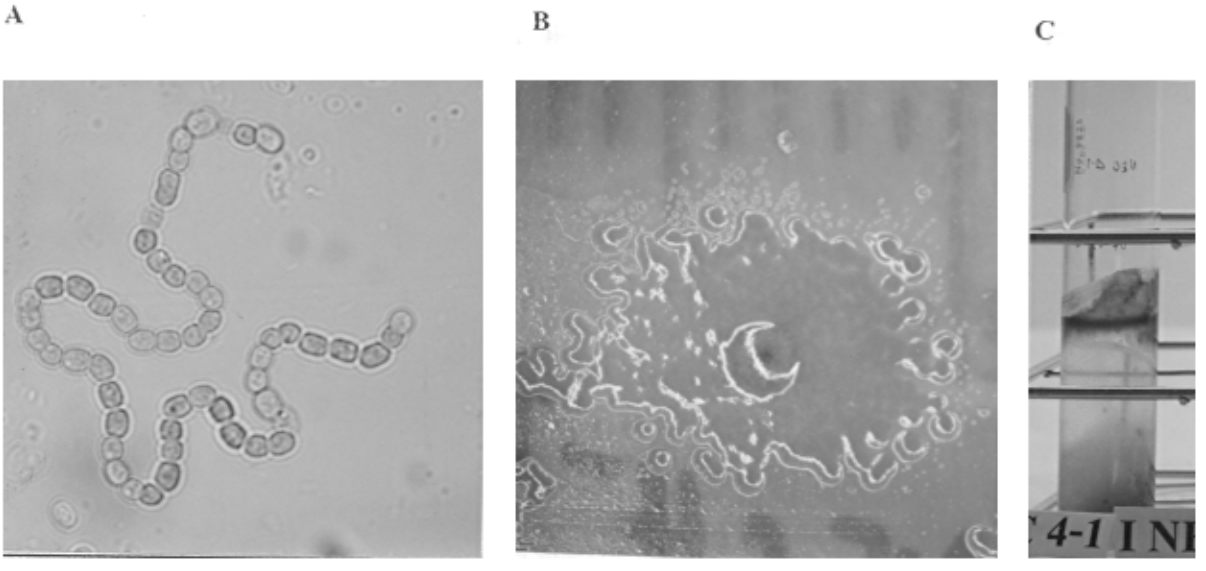

Fig. A49 Cell morphology under microscope with 400X magnification (A), colony forming on BG11 solid (B) and clump forming in BG11 liquid medium (C) of cyanobacterial isolate I NEC4-1

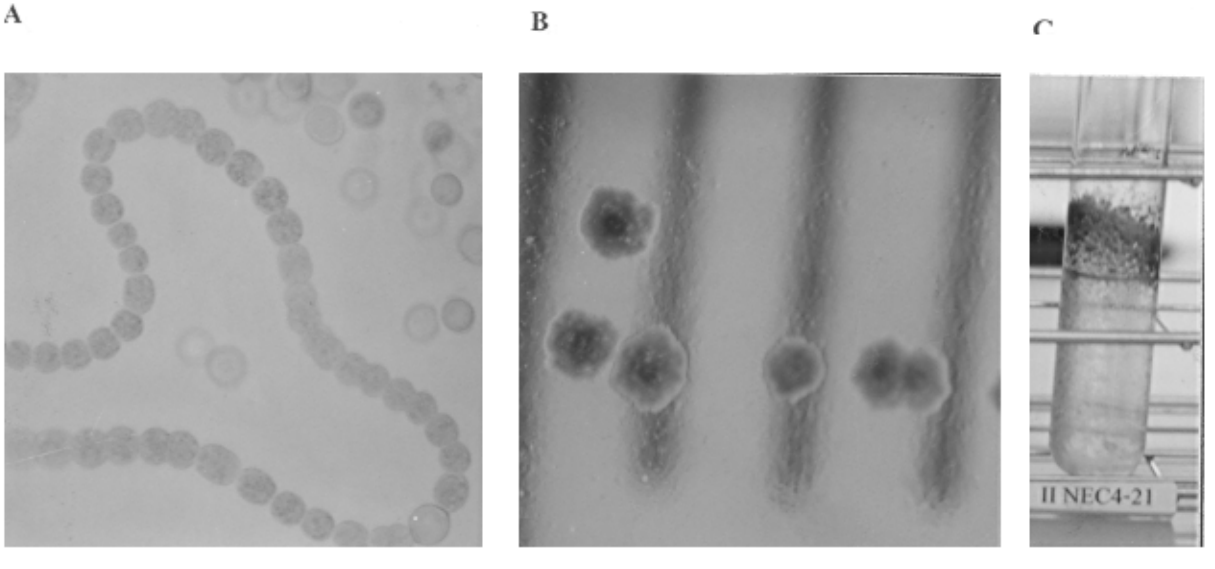

Fig. A50 Cell morphology under microscope with 400X magnification (A), colony forming on BG11 solid (B) and clump forming in BG11 liquid medium (C) of cyanobacterial isolate II NEC4-21

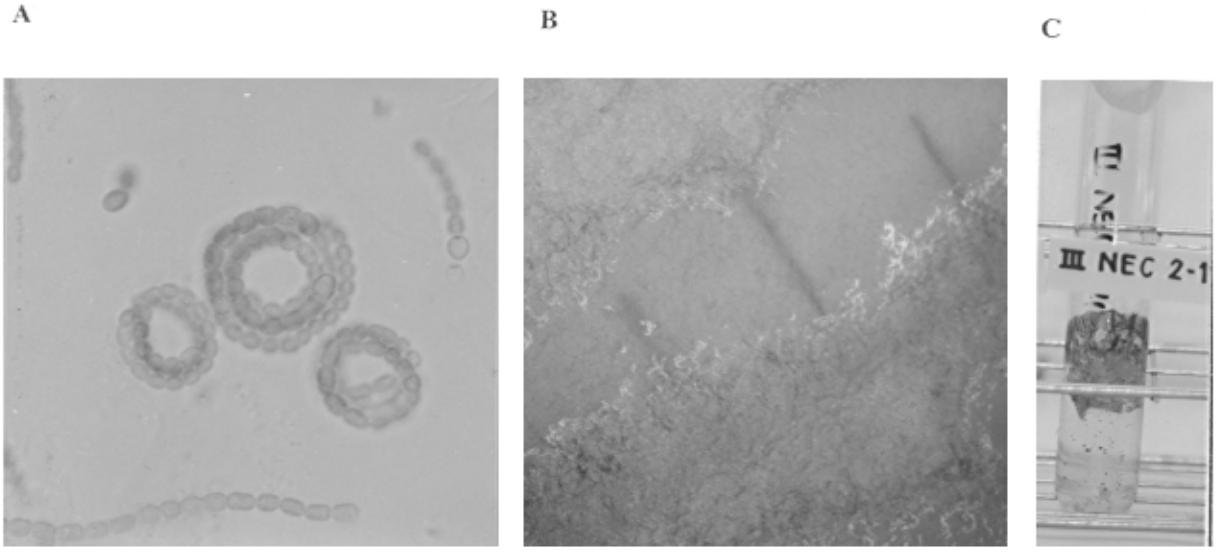

Fig. A51 Cell morphology under microscope with 400X magnification (A), colony forming on BG11 solid (B) and clump forming in BG11 liquid medium (C) of cyanobacterial isolate III NEC2-11

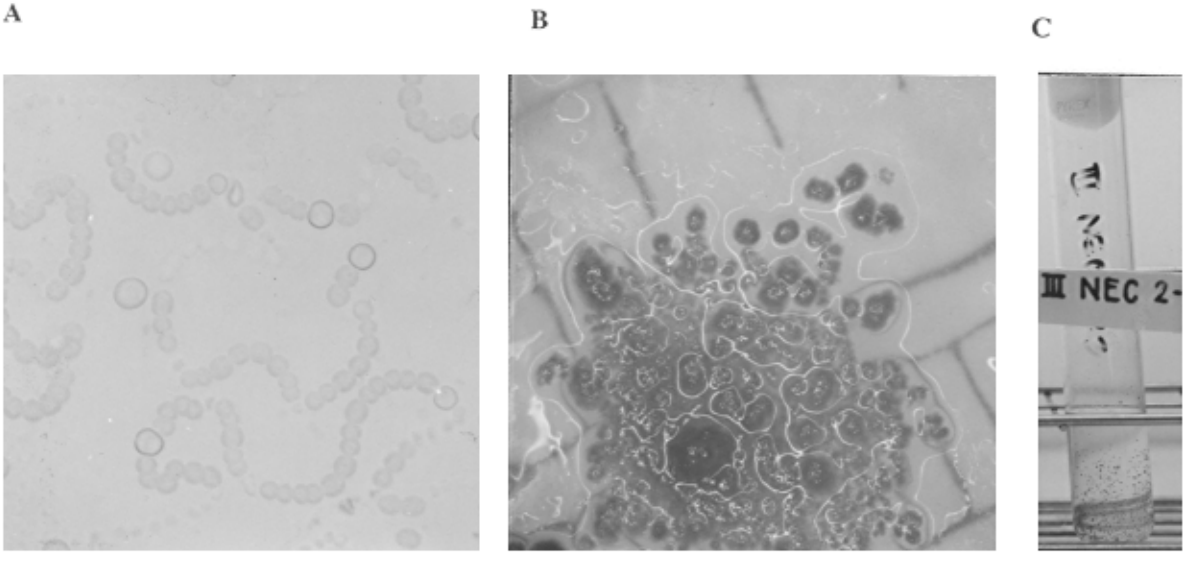

Fig. A52 Cell morphology under microscope with 400X magnification (A), colony forming on BG11 solid (B) and clump forming in BG11 liquid medium (C) of cyanobacterial isolate III NEC2-13

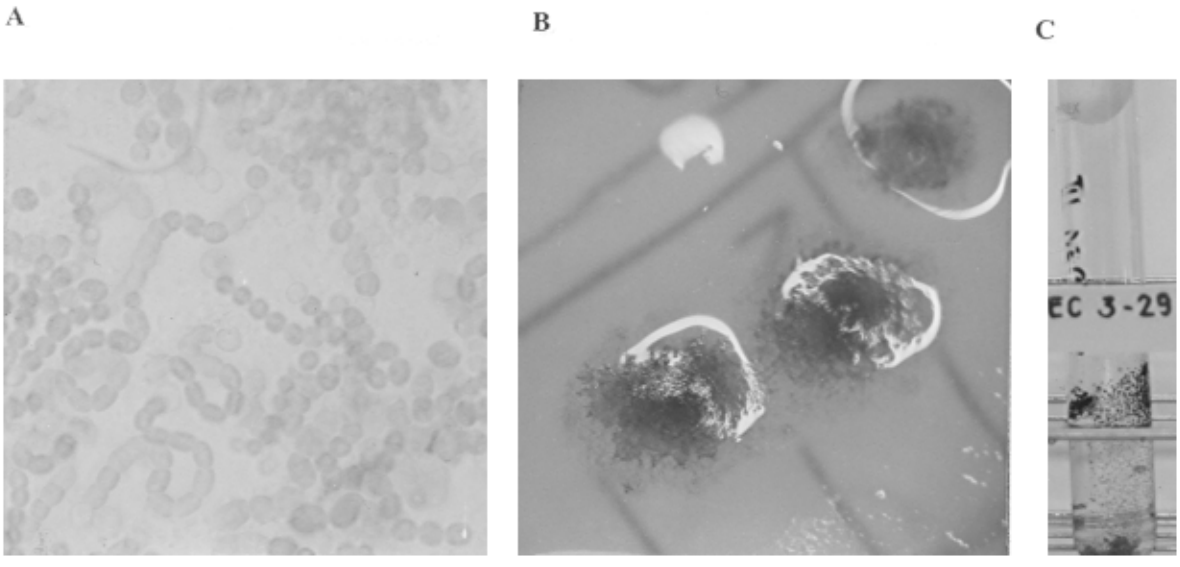

Fig. A53 Cell morphology under microscope with 400X magnification (A), colony forming on BG11 solid (B) and clump forming in BG11 liquid medium (C) of cyanobacterial isolate III NEC3-29

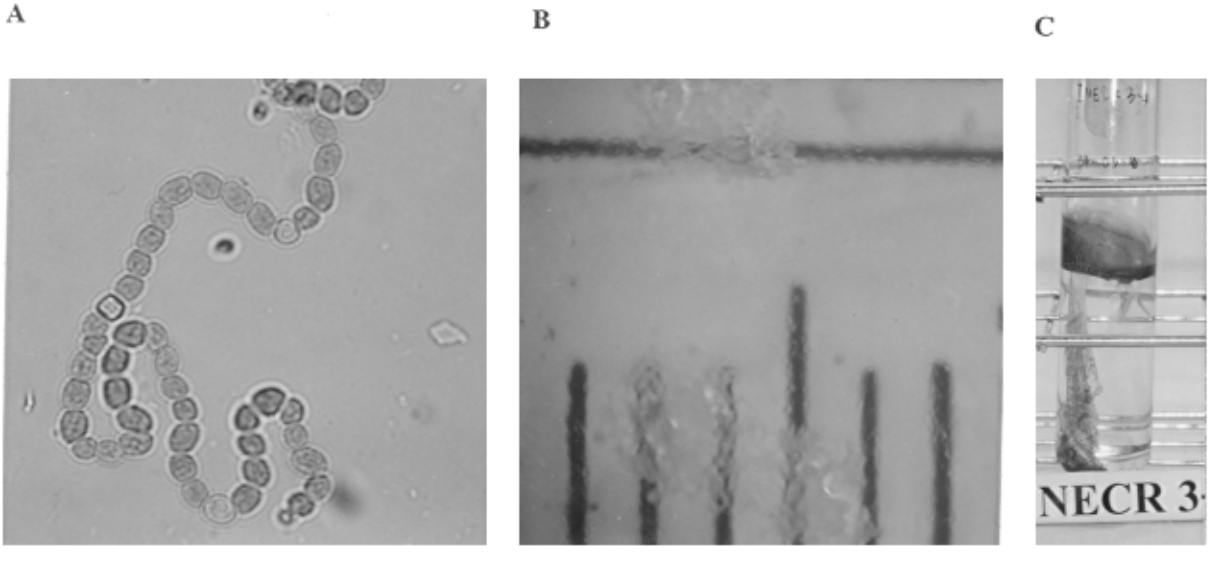

Fig. A54 Cell morphology under microscope with 400X magnification (A), colony forming on BG11 solid (B) and clump forming in BG11 liquid medium (C) of cyanobacterial isolate I NECR3-4

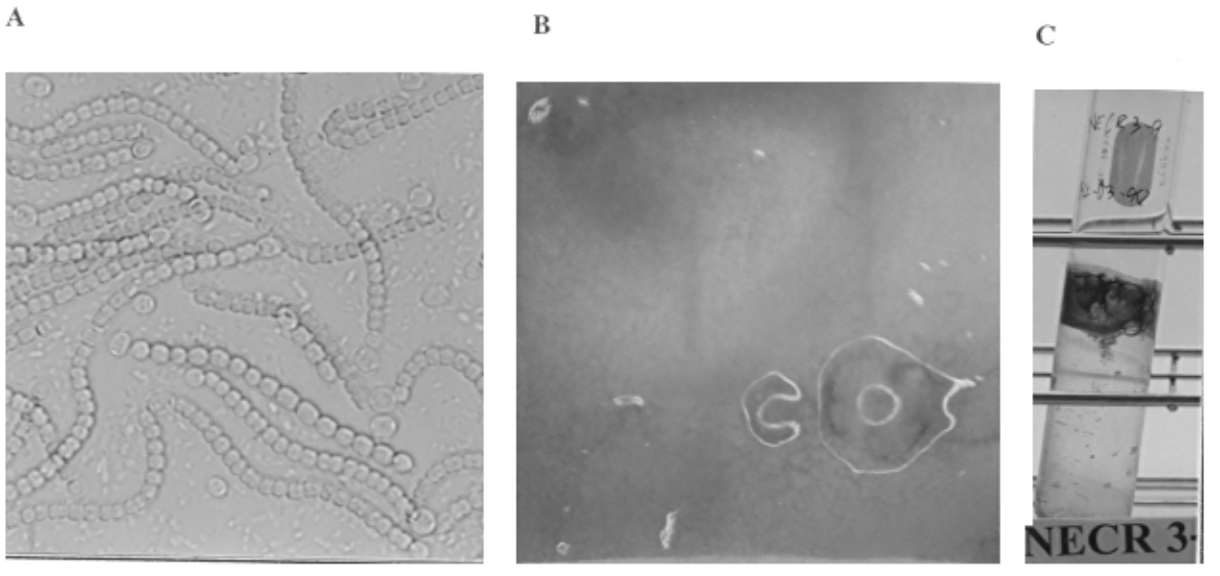

Fig. A55 Cell morphology under microscope with 400X magnification (A), colony forming on BG11 solid (B) and clump forming in BG11 liquid medium (C) of cyanobacterial isolate I NECR3-9

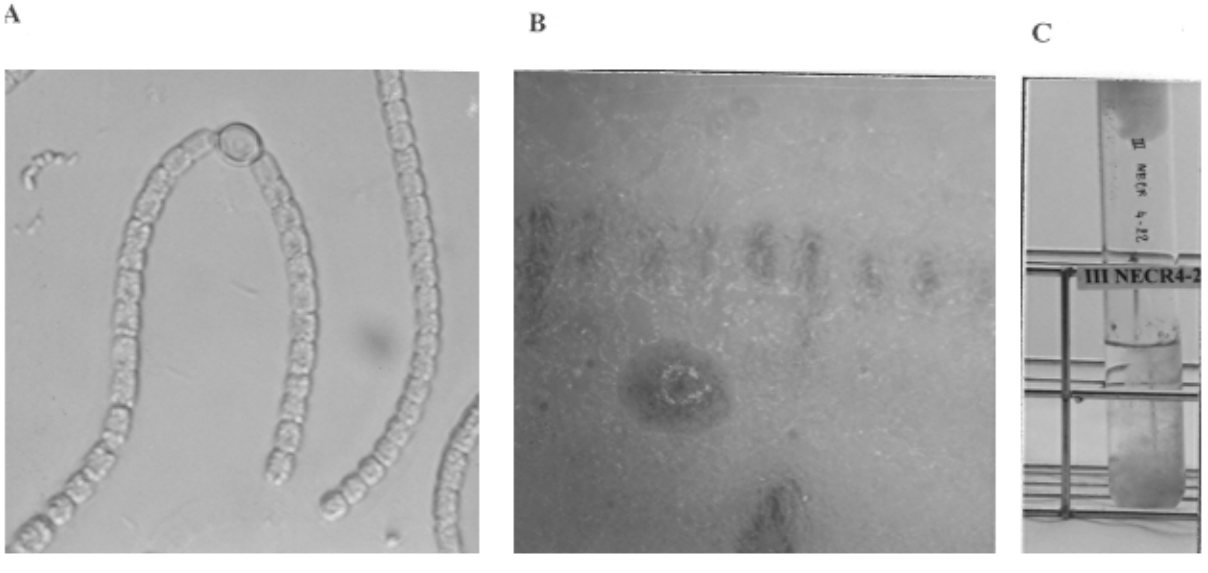

Fig. A56 Cell morphology under microscope with 400X magnification (A), colony forming on BG11 solid (B) and clump forming in BG11 liquid medium (C) of cyanobacterial isolate III NECR4-22

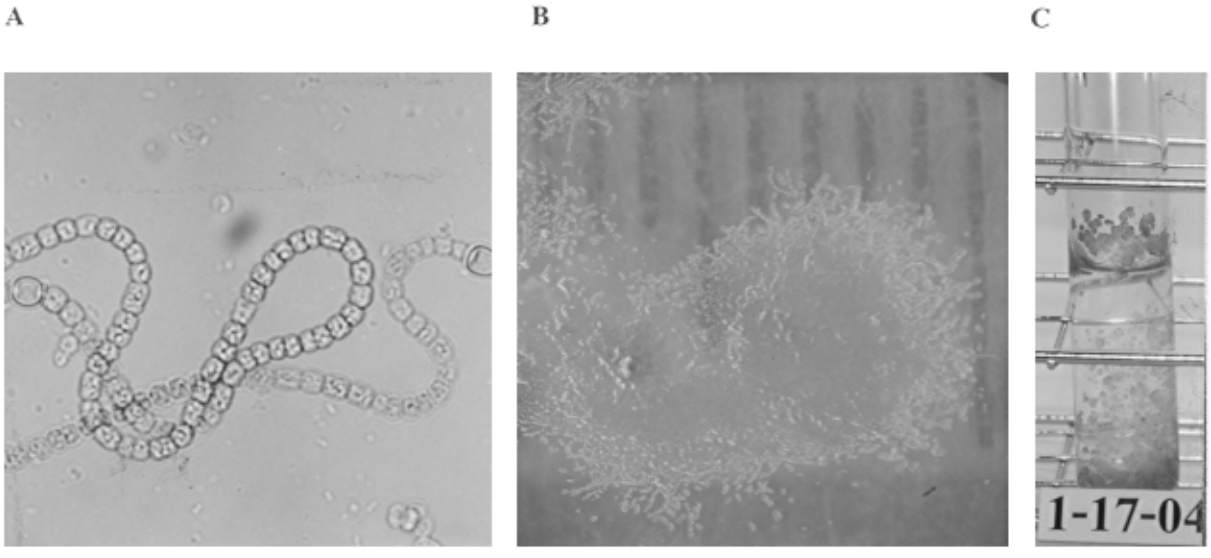

Fig. A57 Cell morphology under microscope with 400X magnification (A), colony forming on BG11 solid (B) and clump forming in BG11 liquid medium (C) of cyanobacterial isolate I NEM3-1-3

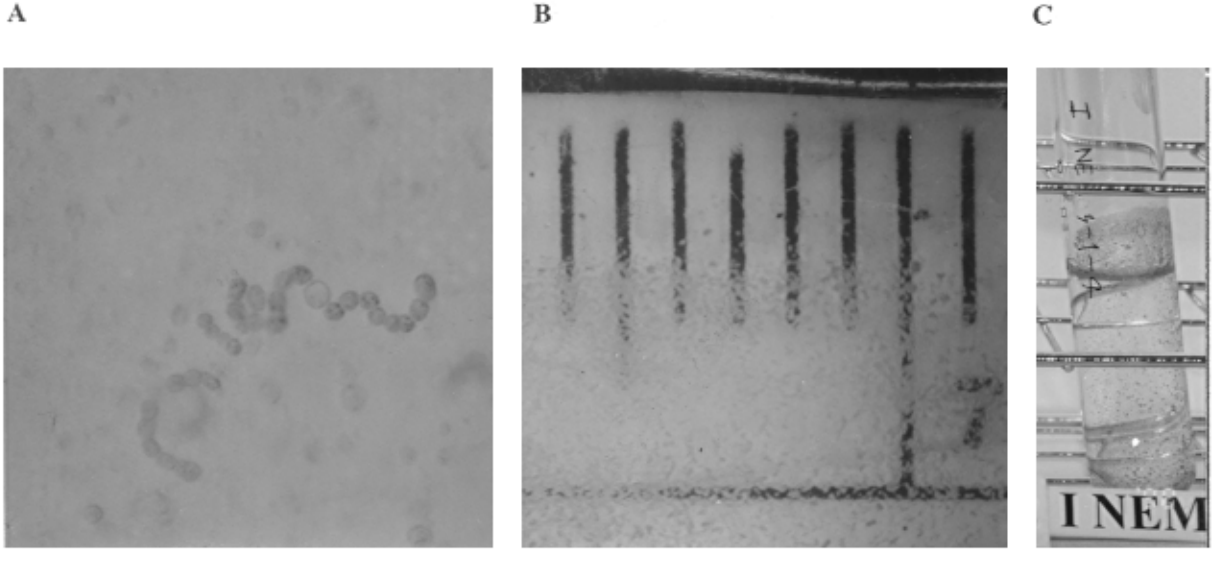

Fig. A58 Cell morphology under microscope with 400X magnification (A), colony forming on BG11 solid (B) and clump forming in BG11 liquid medium (C) of cyanobacterial isolate I NEM3-1-4

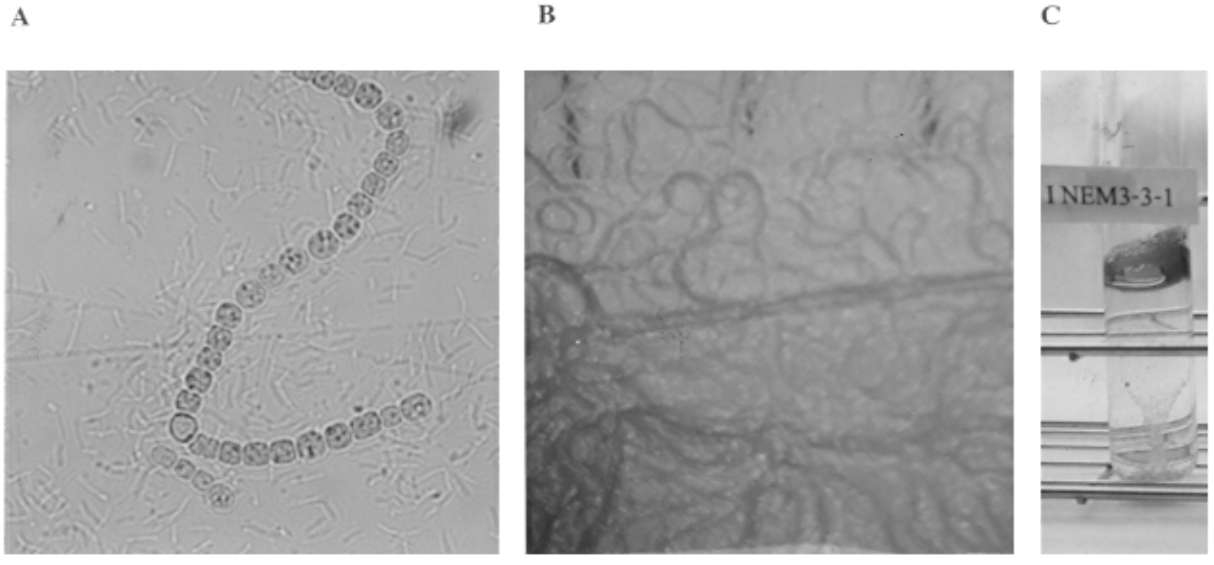

Fig. A59 Cell morphology under microscope with 400X magnification (A), colony forming on BG11 solid (B) and clump forming in BG11 liquid medium (C) of cyanobacterial isolate I NEM3-3-1

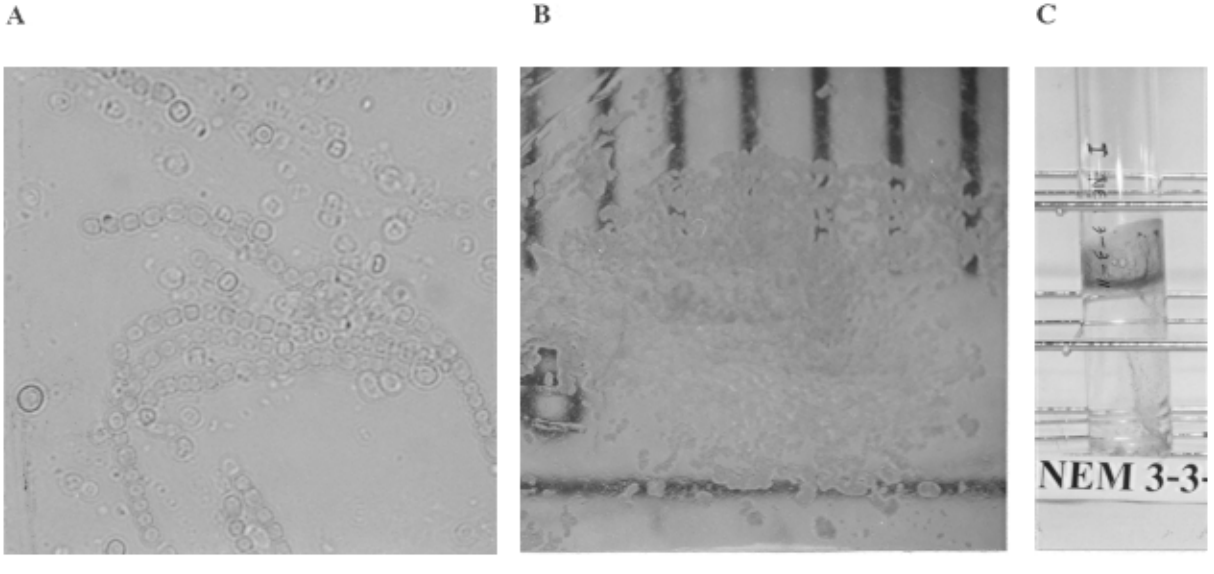

Fig. A60 Cell morphology under microscope with 400X magnification (A), colony forming on BG11 solid (B) and clump forming in BG11 liquid medium (C) of cyanobacterial isolate I NEM3-3-11

A B C

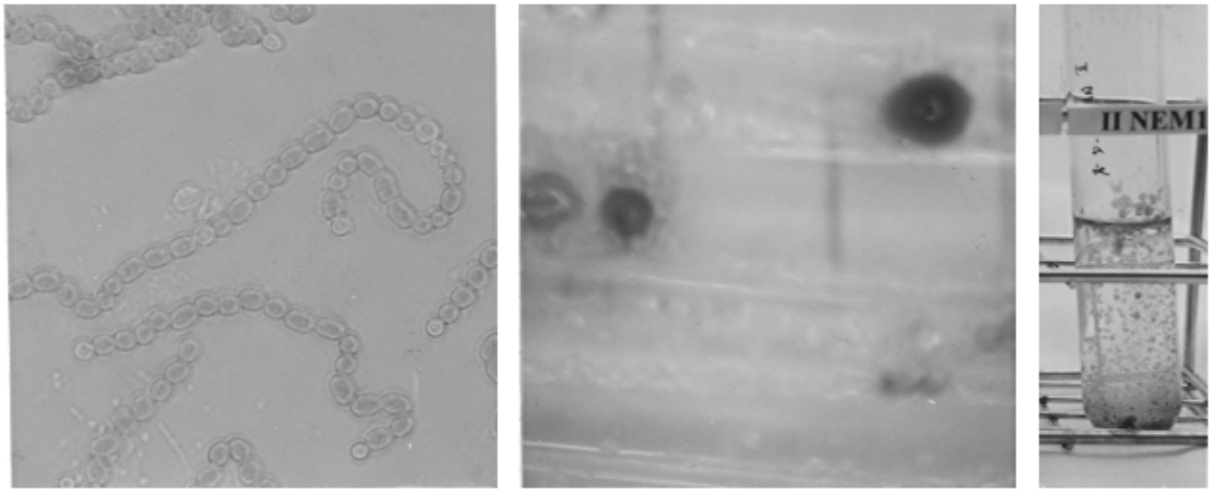

Fig. A61 Cell morphology under microscope with 400X magnification (A), colony forming on BG11 solid (B) and clump forming in BG11 liquid medium (C) of cyanobacterial isolate II NEM1-4-8

A B C

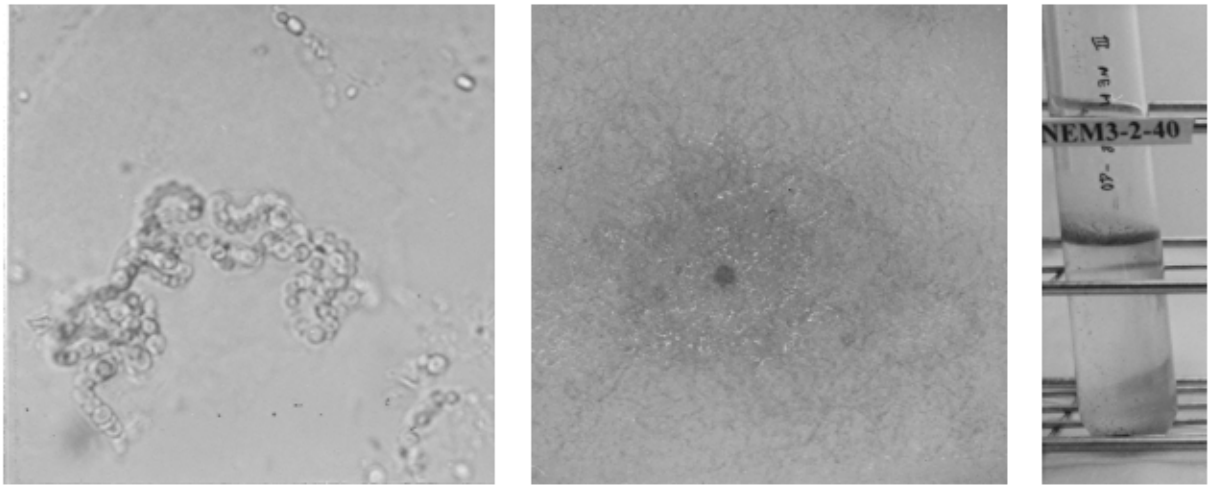

Fig. A62 Cell morphology under microscope with 400X magnification (A), colony forming on BG11 solid (B) and clump forming in BG11 liquid medium (C) of cyanobacterial isolate III NEM3-2-40

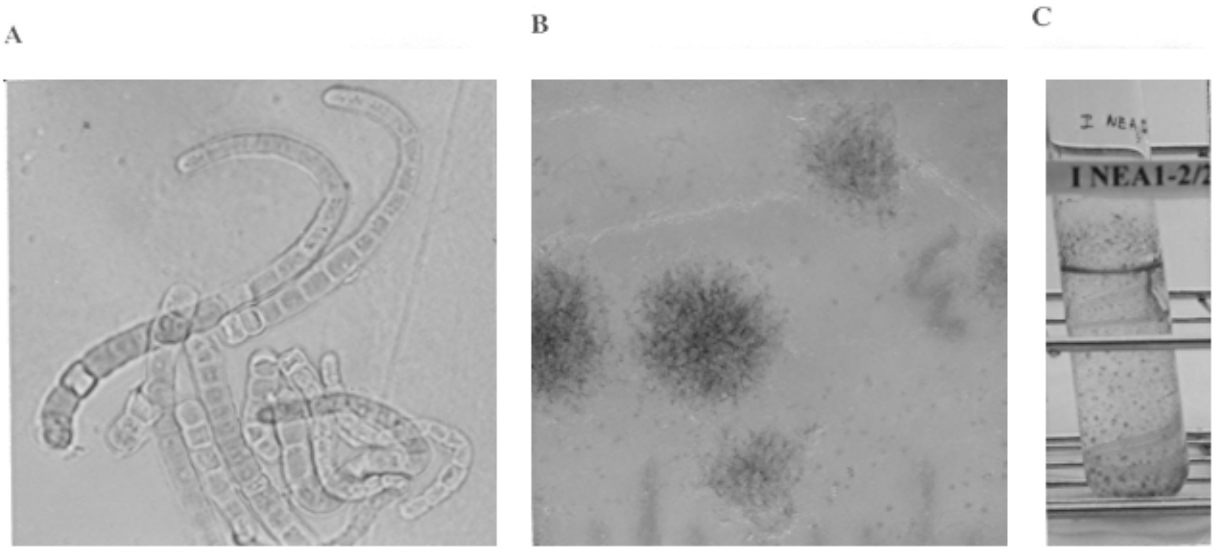

Fig. A63 Cell morphology under microscope with 400X magnification (A), colony forming on BG11 solid (B) and clump forming in BG11 liquid medium (C) of cyanobacterial isolate I NEA1-2/2

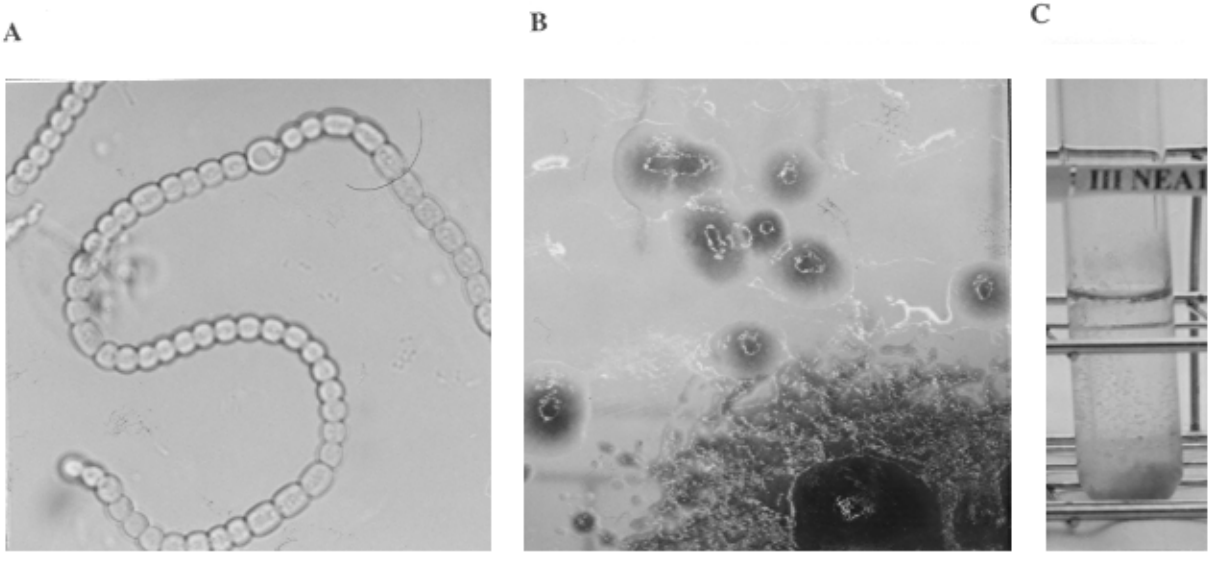

Fig. A64 Cell morphology under microscope with 400X magnification (A), colony forming on BG11 solid (B) and clump forming in BG11 liquid medium (C) of cyanobacterial isolate III NEA1-1

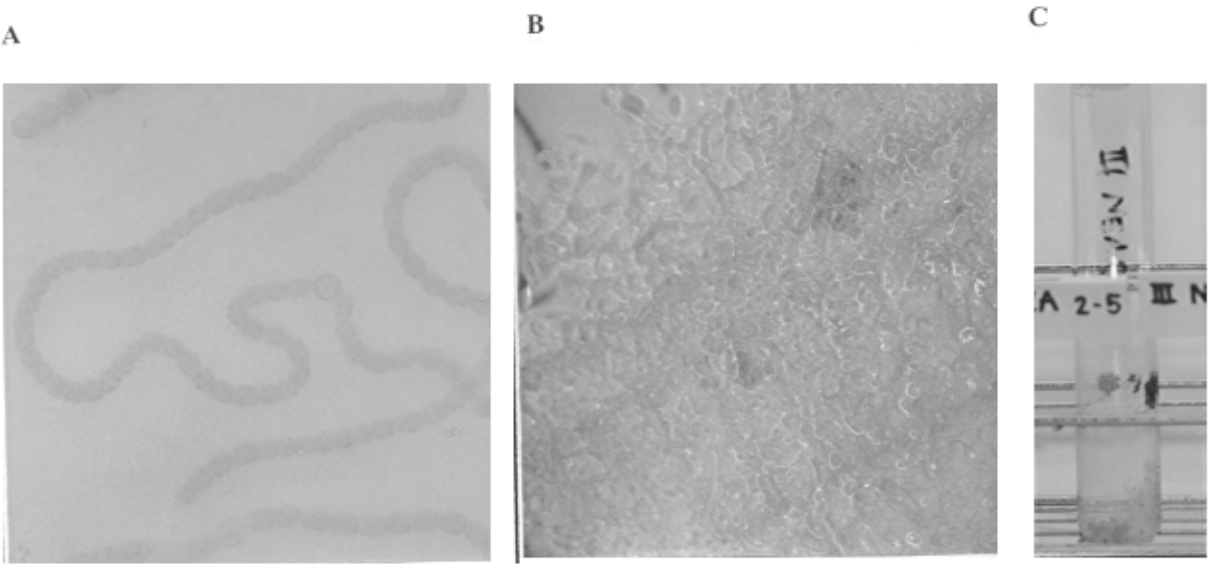

Fig. A65 Cell morphology under microscope with 400X magnification (A), colony forming on BG11 solid (B) and clump forming in BG11 liquid medium (C) of cyanobacterial isolate III NEA2-5

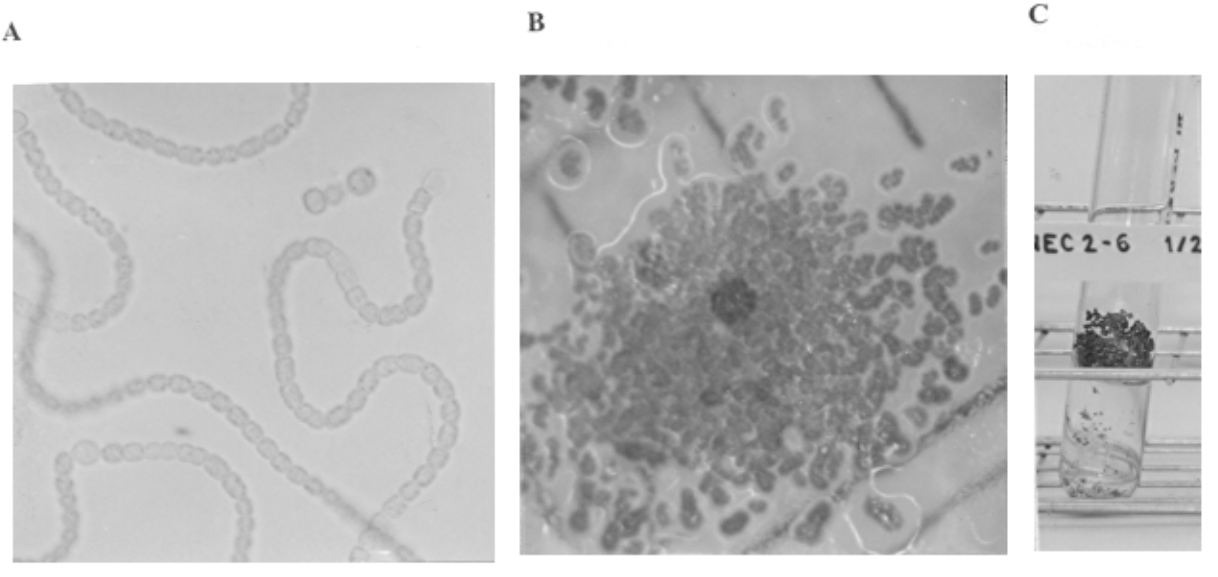

Fig. A66 Cell morphology under microscope with 400X magnification (A), colony forming on BG11 solid (B) and clump forming in BG11 liquid medium (C) of cyanobacterial isolate III NEA2-6 1/2

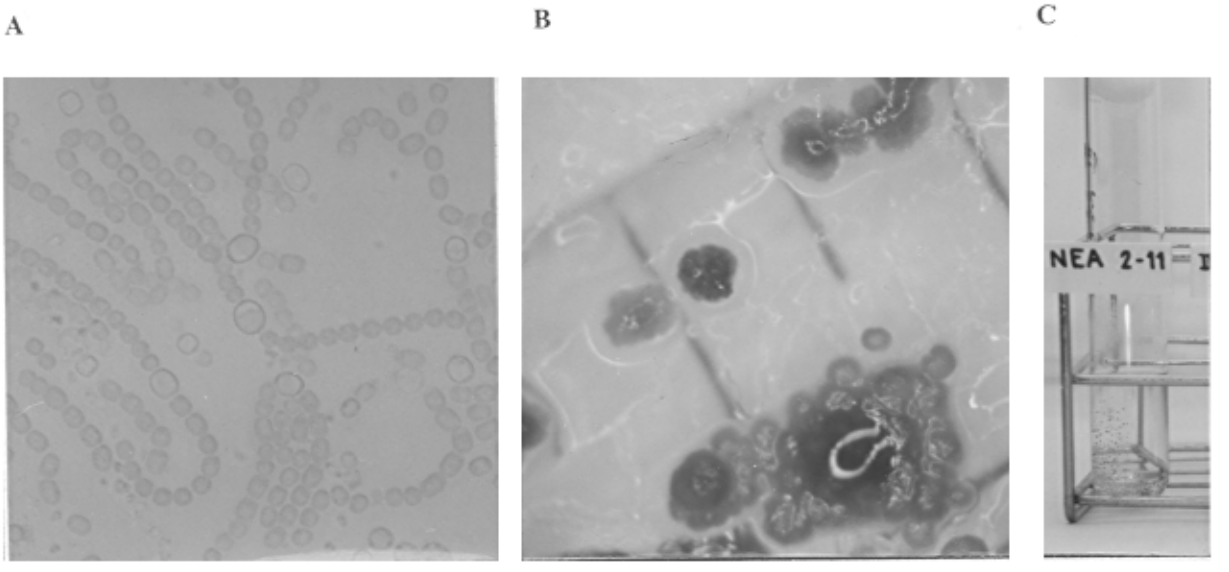

Fig. A67 Cell morphology under microscope with 400X magnification (A), colony forming on BG11 solid (B) and clump forming in BG11 liquid medium (C) of cyanobacterial isolate III NEA2-11

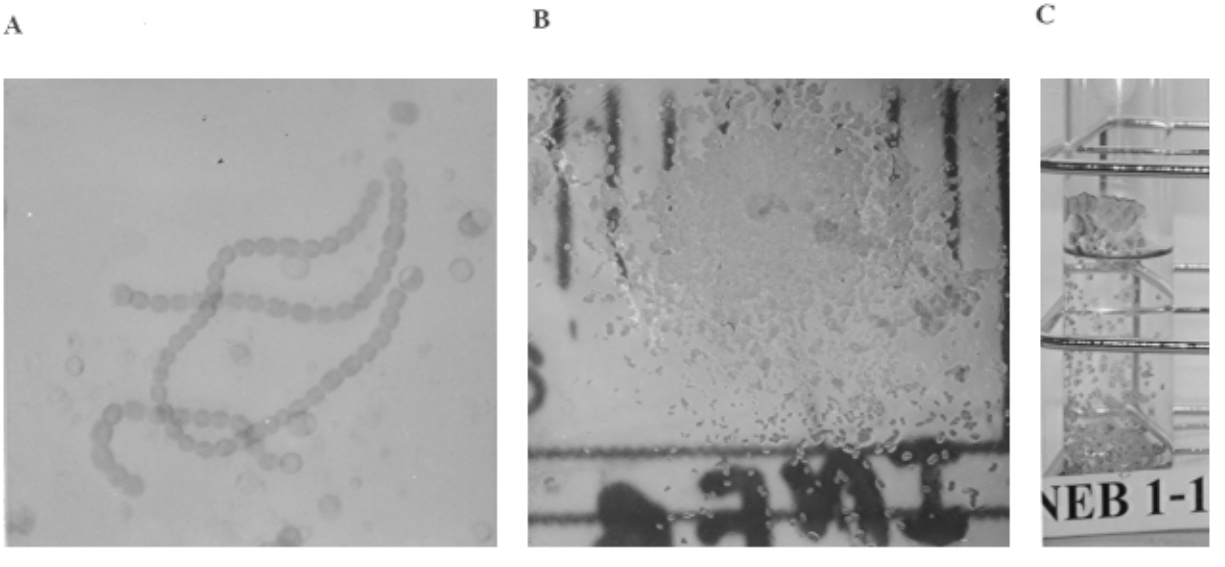

Fig. A68 Cell morphology under microscope with 400X magnification (A), colony forming on BG11 solid (B) and clump forming in BG11 liquid medium (C) of cyanobacterial isolate I NEB1-1

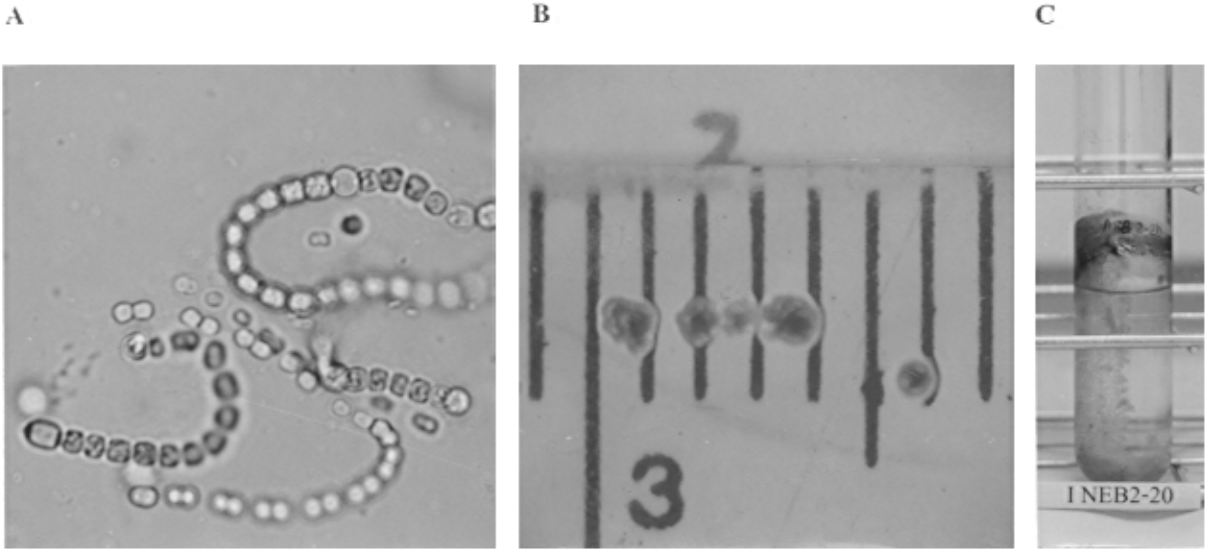

Fig. A69 Cell morphology under microscope with 400X magnification (A), colony forming on BG11 solid (B) and clump forming in BG11 liquid medium (C) of cyanobacterial isolate I NEB2-20

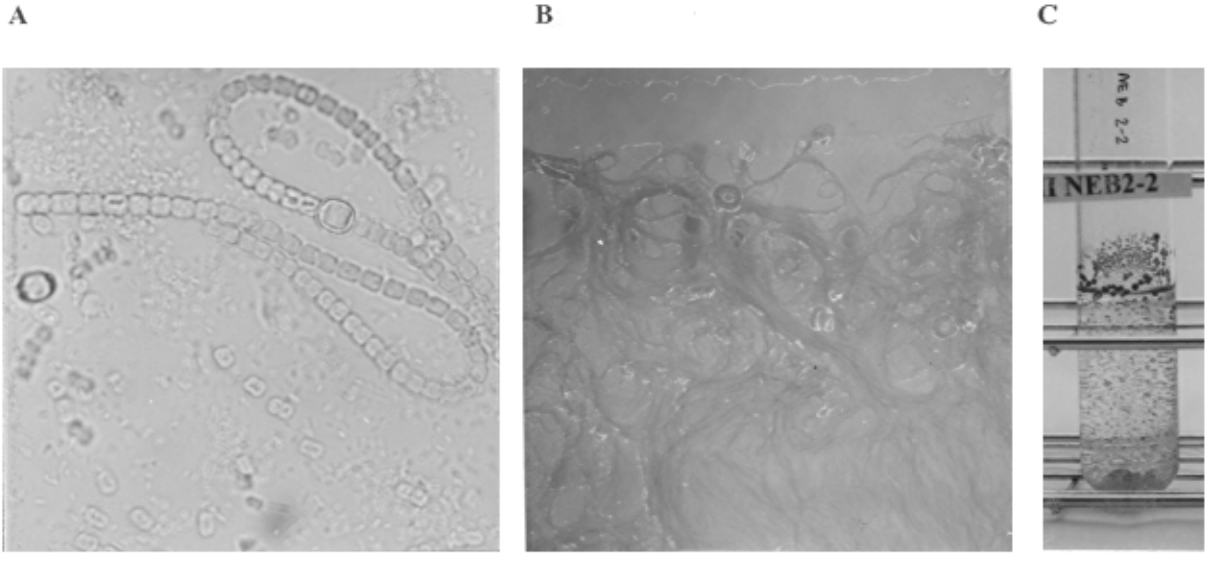

Fig. A70 Cell morphology under microscope with 400X magnification (A), colony forming on BG11 solid (B) and clump forming in BG11 liquid medium (C) of cyanobaceterial isolate III NEB2-2

A B C

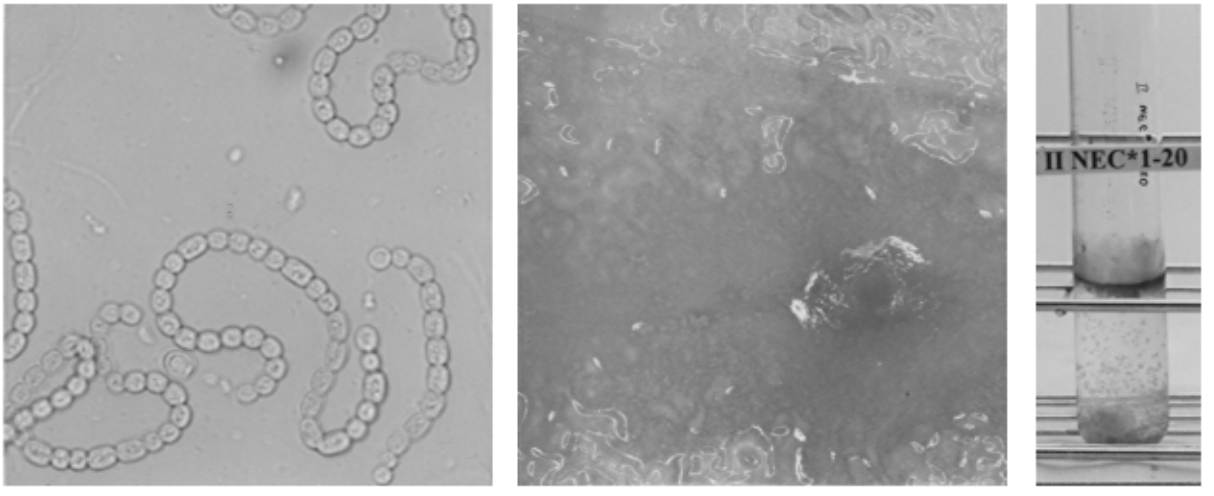

Fig. A71 Cell morphology under microscope with 400X magnification (A), colony forming on BG11 solid (B) and clump forming in BG11 liquid medium (C) of cyanobacterial isolate II NEC*1-20

A B C

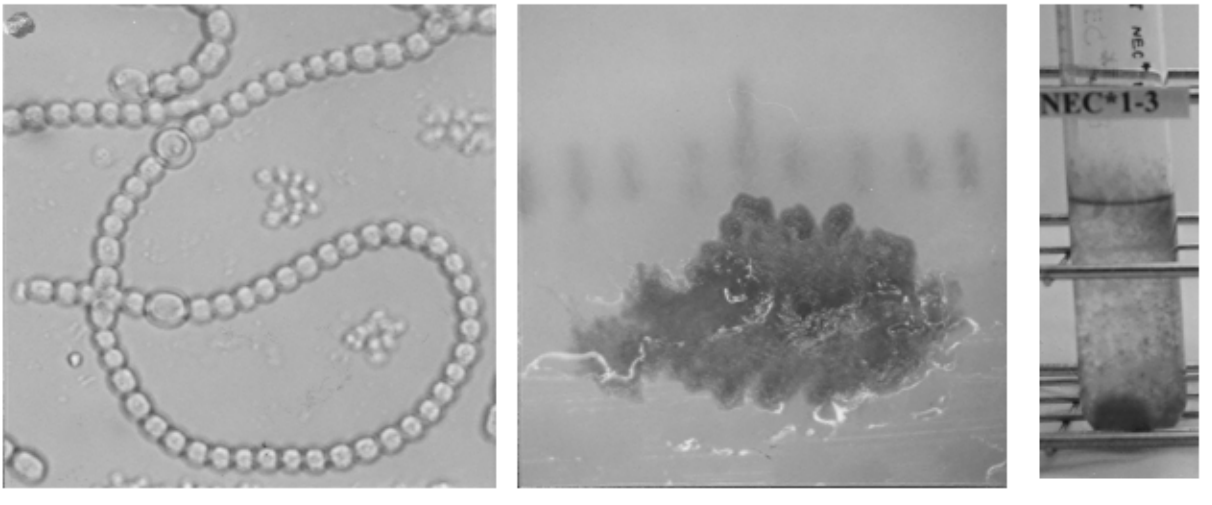

Fig. A72 Cell morphology under microscope with 400X magnification (A), colony forming on BG11 solid (B) and clump forming in BG11 liquid medium (C) of cyanobacterial isolate III NEC*1-3

A B C

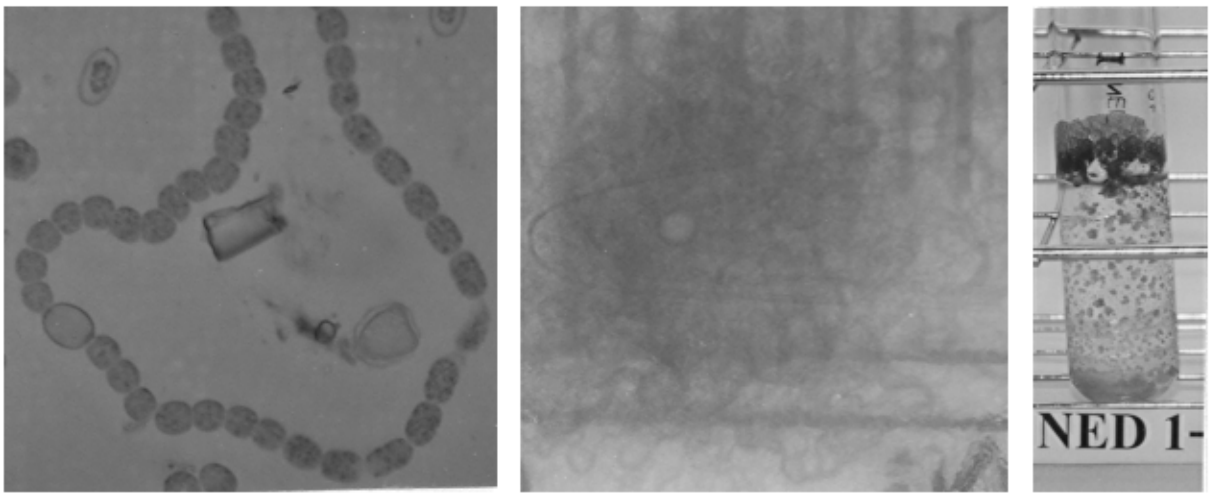

Fig. A73 Cell morphology under microscope with 400X magnification (A), colony forming on BG11 solid (B) and clump forming in BG11 liquid medium (C) of cyanobacterial isolate I NED1-2

A B C

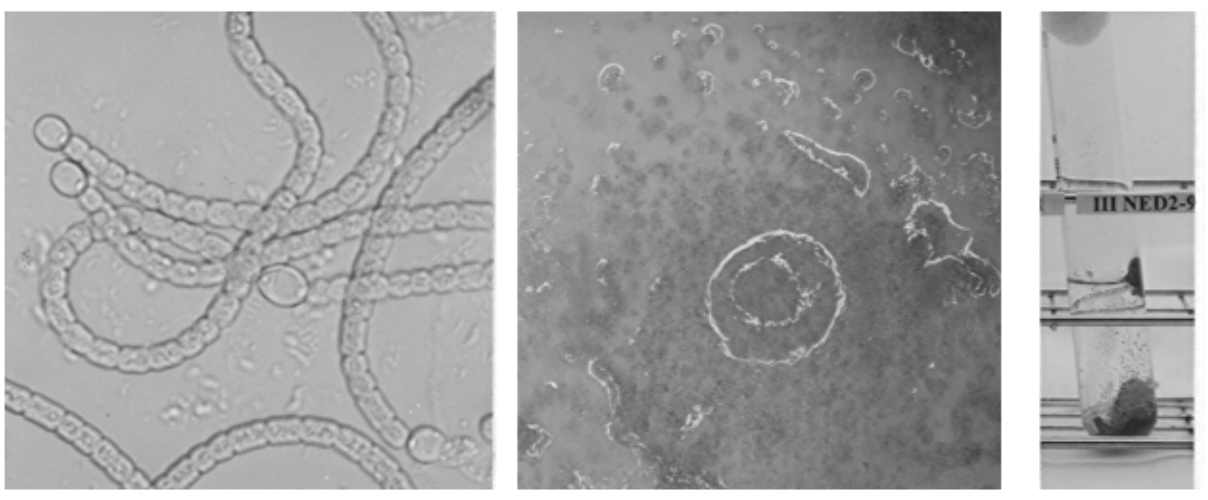

Fig. A74 Cell morphology under microscope with 400X magnification (A), colony forming on BG11 solid (B) and clump forming in BG11 liquid medium (C) of cyanobacterial isolate III NED2-9

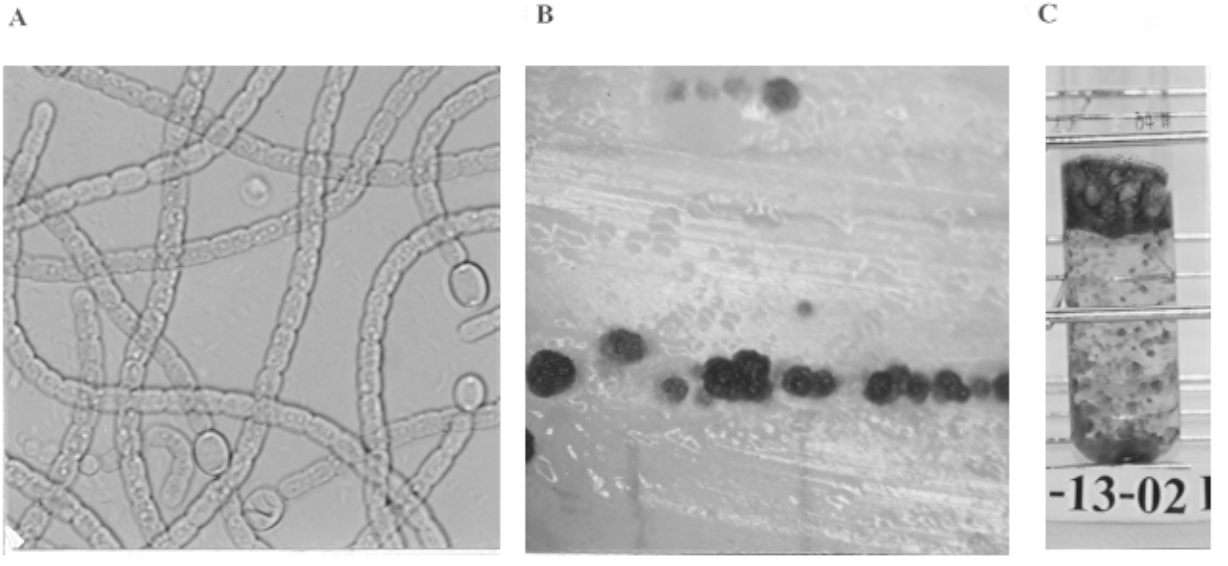

Fig. A75 Cell morphology under microscope with 400X magnification (A), colony forming on BG11 solid (B) and clump forming in BG11 liquid medium (C) of cyanobacterial isolate I CF-2

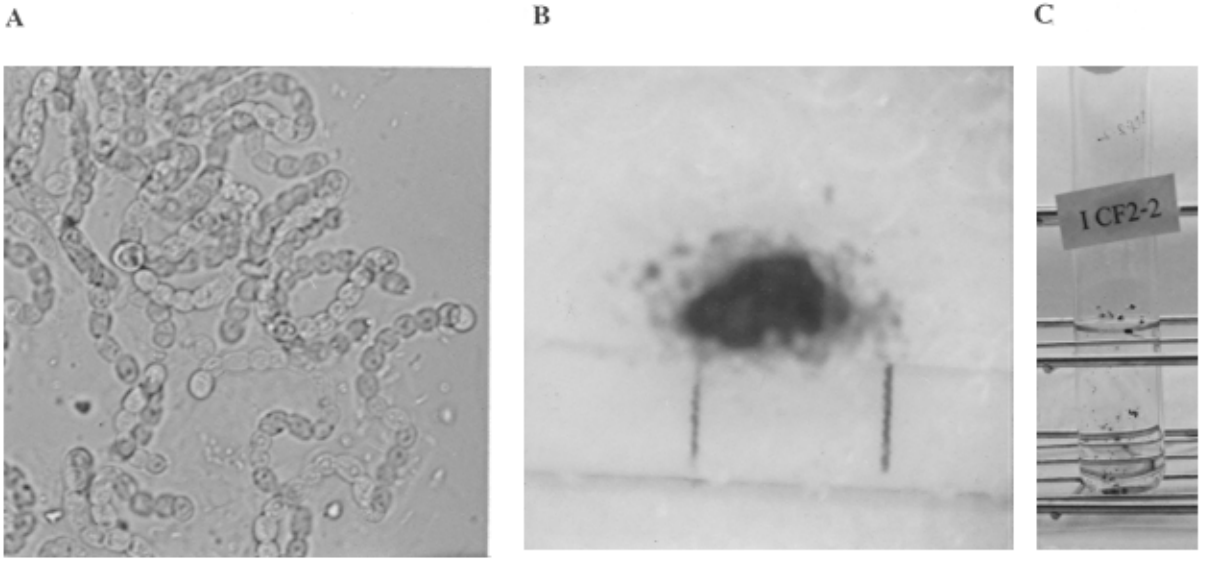

Fig. A76 Cell morphology under microscope with 400X magnification (A), colony forming on BG11 solid (B) and clump forming in BG11 liquid medium (C) of cyanobacterial isolate I CF2-2

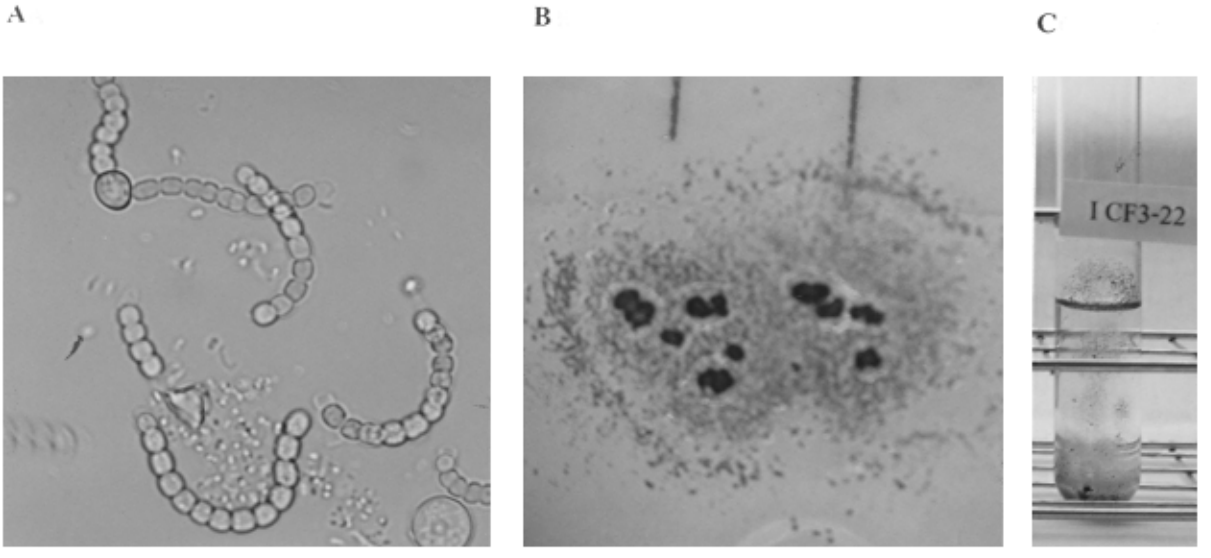

Fig. A77 Cell morphology under microscope with 400X magnification (A), colony forming on BG11 solid (B) and clump forming in BG11 liquid medium (C) of cyanobacterial isolate I CF3-22

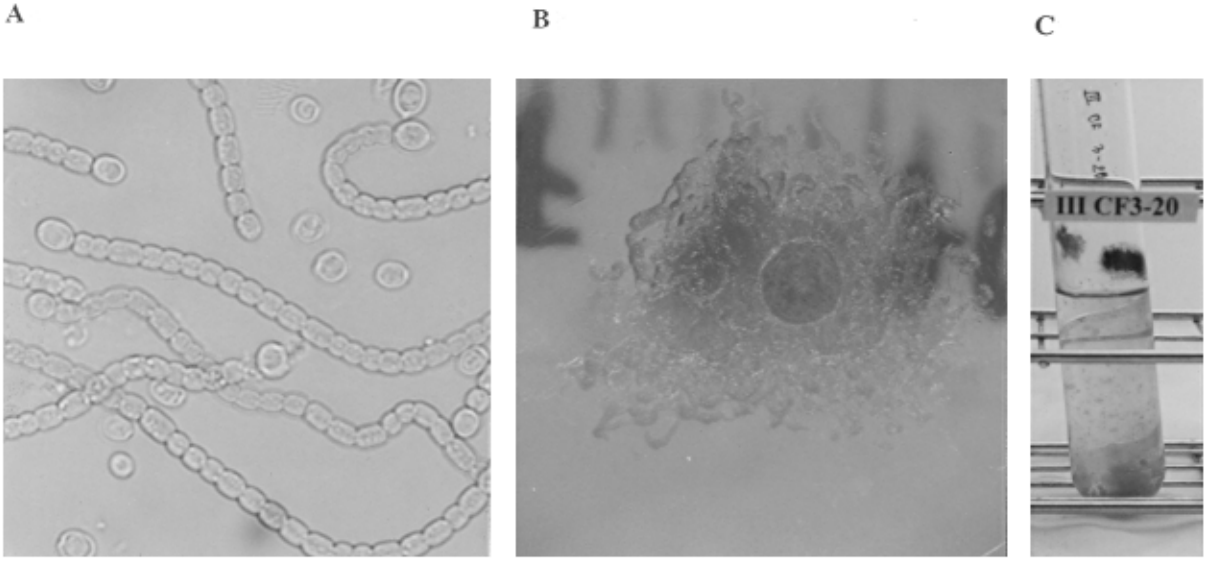

Fig. A78 Cell morphology under microscope with 400X magnification (A), colony forming on BG11 solid (B) and clump forming in BG11 liquid medium (C) of cyanobacterial isolate III CF3-20

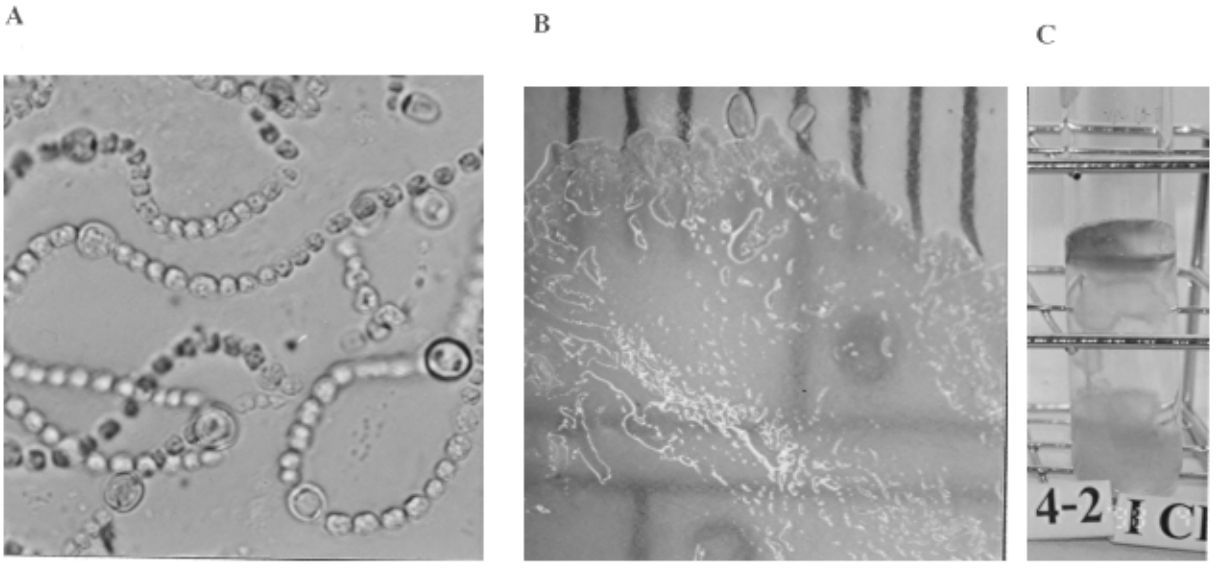

Fig. A79 Cell morphology under microscope with 400X magnification (A), colony forming on BG11 solid (B) and clump forming in BG11 liquid medium (C) of cyanobacterial isolate I CR1-1

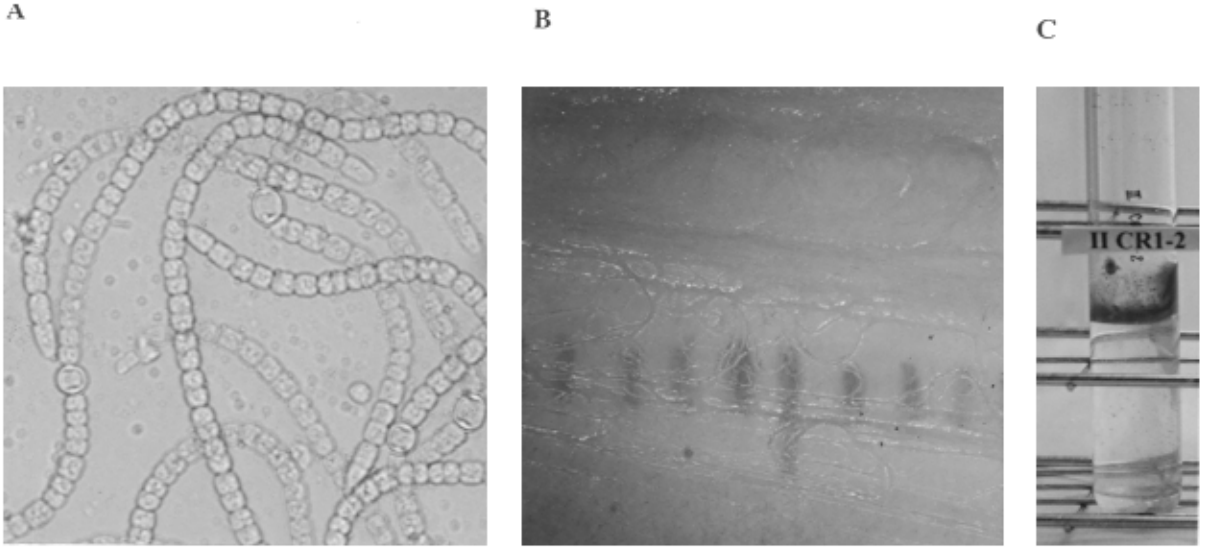

Fig. A80 Cell morphology under microscope with 400X magnification (A), colony forming on BG11 solid (B) and clump forming in BG11 liquid medium (C) of cyanobacterial isolate II CR1-2

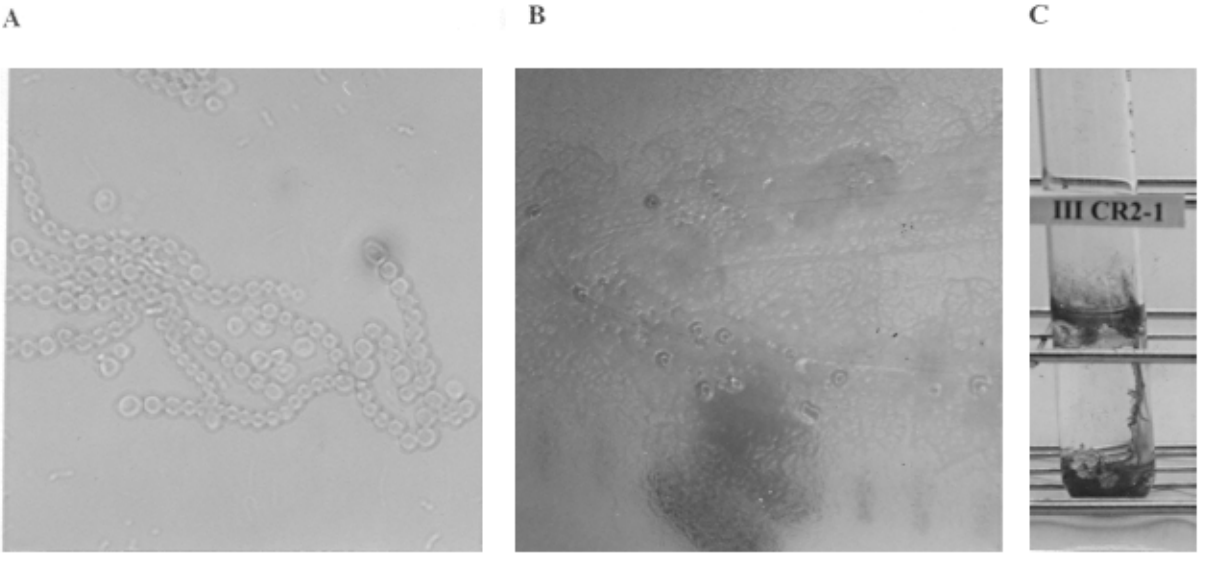

Fig. A81 Cell morphology under microscope with 400X magnification (A), colony forming on BG11 solid (B) and clump forming in BG11 liquid medium (C) of cyanobacterial isolate III CR2-1

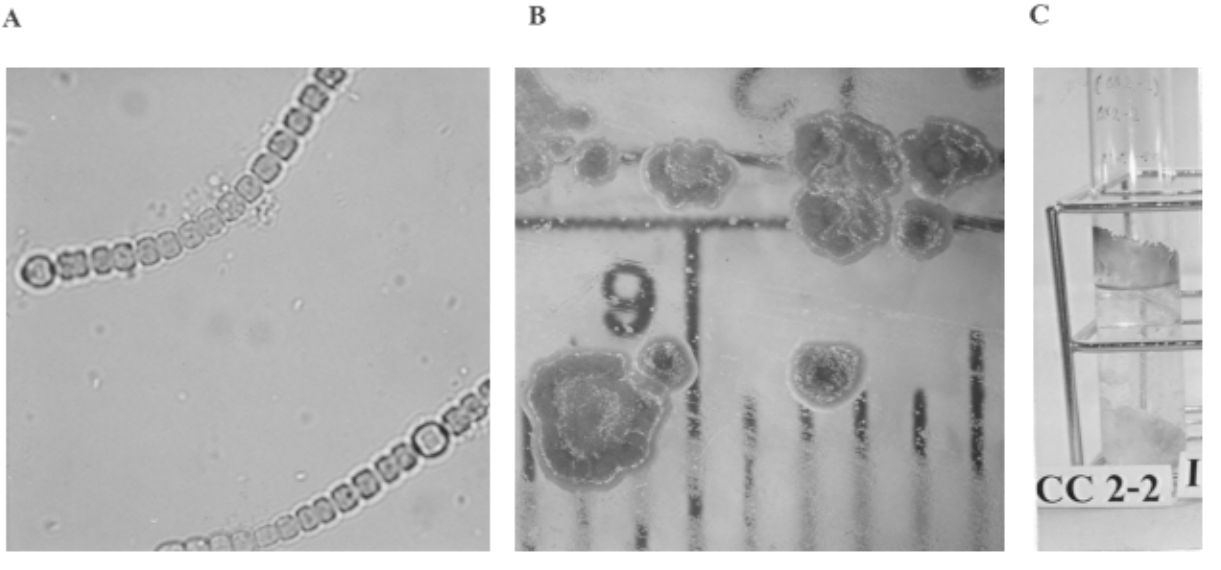

Fig. A82 Cell morphology under microscope with 400X magnification (A), colony forming on BG11 solid (B) and clump forming in BG11 liquid medium (C) of cyanobacterial isolate I CC2-2

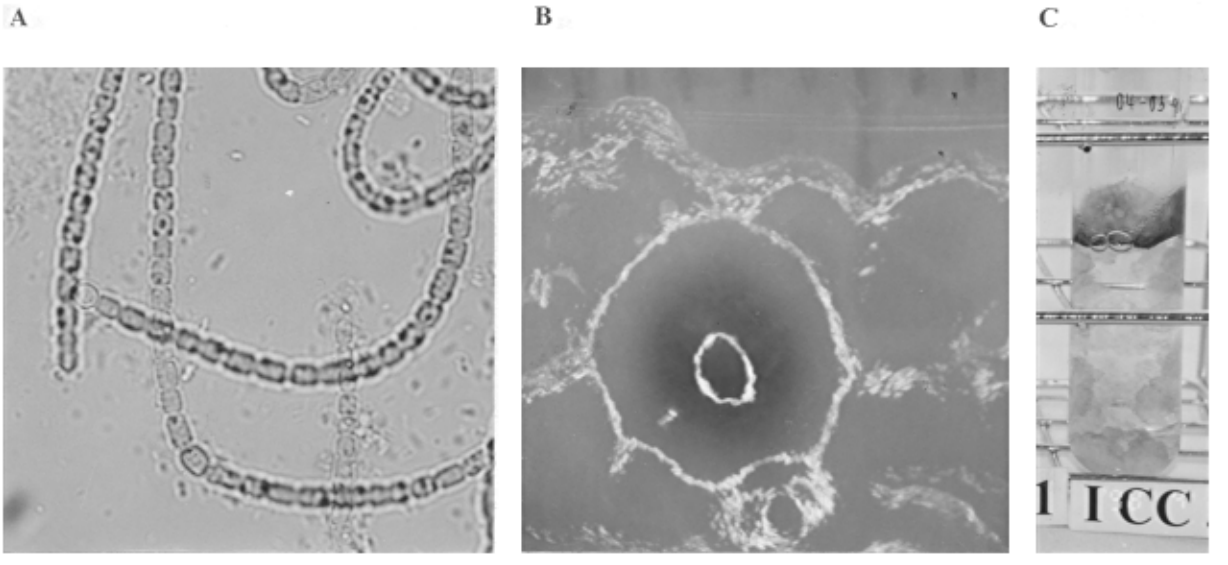

Fig. A83 Cell morphology under microscope with 400X magnification (A), colony forming on BG11 solid (B) and clump forming in BG11 liquid medium (C) of cyanobacterial isolate I CC3-1

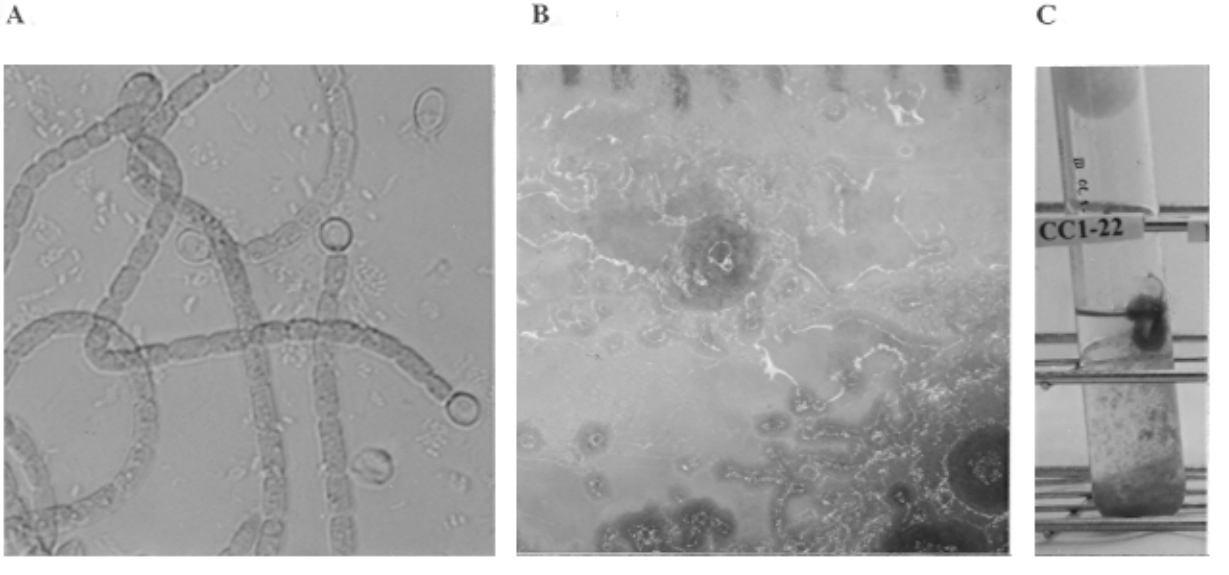

Fig. A84 Cell morphology under microscope with 400X magnification (A), colony forming on BG11 solid (B) and clump forming in BG11 liquid medium (C) of cyanobacterial isolate III CC1-22

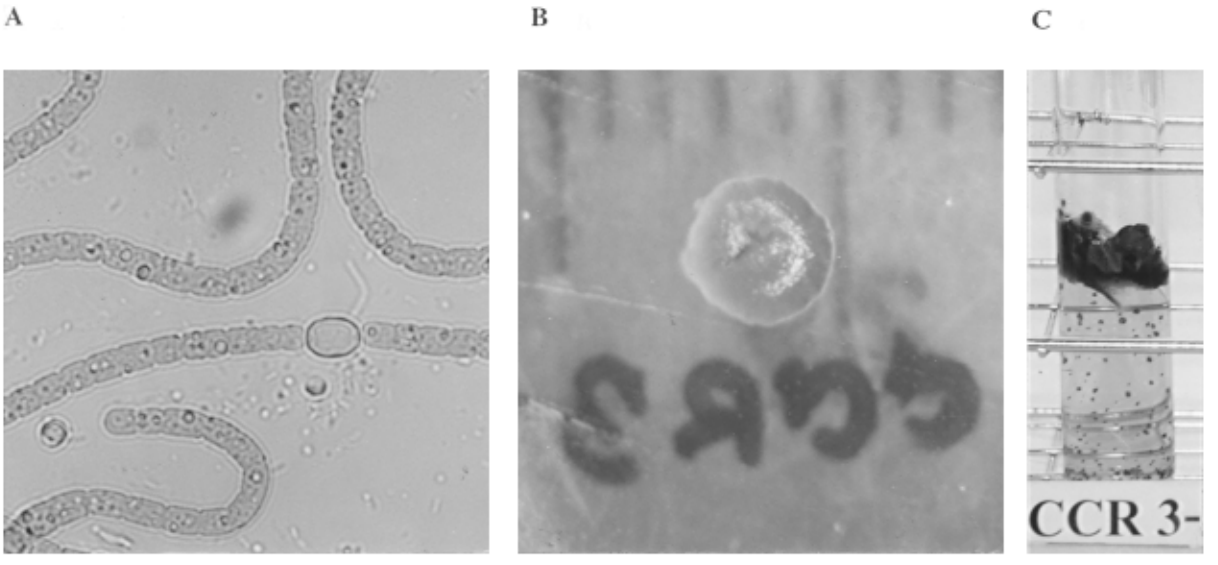

Fig. A85 Cell morphology under microscope with 400X magnification (A), colony forming on BG11 solid (B) and clump forming in BG11 liquid medium (C) of cyanobacterial isolate I CCR3-2

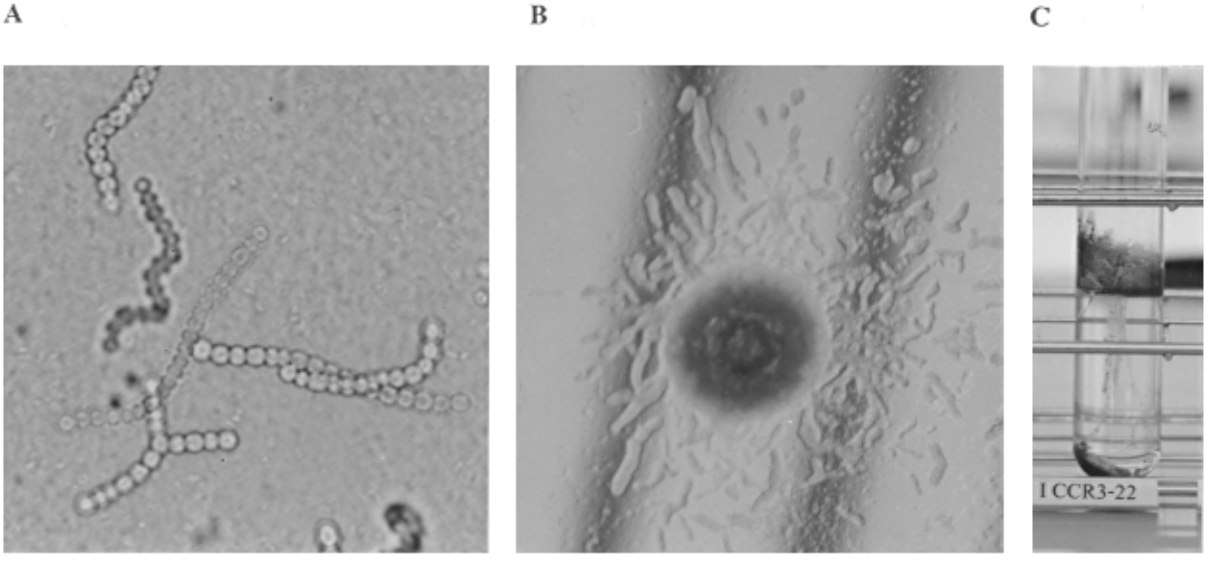

Fig. A86 Cell morphology under microscope with 400X magnification (A), colony forming on BG11 solid (B) and clump forming in BG11 liquid medium (C) of cyanobacterial isolate I CCR3-22

A B C

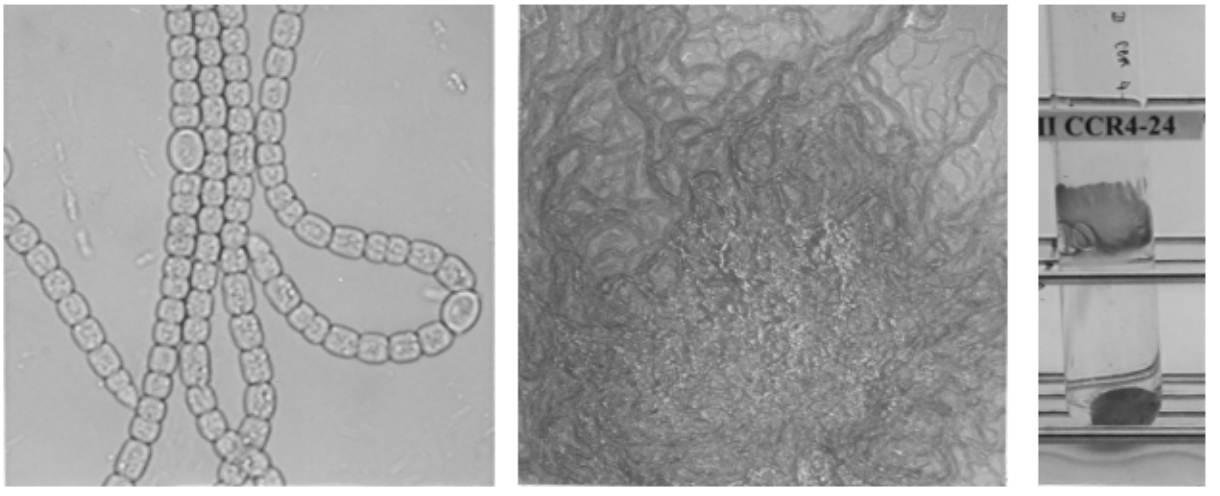

Fig. A87 Cell morphology under microscope with 400X magnification (A), colony forming on BG11 solid (B) and clump forming in BG11 liquid medium (C) of cyanobacterial isolate III CCR4-24

A B C

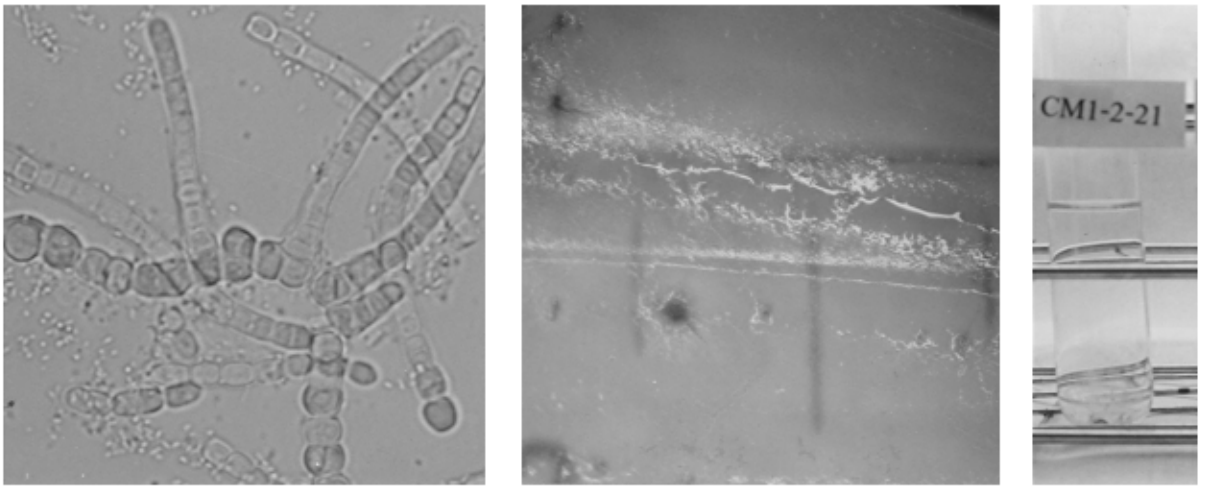

Fig. A88 Cell morphology under microscope with 400X magnification (A), colony forming on BG11 solid (B) and clump forming in BG11 liquid medium (C) of cyanobacterial isolate I CM1-2-21

A B C

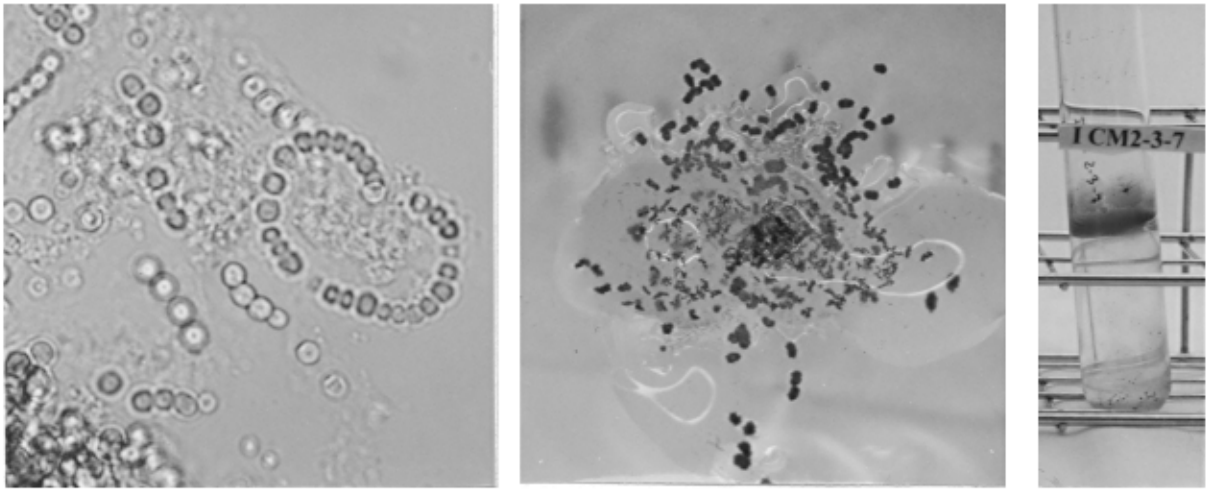

Fig. A89 Cell morphology under microscope with 400X magnification (A), colony forming on BG11 solid (B) and clump forming in BG11 liquid medium (C) of cyanobacterial isolate I CM2-3-7

A B C

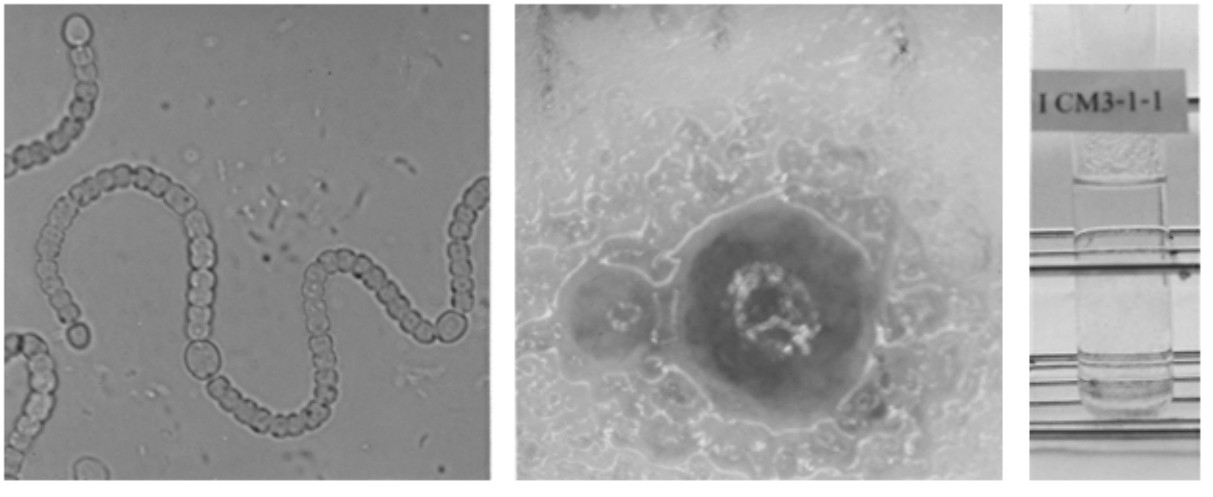

Fig. A90 Cell morphology under microscope with 400X magnification (A), colony forming on BG11 solid (B) and clump forming in BG11 liquid medium (C) of cyanobacterial isolate I CM3-1-1

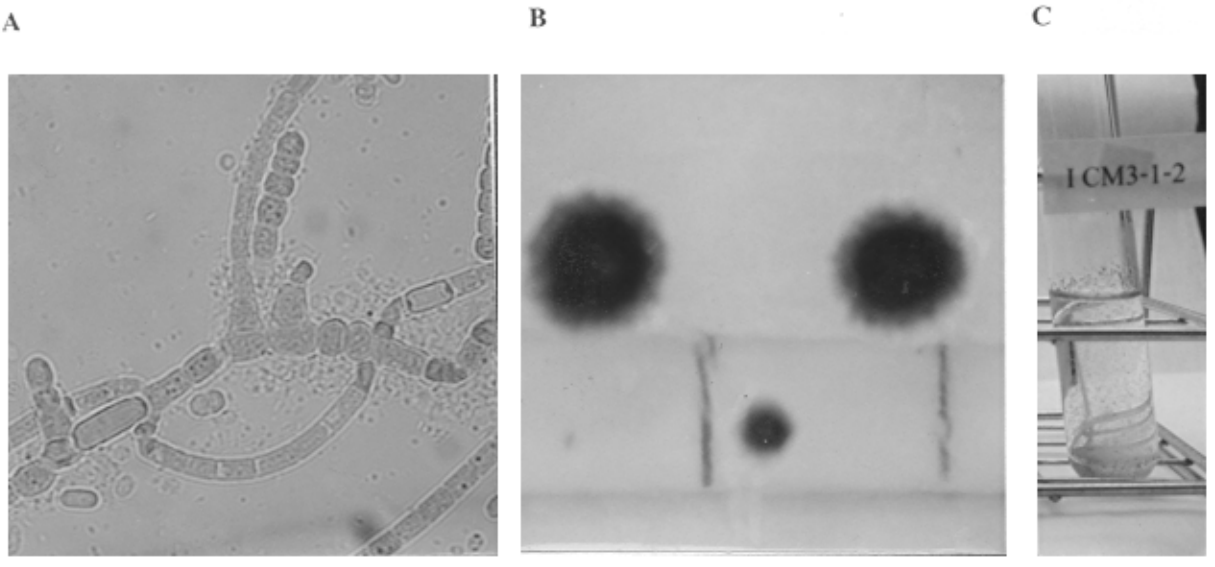

Fig. A91 Cell morphology under microscope with 400X magnification (A), colony forming on BG11 solid (B) and clump forming in BG11 liquid medium (C) of cyanobacterial isolate I CM3-1-2

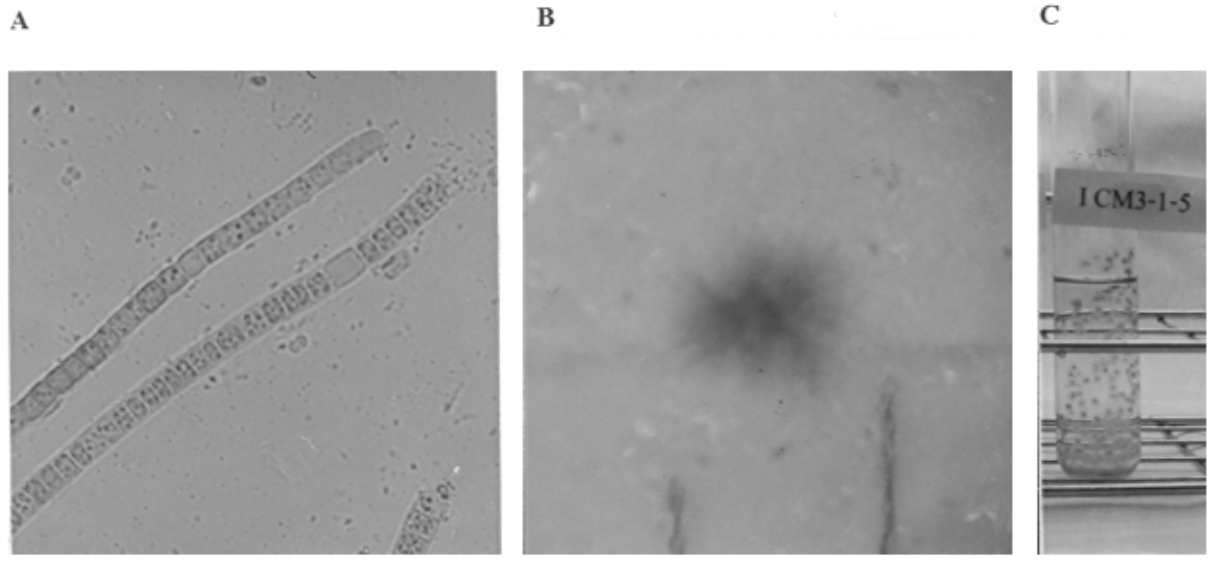

Fig. A92 Cell morphology under microscope with 400X magnification (A), colony forming on BG11 solid (B) and clump forming in BG11 liquid medium (C) of cyanobacterial isolate I CM3-1-5

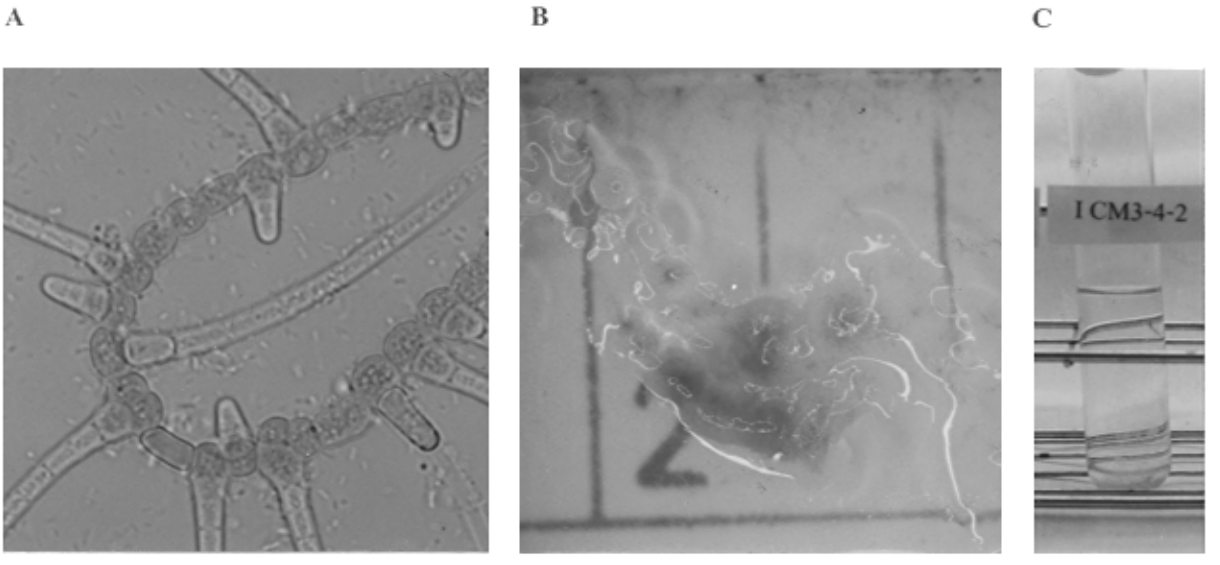

Fig. A93 Cell morphology under microscope with 400X magnification (A), colony forming on BG11 solid (B) and clump forming in BG11 liquid medium (C) of cyanobacterial isolate I CM3-4-2

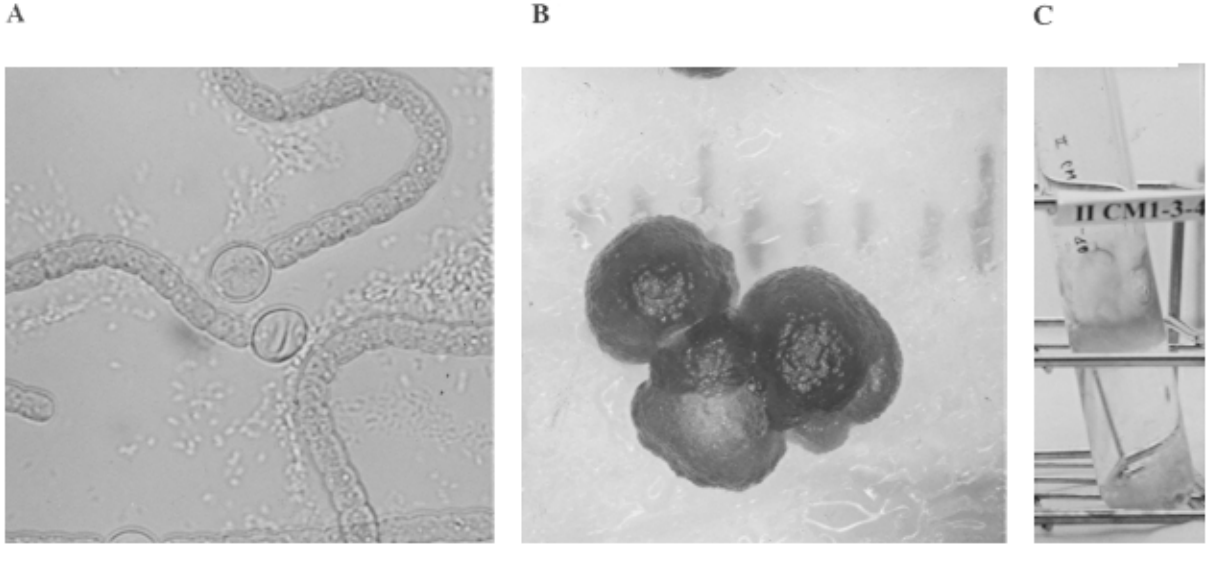

Fig. A94 Cell morphology under microscope with 400X magnification (A), colony forming on BG11 solid (B) and clump forming in BG11 liquid medium (C) of cyanobacterial isolate II CM1-3-40

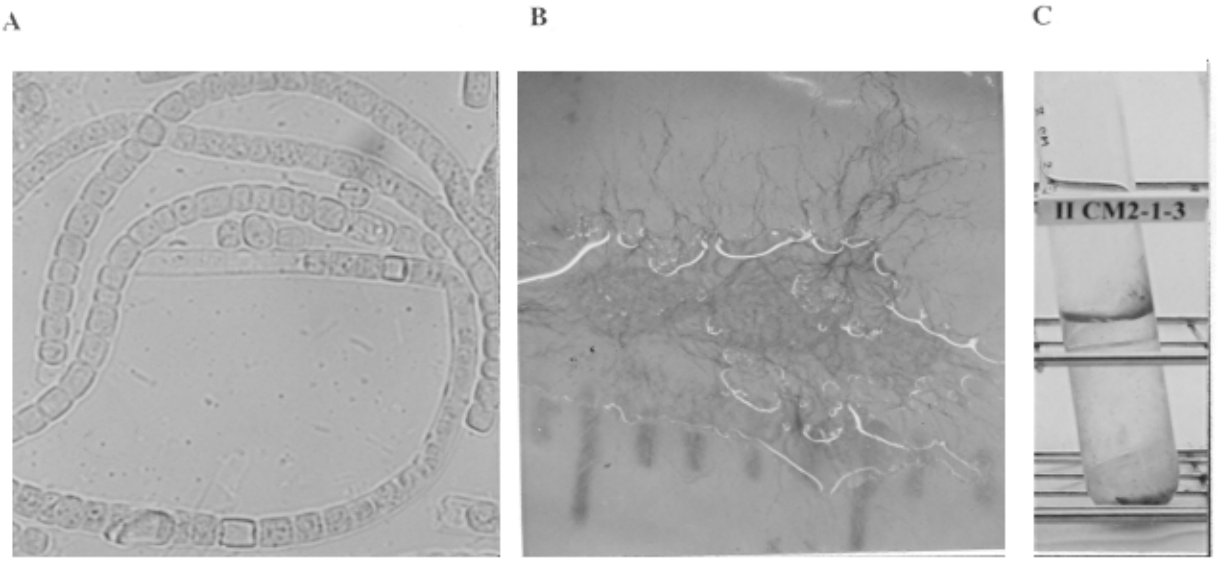

Fig. A95 Cell morphology under microscope with 400X magnification (A), colony forming on BG11 solid (B) and clump forming in BG11 liquid medium (C) of cyanobacterial isolate II CM2-1-3

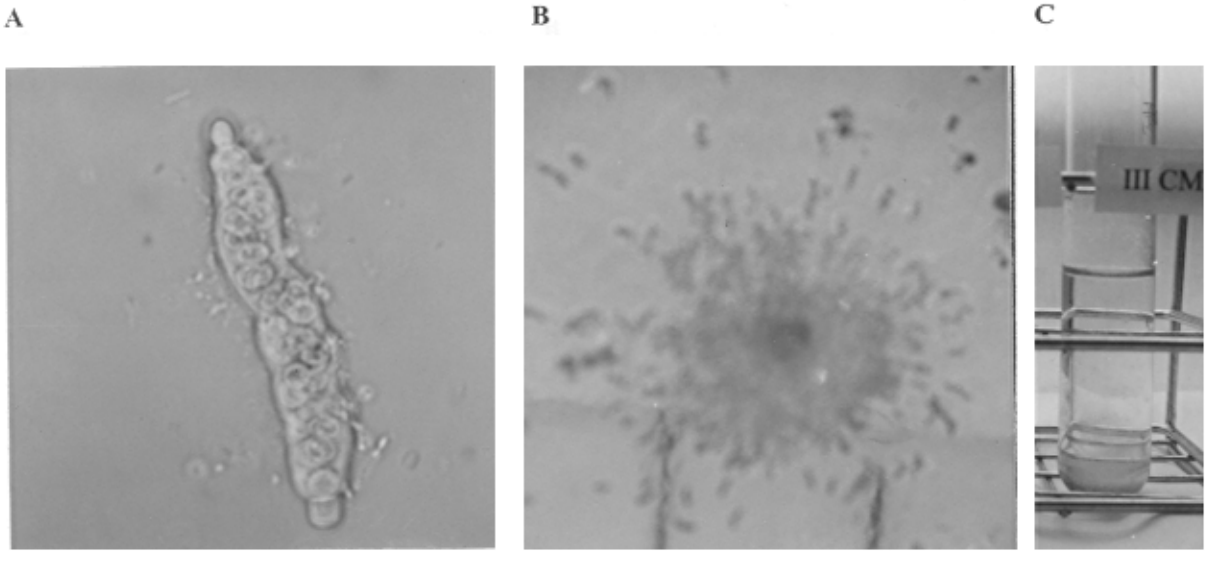

Fig. A96 Cell morphology under microscope with 400X magnification (A), colony forming on BG11 solid (B) and clump forming in BG11 liquid medium (C) of cyanobacterial isolate III CM3-1

A B C

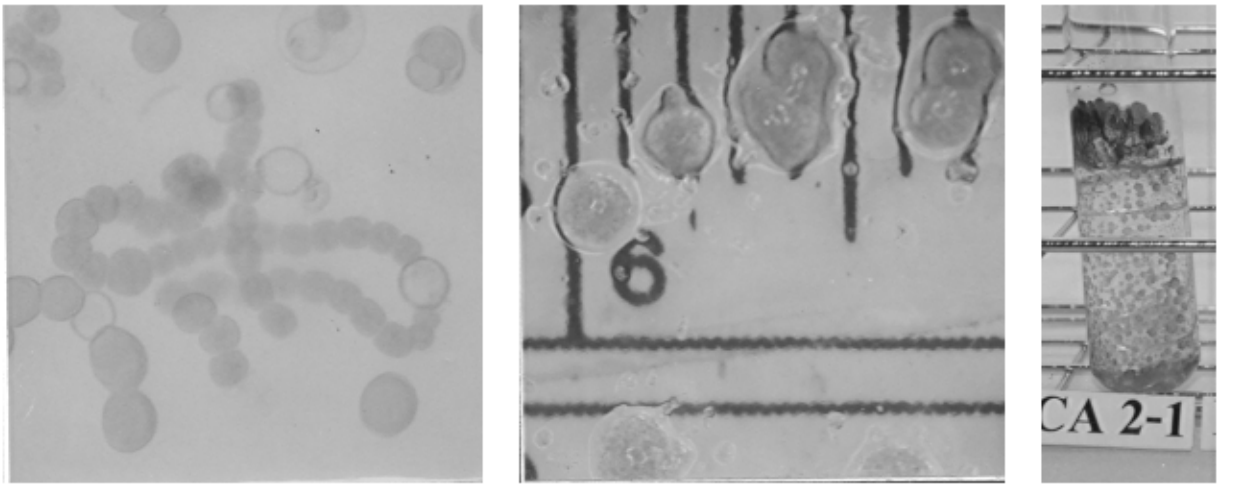

Fig. A97 Cell morphology under microscope with 400X magnification (A), colony forming on BG11 solid (B) and clump forming in BG11 liquid medium (C) of cyanobacterial isolate I CA2-1

A B C

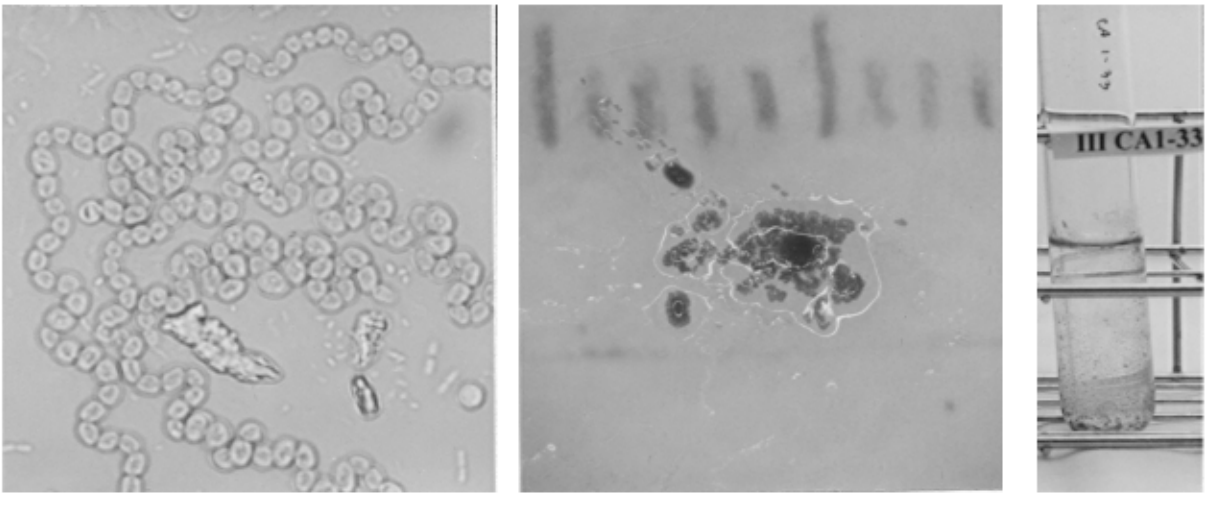

Fig. A98 Cell morphology under microscope with 400X magnification (A), colony forming on BG11 solid (B) and clump forming in BG11 liquid medium (C) of cyanobacterial isolate III CA1-33

A B C

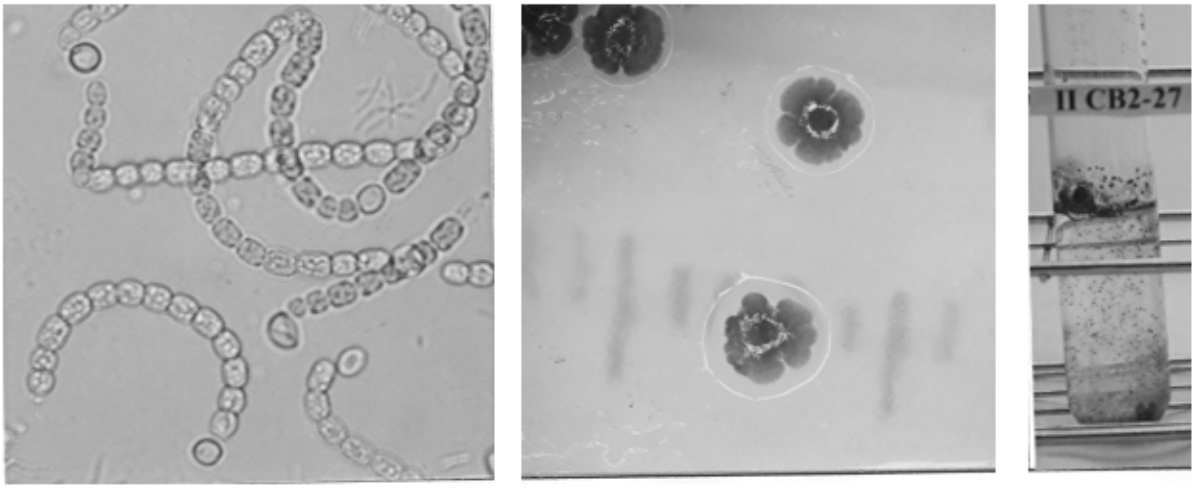

Fig. A99 Cell morphology under microscope with 400X magnification (A), colony forming on BG11 solid (B) and clump forming in BG11 liquid medium (C) of cyanobacterial isolate II CB2-27

A B C

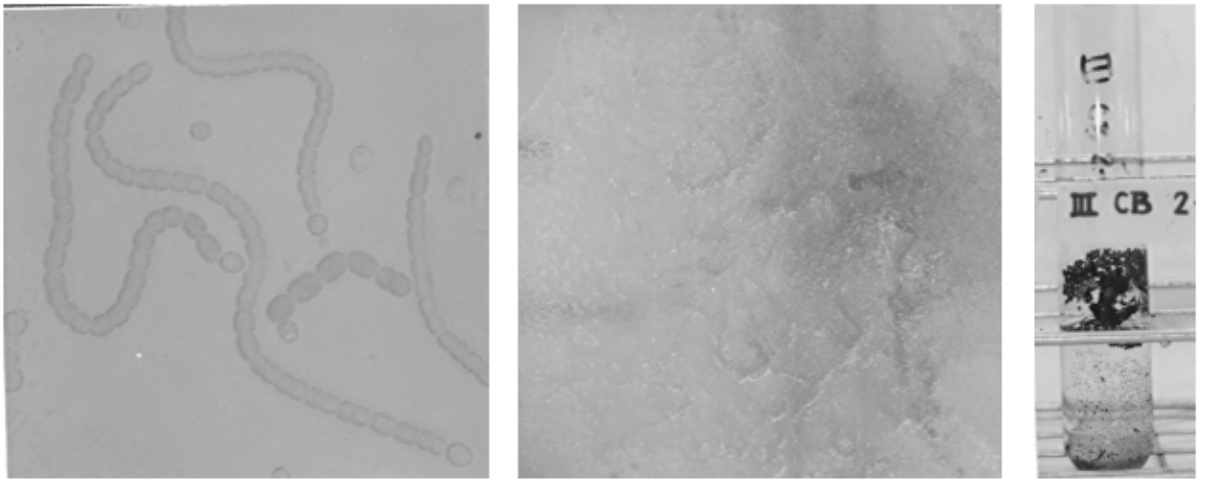

Fig. A100 Cell morphology under microscope with 400X magnification (A), colony forming on BG11 solid (B) and clump forming in BG11 liquid medium (C) of cyanobacterial isolate III CB2-2

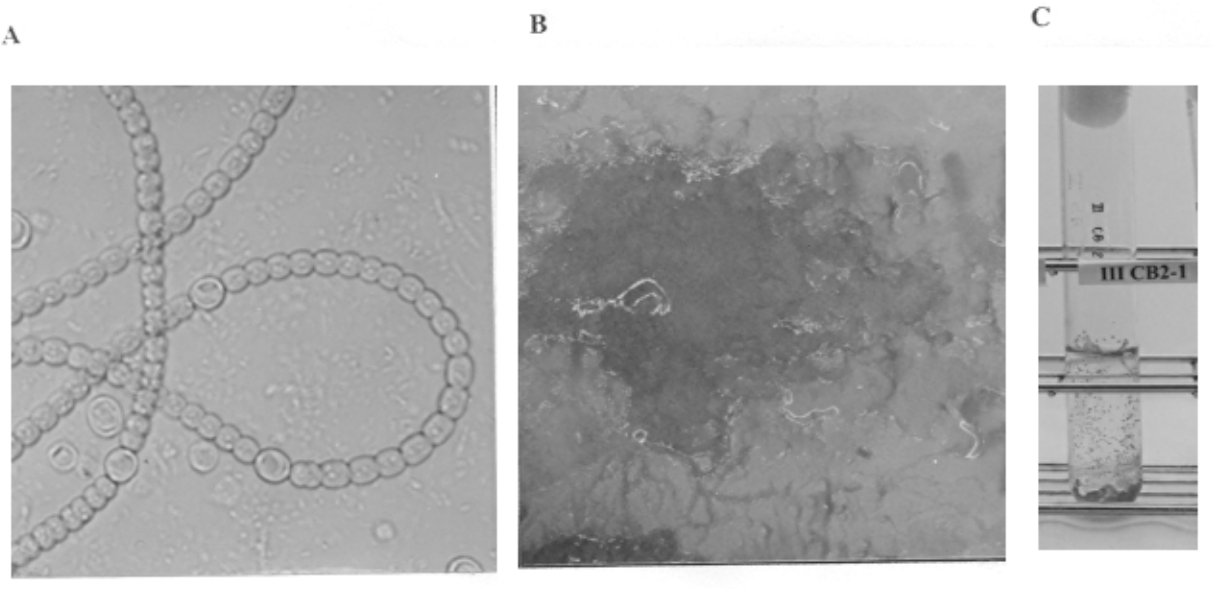

Fig. A101 Cell morphology under microscope with 400X magnification (A), colony forming on BG11 solid (B) and clump forming in BG11 liquid medium (C) of cyanobacterial isolate III CB2-1

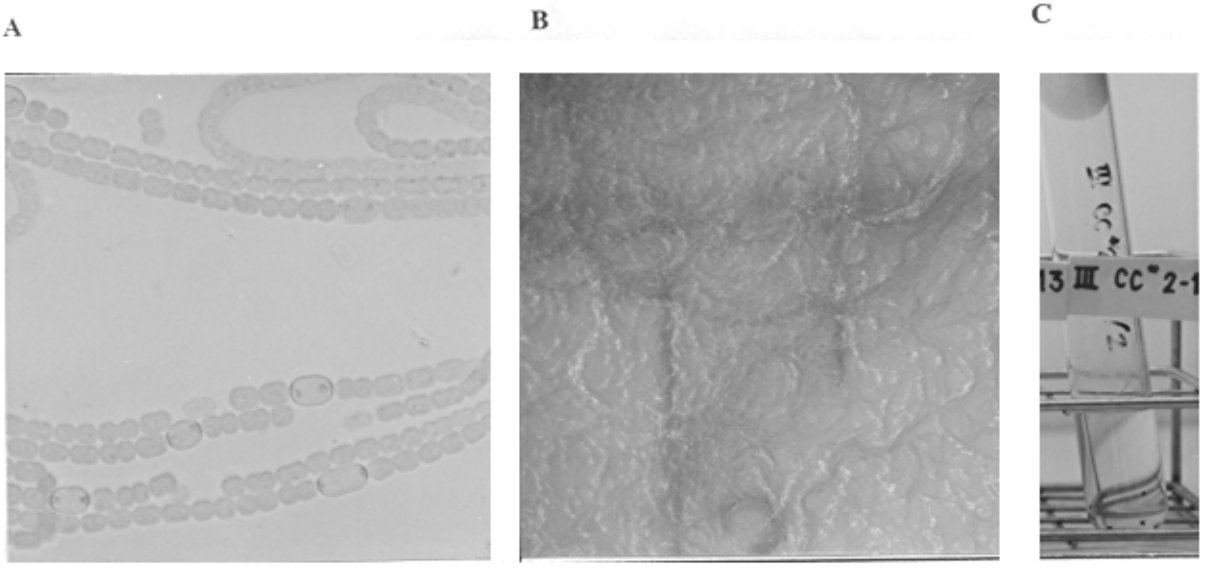

Fig. A102 Cell morphology under microscope with 400X magnification (A), colony forming on BG11 solid (B) and clump forming in BG11 liquid medium (C) of cyanobacterial isolate III CC*2-1 1/2

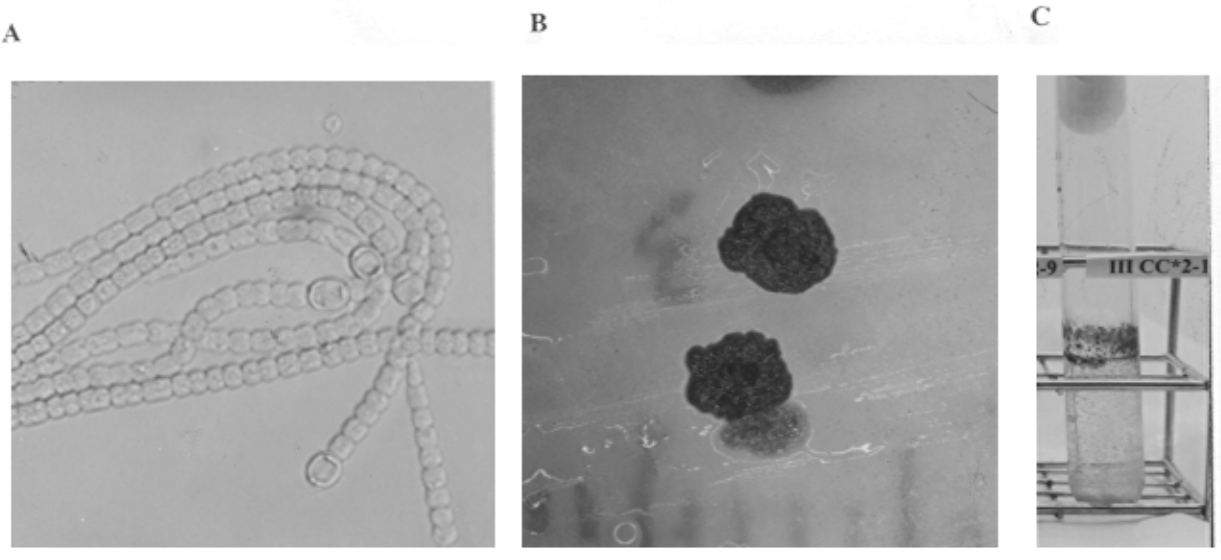

Fig. A103 Cell morphology under microscope with 400X magnification (A), colony forming on BG11 solid (B) and clump forming in BG11 liquid medium (C) of cyanobacterial isolate III CC*2-10

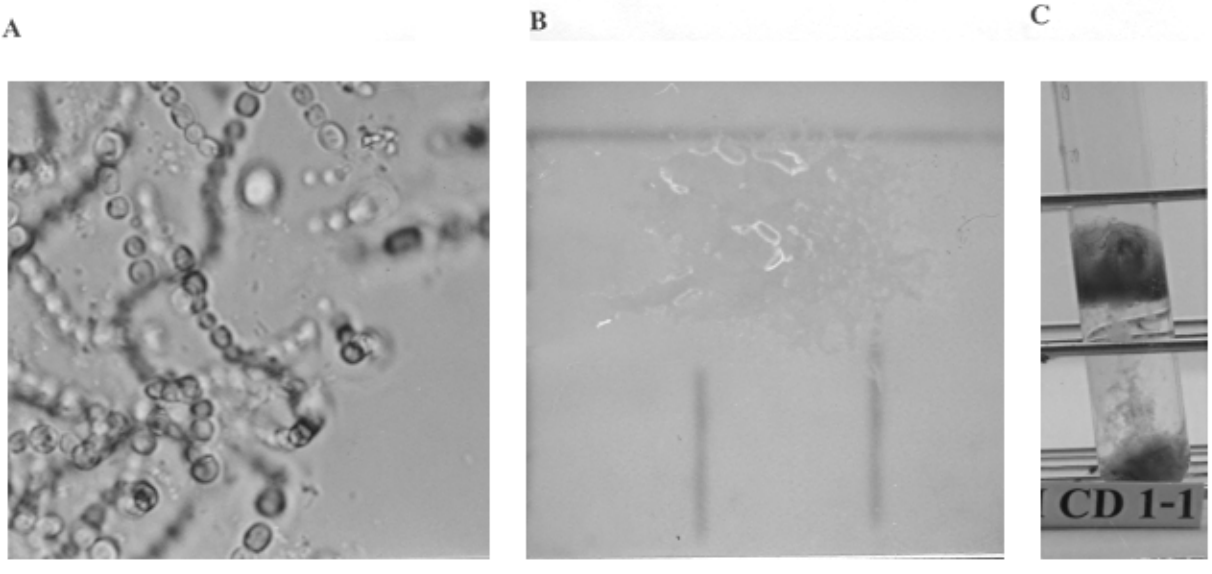

Fig. A104 Cell morphology under microscope with 400X magnification (A), colony forming on BG11 solid (B) and clump forming in BG11 liquid medium (C) of cyanobacterial isolate I CD1-1

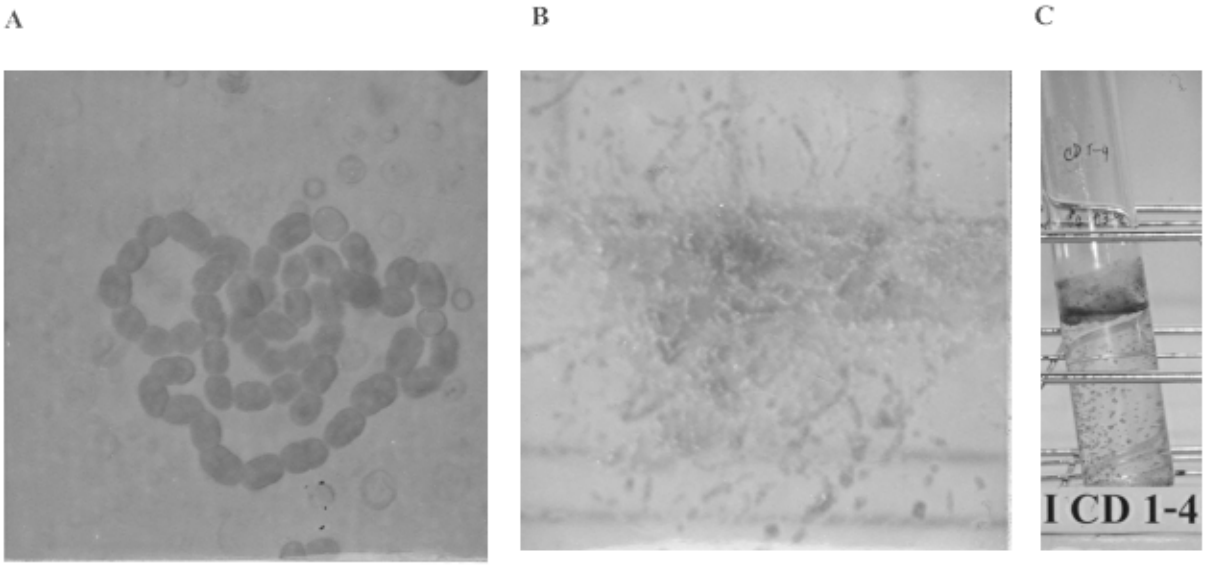

Fig. A105 Cell morphology under microscope with 400X magnification (A), colony forming on BG11 solid (B) and clump forming in BG11 liquid medium (C) of cyanobacterial isolate I CD1-4

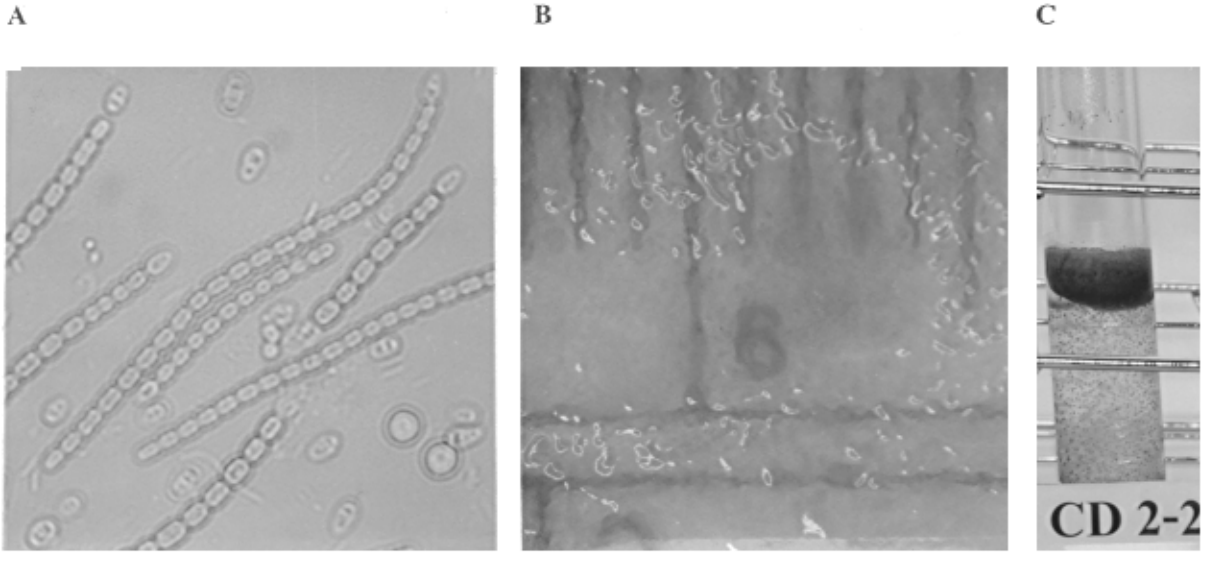

Fig. A106 Cell morphology under microscope with 400X magnification (A), colony forming on BG11 solid (B) and clump forming in BG11 liquid medium (C) of cyanobacterial isolate I CD2-2

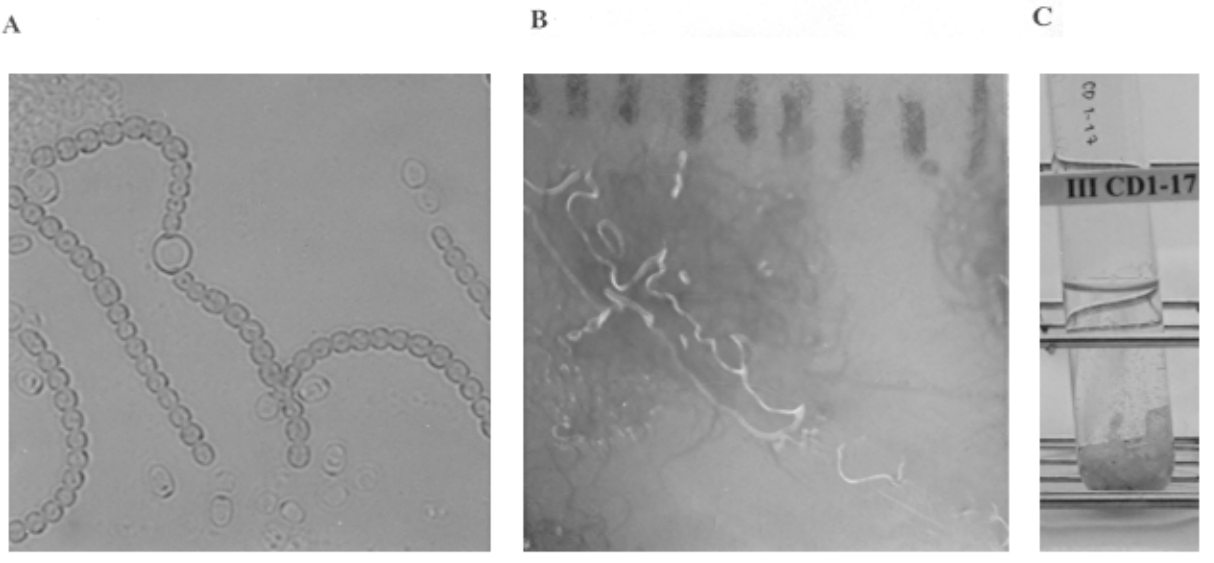

Fig. A107 Cell morphology under microscope with 400X magnification (A), colony forming on BG11 solid (B) and clump forming in BG11 liquid medium (C) of cyanobacterial isolate III CD1-17

BIBLIOGRAPHY

Miss Sasidhorn Innok was born on October 12, 1973 in Lopburi, Thailand. She graduated with the Bachelor degree of Science in Associated Medical Science, Khon Kaen University in 1995. She had been working in Saint's Mary Hospital and Regional Blood Center IV Thai Red Cross Society for two years. During her Master degree enrollment in school of Biotechnology, Institute of Agricultural, Suranaree University of Technology (1997-1999). She presented research work in Annual Meeting of Biodiversity Research & Training Program in 1998 and 1999 at Khon Kaen and Songkla provinces, the 10th Annual meeting of the Thai society for Genetic Engineering and Biotechnology on Biotechnology for a self-sufficient economy, 25-27 November 1998, Bankok, Thailand, poster presentation at the 5th Asia-Pacific Biochemical Engineering Conference 1999 and the 11th Annual Meeting of the Thai Society for Biotechnology on 15-18 November 1999 Phuket, Arcadia Hotel & Resort Phuket, Thailand, International Conference on Asian Network on Microbial Research, 29 November-1 December 1999 and Nitrogen Fixation Seminar Under Programme of Large Scale Cooperation (NRCT-JSPS/DOST/LIPI/VCC), 1-5 December 1999, Chiang Mai, Thailand.