APPLICATION OF DNA TECHNIQUES TO MONITOR CYANOBACTERIAL BEHAVIOUR IN ENVIRONMENTS

Sasidhorn Innok

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การประยุกต์ใช้เทคนิคทางดีเอ็นเอเพื่อติดตามพฤติกรรมของไซยาโนแบคทีเรียในสิ่งแวดล้อม

นางสาวศศิธร อินทร์นอก

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต

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Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy.

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ทำการตรวจสอบพฤติกรรมของไซยาโนแบคทีเรียโดยใช้เทคนิคทางดีเอ็นเอ โดยการศึกษาครั้งนี้ แบ่งไซยาโนแบคทีเรียเป็น 2 กลุ่ม ได้แก่ ไซยาโนแบคทีเรียที่ผลิตสารพิษ microcystins และไซยาโนแบคทีเรียที่สามารถตรึงไนโตรเจนได้ โดยใช้เทคนิคทางดีเอ็นเอในการติดตามความอยู่รอดของไซยาโนแบคทีเรียทั้ง 2 กลุ่ม กลุ่มที่ 1 *Microcystis viridis* สามารถผลิตสารพิษที่ชื่อว่า microcystins ในทะเลสาบเซนบะ ประเทศญี่ปุ่น จากการศึกษาการบำบัดน้ำโดยใช้ ultrasonic radiation และ jet circulation ร่วมกับการกำจัดน้ำออกโดยอาศัยน้ำในแม่น้ำ ซึ่งวิธีดังกล่าวจะทำลาย gas vacuole ของไซยาโนแบคทีเรีย ทั้งนี้ได้สกัดดีเอ็นเอโดยตรงจากดินตะกอน เพื่อใช้ในการติดตามความคงอยู่ของ *M. viridis* ภายหลังจากการบำบัดน้ำแล้ว โดยใช้ primer 3 คู่ได้แก่ rRNA intergenic spacer (RISA), DNA dependent RNA polymerase (*rpo*C1) และ *rpo*C1 fragment ที่จำเพาะเจาะจงกับ *Microcystis* มาใช้ในการเพิ่มจำนวนดีเอ็นเอที่สนใจโดยเทคนิค PCR ผลการศึกษาจากแต่ละ primer จะแสดงโดยอาศัยพื้นฐานของ single strand conformation polymorphisms (SSCP) นอกจากนี้ยังใช้ *rpo*C1 fragment ที่จำเพาะกับ *Microcystis* มาทำการศึกษาโดยใช้เทคนิค denaturing gradient gel electrophoresis (DGGE) จากการศึกษา ไม่พบแบบแผน DNA ของ *Microcystis* ในตัวอย่างดินตะกอนที่นำมาศึกษา อย่างไรก็ตามยังได้ใช้เทคนิค terminal restriction fragment length polymorphisms (T-RFLP) โดยใช้ชุดยีน 16S rRNA พบว่าแบบแผน DNA ของ 16S rRNA ในดินตะกอนมีขนาด 91 และ 477 bp ซึ่งพบในแบบแผน DNA ของ *Microcystis* เมื่อตัดด้วยเอนไซม์ตัดจำเพาะ *Hha*I และ *Msp*I แต่ไม่พบแบบแผน DNA ที่ขนาด 75 bp ซึ่งเป็น DNA หลักของ *Microcystis* โดยพบทั้งที่ตัดด้วยเอนไซม์ตัดจำเพาะ *Hha*I และ *Msp*I ผลการศึกษาแสดงให้เห็นว่า *M. viridis* น่าจะสลายไปจากทะเลสาบแห่งนี้ หลังจากมีการบำบัดด้วย ultrasonic radiation และ jet circulation

กลุ่มที่ 2 ศึกษา *Nostoc* sp. สายพันธุ์ VICCR1-1 ที่มีความสามารถในการตรึงไนโตรเจนได้สูงถึง 11 $\mu molC_2H_4/h/mg$ chlorophyll a ซึ่งแยกได้จากพื้นที่ปลูกข้าวสลับกับพืชไร่ โดยนำมากระตุ้นให้มีการสร้างเซลล์ heterocysts และ akinetes โดยอาศัยการปรับเปลี่ยนองค์ประกอบของอาหารเลี้ยงเชื้อ พบว่าในสภาวะที่ขาด $CaCl_2$ จะทำให้มีการเปลี่ยนแปลงเป็นเซลล์ heterocysts สูงถึง 46.61% และหากนำเซลล์ที่เลี้ยงในอาหารที่มีแหล่งไนโตรเจน มาเลี้ยงในอาหารที่ไม่มีไนโตรเจนและ $CaCl_2$ พบว่าจะมีเซลล์ heterocysts เพิ่มสูงถึง 62.59% จากการศึกษาโปรตีนที่สร้างภายในเซลล์ในสภาวะที่ส่งเสริมการสร้างเซลล์ heterocysts พบ โปรตีนขนาด 72 KDa ซึ่งมี

เปอร์เซ็นต์ความเหมือนกับ chaperonin GroEL (HSP60 family) และขนาด 140 KDa ซึ่งมีเปอร์เซ็นต์ความเหมือนกับ phycobilisome core-linker protein ในสภาวะที่มี และไม่มีแหล่งอาหารไนโตรเจน ส่วนโปรตีนขนาด 45 KDa ซึ่งมีเปอร์เซ็นต์ความเหมือนกับ outer membrane protein (porin) จะสูงขึ้นเมื่อเลี้ยงในอาหาร BG11 ที่ปราศจากแหล่งอาหารไนโตรเจนและ$CaCl_2$ เมื่อทำการศึกษาการกระตุ้นให้มีการสร้างเซลล์ akinetes พบว่าฟอสฟอรัสและเหล็ก มีผลต่อการเปลี่ยนแปลงรูปร่างเป็นเซลล์ akinetes โดยเฉพาะในสภาวะที่ขาดธาตุอาหารทั้งสองพร้อมกัน โดยจะมีการสร้างเซลล์ akinetes สูงถึง 21.17% เมื่อเปรียบกับการเพาะเลี้ยงในอาหารสภาวะปกติ (อาหาร $BG11_0$)อย่างไรก็ตามการแสดงออกของยีนที่เกี่ยวข้องกับการสร้างเซลล์ heterocysts (*het*R) และ akinetes (*sod*F และ *ava*K) พบว่าการศึกษาการแสดงออกของ *het*R เพียงยีนเดียวไม่เพียงพอต่อการศึกษาการแสดงออกของยีนที่เกี่ยวข้องกับการสร้าง heterocysts ส่วน *sod*F และ *ava*K ซึ่งมีความสัมพันธ์กับการสร้าง akinetes ไม่สามารถตรวจวัดได้ในการศึกษานี้

ได้ทำการทดสอบการงอกของเซลล์ akinetes ในสภาวะเครียด พบว่าเซลล์ดังกล่าวสามารถงอกได้ที่ pH ระหว่าง 3-10 อุณหภูมิสูงถึง 40°C และความเข้มข้นของเกลือที่ 0.5 M NaCl แม้จะอยู่ในสภาวะดังกล่าวเป็นเวลา 7 วัน หากนำเซลล์ akinetes ไปผสมกับแร่ดินมอนต์มอริลโลไนต์ในอัตรา 4.0×10^6 cfu/กรัมของแร่ดินมอนต์มอริลโลไนต์ ซึ่งสามารถเก็บรักษาไว้ในรูปดังกล่าวได้นานเป็นปี โดยในสัปดาห์ที่ 56 จะมีจำนวนเซลล์ที่รอดชีวิตอยู่ 3.3×10^5 cfu/กรัมของแร่ดินมอนต์มอริลโลไนต์ เมื่อนำไปใช้ในพื้นที่นาด้วยจำนวน 2.8×10^6 cfu/ตารางเมตร (ตร.ม.) ผลผลิตหลังเก็บเกี่ยวไม่มีความแตกต่างอย่างมีนัยสำคัญเมื่อเปรียบเทียบกับในแปลงที่มีปุ๋ยเคมีไนโตรเจน แปลงที่มีไซยาโนแบคทีเรีย และแปลงที่มีหัวเชื้อไซยาโนแบคทีเรีย เมื่อติดตามปริมาณของหัวเชื้อที่ใส่ในพื้นที่นาโดยวิธี MPN-DGGE บนชุดของยีน 16 S rRNAพบว่า หลังเก็บเกี่ยวจะยังคงมีเชื้อดังกล่าวอยู่ในจำนวน 1.0×10^7 cfu/ตร.ม., 2.5×10^5 cfu/ตร.ม. และ 1.62×10^6 cfu/ตร.ม. ในแปลงทดลองที่มีปุ๋ยเคมีไนโตรเจน แปลงที่มีไซยาโนแบคทีเรีย และแปลงที่มีหัวเชื้อไซยาโนแบคทีเรีย ตามลำดับ

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TOXIN-PRODUCING CYANOBACTERIA/N_2-FIXING CYANOBACTERIA/ DENATURING GRADIENT GEL ELECTROPHORESIS (DGGE)/TERMINAL RESTRICTION FRAGMENT LENGTH POLYMORPHISM (T-RFLP)/CYANOBACTERIAL INOCULUM

The cyanobacterial behaviours in environment were investigated on the basis of DNA techniques. Two groups of cyanobacteria were selected: microcystin producing cyanobacteria and N_2-fixing cyanobacteria. Firstly, *Microcystis*, known as toxic microcystin producing cyanobacteria, normally blooms in Senba Lake, Japan. Recently, this lake has been treated by ultrasonic radiation and jet circulation which were integrated with flushing with river water. This treatment was most likely sufficient for the destruction of cyanobacterial gas vacuoles. In order to confirm whether *M. viridis* still existed, a molecular genetic monitoring technique on the basis of DNA direct extraction from the sediment was applied. Three primer sets were used for polymerase chain reaction (PCR) based on a rRNA intergenic spacer analysis (RISA), the DNA dependent RNA polymerase (*rpo*C1) and a *Microcystis* sp.-specific *rpo*C1 fragment. The results from each primer were demonstrated on the basis of single strand conformation polymorphisms (SSCP). Moreover, the *Microcystis* sp.-specific *rpo*C1 fragment was further analyzed by denaturing gradient gel electrophoresis (DGGE). The DNA pattern representing *M. viridis* could not be

detected in any of the sediment samples. However, the results were confirmed with another technique, terminal restriction fragment length polymorphisms (T-RFLP). Although T-RFLP patterns of 16S rDNA in sediment at 91 bp and 477 bp lengths were matched with the T-RFLP of *M. viridis* (*Hha*I and *Msp*I endonuclease digestion, respectively), the T-RFLP pattern of 75 bp length was not matched with *M. viridis* (both of *Hha*I and *Msp*I endonuclease digestion) which were the major T-RFLP pattern of *M. viridis*. Therefore, the results most likely indicated that *M. viridis* seems to have disappeared because of the addition of the ultrasonic radiation and jet circulation to the flushing treatment.

Secondly, *Nostoc* sp. strain VICCR1-1 was isolated from rice in rotation of other crops cultivation showed the highest nitrogen fixation efficiency about 11 µmol C_2H_4/h/mg total chlorophyll a. The *Nostoc* sp. strain VICCR1-1 was induced in order to form heterocysts and akinetes on basis of nutrient modification. Absence of $CaCl_2$ played the role of heterocyst differentiation which was induced as high as 46.61%. The number of heterocyst was induced up to 62.59% when transferred the cyanobacterial cells from BG11 to $BG11_0$ (without $CaCl_2$) medium. Proteins were extracted after heterocyst induction. There were 72 KDa and 140 KDa proteins expected to be chaperonin GroEL (HSP60 family) and phycobilisome core-membrane linker protein, respectively in both the medium with and with out N-source. Besides protein in size 45 KDa (expected to be outer membrane protein, porins) was up-regulated only when grown in $BG11_0$ (without $CaCl_2$) medium. In case of akinetes induction, phosphorus and iron were found to be the critical composition in akinete differentiation, especially when lack of both elements. The number of akinete cells could be increased up to 21.17% compared with culturing in normal condition ($BG11_0$ medium). The gene expression which involved heterocysts and akinetes

differentiation was observed based on *het*R (heterocyst differentiation), *sod*F and *ava*K (akinete development). The results suggested that only *het*R expression alone could not be the indicator for heterocyst development and *sod*F and *ava*K were not detected during akinete differentiation.

The germination of akinete cell was tested under various stress conditions. Cells could well germinate under the broad range of pH from 3 to 10, at high temperature as 40°C and high salinity as 0.5 M NaCl, eventhough grown on these conditions for 7 days. In order to prepare akinete inoculum, akinete cells were homogeneously mixed with montmorillonite clay at 4.0×10^6 cfu/g of montmorillonite clay. The akinetes could survive in the montmorillonite clay in constant number up to 1 year. The cells were still survived for 3.3×10^5 cfu/g of montmorillonite clay. To test the effect of *Nostoc* sp. strain VICCR1-1 as biofertilizer with rice, inoculum was applied in amount 2.8×10^6 cfu/m^2 in the field. After harvesting, the grain yields from chemical-N fertilizer, vegetative cells and akinete inoculum treatments were not significantly different. To monitor the persistence of *Nostoc* sp. strain VICCR1-1 after harvesting, the MPN-DGGE technique using 16S rRNA gene was employed. The results indicated that the remaining population is at 1.0×10^7, 2.5×10^5 and 1.62×10^6 cell/m^2 in treatments supplied N-fertilizer, vegetative cells and akinete inoculum, respectively.

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LIST OF ABBREVIATIONS

ARA	=	acetylene reduction activity
bp	=	base pair
BS	=	blooming season
cfu	=	colony forming unit
d	=	day
°C	=	degree celcius
DGGE	=	denaturing gradient gel electrophoresis
DNA	=	deoxyribonucleic acid
dNTPs	=	deoxynucleosine 5’ phosphate
g	=	gram
h	=	hour
ha	=	hectare
Kb	=	kilobase pair
kDa	=	kilodalton
kg	=	kilogram
l	=	liter
LC/MS	=	liquid chromatography/mass spectrometry
LD_{50}	=	median lethal dose
M	=	molarity
m	=	meter

LIST OF ABBREVIATIONS (Continued)

µg	=	microgram
µmol	=	micromole
m^2	=	square meter
MC/MCs	=	microcystin/microcystins
mg	=	milligram
mM	=	millimolar
MPN	=	most probable number
mRNA	=	messenger ribonucleic acid
MW	=	molecular weight
N	=	normal
ng	=	nanogram
nM	=	nanomolar
NS	=	non-blooming season
PCR	=	polymerase chain reaction
RISA	=	rRNA intergenic spacer analysis
RNaseA	=	ribonuclease A
RT-PCR	=	reverse transcriptase polymerase chain reaction
s	=	second
SDS-PAGE	=	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SOD	=	superoxide dismutase
SSCP	=	single strand conformation polymorphisms
t	=	ton

LIST OF ABBREVIATIONS (Continued)

T-RFLP	=	terminal restriction fragment length polymorphisms
U	=	unit
USRS	=	ultrasonic radiation system
v	=	volt
v/v	=	volume per volume
w/v	=	weight per volume
wt	=	weight

CHAPTER I

GENERAL INTRODUCTION

Cyanobacteria (blue-green algae) are unique photosynthetic organisms of great importance because of their existence of some 3.5 billion years (Singh *et al*., 1999). They were an ancient group of prokaryotic microorganisms exhibiting the general characteristics of gram negative bacteria with a 4 layered cell wall; murein (peptidoglycan) as rigid structure (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). Ample evidences indicated that chloroplasts, which confer photoautography to plants and algae, were derived from symbiotic cyanobacteria that were engulfed by primitive eukaryotic cells. Because of the variety of their physiological, morphological and developmental features, cyanobacteria constitute an extremely diverse groups of prokaryotes which have colonized a wide range of habitats, they occurred 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).

Cyanobacteria are belonged to division Cyanophyta, there are about 150 genera with about 2000 species (www, 2005). The classically distinguishing of different species of cyanobacteria has relied upon identifying morphological, developmental and

biochemical characteristics (Mazel *et al*., 1990). The external morphology shows various forms such as single cells, colonies, filaments, colonial forms-flat, spherical, elongate, cubical or amorphous. Some cyanobacteria cover with gelatinous sheet which may remain distinct or may fuse into gelatinous matrix. A filament may consist of one or more chains of cells, each chain is termed a trichome. There are no organelles for locomotion, however, some cyanobacteria can move forward and backward dimensions for example, *Oscillaoria*. Cyanobacteria have chlorophyll a, carotenoids and additional accessory pigments called phycobiliproteins (consists of phycocyanins and phycoerythrins) assembled as a phycobilisome on thylakoid membranes. Thus, the color characteristics of cyanobacteria are varied from green, blue-green or olive green to various shades of red to purple or even black. Typically a protoplast surrounded by a cell wall enclosed in a gelatinous sheath. Protoplast consists of colorless central body (rich in chromatin-resembles a nucleus), contains most of the ribosomes and pigments which are scattered through the chromoplasm. Cyanobacteria are no chloroplasts, however, the photosynthetic pigments are arranged in flattened disc, known as thylakoids which are no stack and no grana (www, 2005). The storage product is cyanophycin starch which consists of glycogen and protein. For reproduction, they were asexual reproduction by using fragmentation of colonies, spore (akinete and sporangiospore) and other structures such as heterocysts, separation disc or hormogonia to be fragmentation point and fragmentation area (www, 2002).

Rippka's classification divided N_2-fixing cyanobacteria 178 living strains into 22 genera, based on morphological and developmental features which could readily be determined in cultured material and which were, as far as possible, constant for a given strain (because the previous classification had been botanical in nature, it had been

based on descriptions of dead type-specimens). These 22 genera were placed in five main sections as described in Table 1.1. As indicated, one of the primary morphological criteria used in this classification is whether the strains are unicellular or filamentous in nature (Rippka *et al*., 1979).

Table 1.1. The major taxonomic group of cyanobacteria (Rippka *et al*., 1979; Castenholz and Waterbury, 1989)

Cell arrangement	Group	Reproduction	Heterocysts	Division	Genera
Unicellular forms (Fig. 1.1)	I				*Gloeothece* *Gloeobacter* *Gloeocapsa* *Synechococcus* *Synechocystis* *Chamaesiphon*
	II	Multiple fission, possibly also with binary fission			*Dermocarpa* *Xenococcus* *Dermocarpella* *Myxosarcina* *Chroococcidopsis* *Pleurocapsa* group

Table 1.1. (continued)

Cell arrangement	Group	Reproduction	Heterocysts	Division	Genera
Filamentous forms (cells form a trichome) (Fig. 1.2)	III	Intercalary cell division and trichome breakage	No	One plane	*Spirulina* *Oscillatoria* *Pseudonabaena* *Lyngbya*
	IV	As above, plus may form hormogonia	Yes	One plane	*Phormidium* *Plectonema* *Anabaena* *Nodularia* *Cylindrospermum* *Nostoc*
	V	As section IV	Yes	More than one plane	*Scytonema* *Calothrix* *Chlorogloeopsis* *Fischerella*

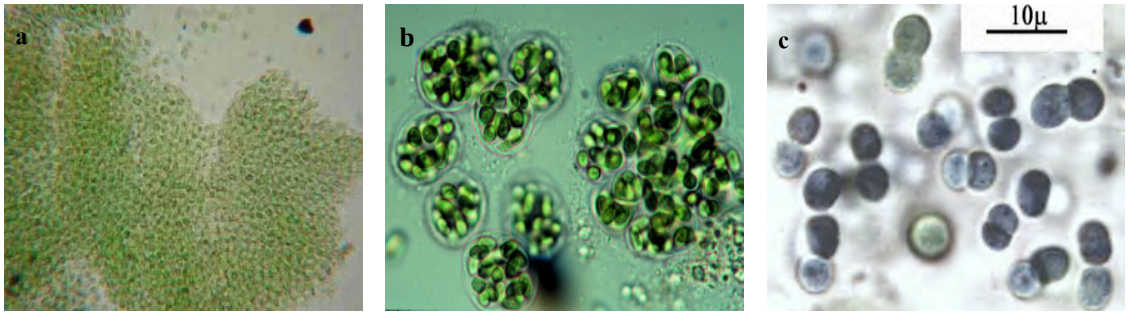

Figure 1.1 Non-filamentous forms: (a) *Microcystis* sp. (www, 2005), (b) *Gloeocapsa* sp. (www, 2005) and (c) *Synechococcus* sp. (www, 2005).

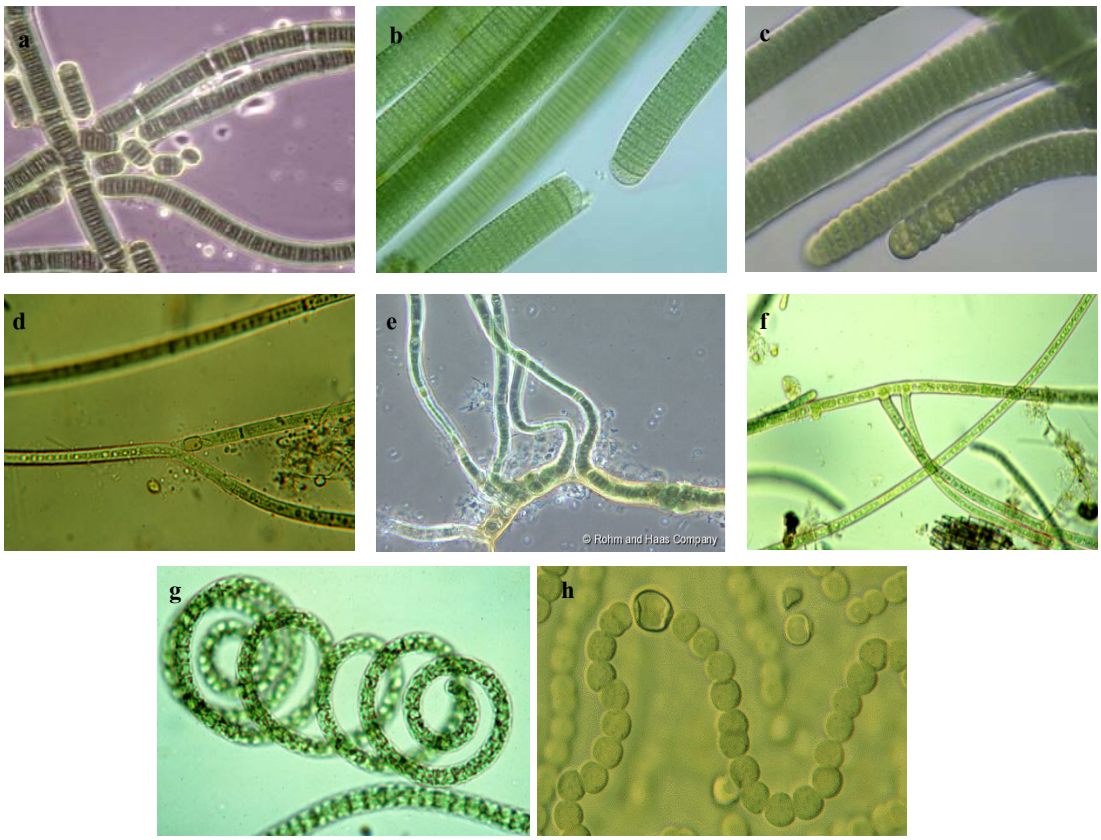

Figure 1.2 Filamentous form: a; *Lyngbya* sp. (www, 2005), b; *Oscillatoria* sp. (www, 2005), c; *Plectonema* sp. (www, 2005), d; *Tolypothrix* sp. (www, 2005), e; *Scytonema* sp. (www, 2005), f; *Hapalosiphon* sp. (www, 2005), g; *Spirulina* sp. (www, 2005) and h; *Nostoc* sp. (www, 2005).

1.1 Differentiated Cell Types and Multicellular Structures

1.1.1 Vegetative cell

Vegetative cell are typical of those of group as a whole, possessing all the requirement for the higher plant type of photosynthesis (Fig. 1.3), e. g. use of water as an ultimate source of reductant, carbon dioxide fixation by Calvin-cycle enzymes and evolution of oxygen. Photosynthesis pigments are located in thylakoids in the outer regions of the cells. In addition to chlorophyll a, carotenes and xanthophylls, they

have characteristic accessory pigments called phycobiliproteins. These have a role as interceptors, but appear to function also as a readily usable nitrogen store. Vegetative cells may also contain polyhedral bodies termed carboxysomes. These are molecular aggregates of the Calvin-cycle enzyme, ribulose biphosphate carboxylase/oxygenase (Rubisco). As carbon stores, polyglucoside bodies may be found among the thylakoid. Excess nitrogen is stored in structured granules as a 1:1 copolymer of aspartic acid and arginine known as cyanophycin. In addition, vegetative cells may contain gas vacuoles, polyphosphate bodies and lipid bodies. They are thus well equipped to accumulate essential metabolites. The various inclusions are almost abundant in slow-growing phases and become depleted during rapid growth (Castenholz and Waterbury, 1989).

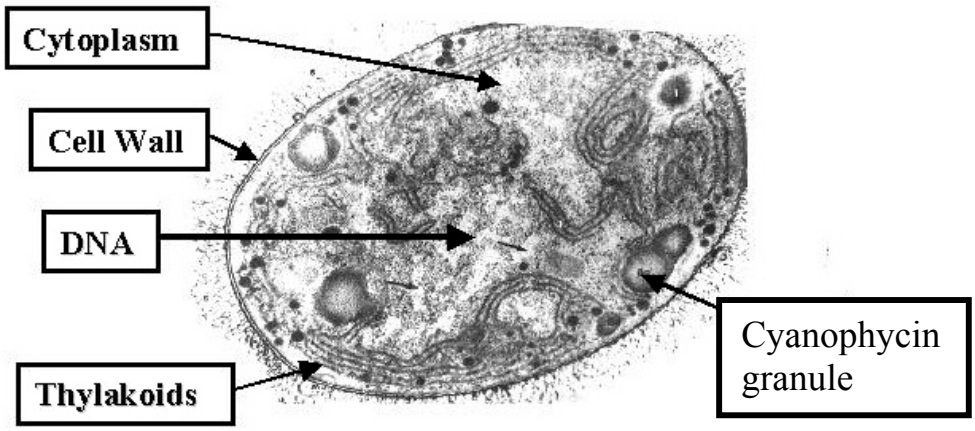

Figure 1.3 Vegetative cell organization (www, 2005).

1.1.2 Heterocyst cell

Heterocysts are morphologically distinct cells that develop in response to a lack of combined sources in the environment (Fig. 1.4). They are easy to recognize under the microscope since (i) they are usually larger than vegetative cells, (ii) they develop

thick tegumentary layers, (iii) they develop intracellular hyaline buttons at the points of attachment to the vegetative cells and (iv) they are usually pale yellow in coloration and have reduced autofluorescence. The ability to develop heterocysts occurs without exception within a monophyletic group of filamentous cyanobacteria (heterocystous), corresponding to the orders Nostocales and Stigonematales in the bacterial taxonomy. They may differentiate from end-cells (terminal heterocysts, as in *Calothix*) or from cells within the trichome (regularly-spaced intercalary heterocysts, as in *Anabaena*). Heterocysts are highly specialized in the fixation of gaseous dinitrogen under aerobic conditions. They represent a successful solution to the non-trivial problem of avoiding nitrogenase inactivation by free oxygen in oxygen evolving organisms. Heterocyst biology has been relatively well studied at the biochemical and molecular level. In heterocystous cyanobacteria, heterocysts are the only cells which express *nif* (nitrogen fixation) genes and synthesize nitrogenase. Apparently, heterocysts do not evolve oxygen themselves (Photosystem II activity is absent or restricted) but a functional Photosystem I provides ATP. The source of reductant for nitrogen fixation is provided (as organic carbon) by the adjacent vegetative cells, which in turn obtain fixed nitrogen from the heterocyst in the form of amino acids (mostly glutamine). The heterocyst protect their nitrogenase from oxygen inactivation by maintaining reduced internal partial pressures of oxygen, a situation that is attained by means of increased rates of cellular respiration and, apparently, by restricting diffusive entry of oxygen from the environment thanks to their thick envelope. The developmental regulation of heterocysts is beginning to be understood at the genetic level. The autoregulated gene *het*R, activated by the deficiency in combined nitrogen seems to play a crucial role in initiation of heterocyst development (www, 2000).

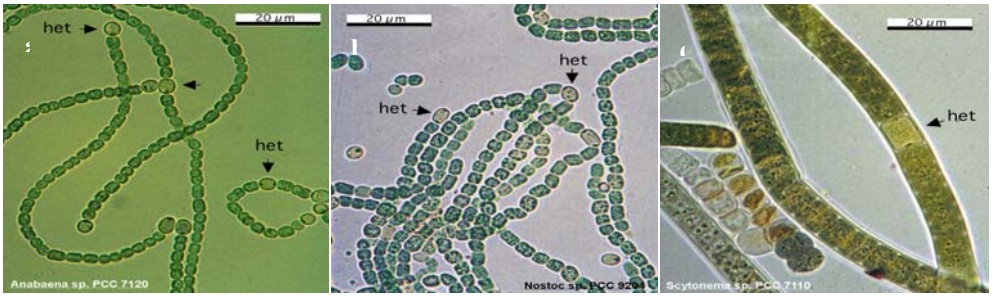

Figure 1.4 Heterocyst cell of some cyanobacteria; (a) *Anabaena* sp. PCC7120, (b) *Nostoc* sp. PCC9204 and (c) *Scytonema* sp. PCC7110 (www, 2005).

1.1.3 Akinete cell

They are formed exclusively by heterocystous filamentous cyanobacteria, but not by all, and may differentiate en masse (in a body: as a whole) or at special locations within the filaments (usually close or next to a heterocyst) (Fig. 1.5). Akinetes are resistant to desiccation, to low temperature (including freeze-thaw cycling), and to digestion in animal guts, and they are considered to be resting stages. However, they are fundamentally different from typical bacterial spores in that they are not heat-resistant. These non-motile cells characterized by (i) their enlarged size with respect to the vegetative cells, (ii) their thick cell wall and additional tegumentary (an enveloping layer as membrane) layers and (iii) their high content of nitrogen reserves in the form of cyanophycin granules. The process of differentiation may be mediated by specific hormone-like compounds produced by vegetative cells. Germination of akinetes into vegetative cells occurs when the environmental conditions (light intensity, phosphate availability) become favourable for the growth of vegetative filament. Genetic evidence suggests that the early regulatory process of akinete development is common to that of heterocysts (www, 2000).

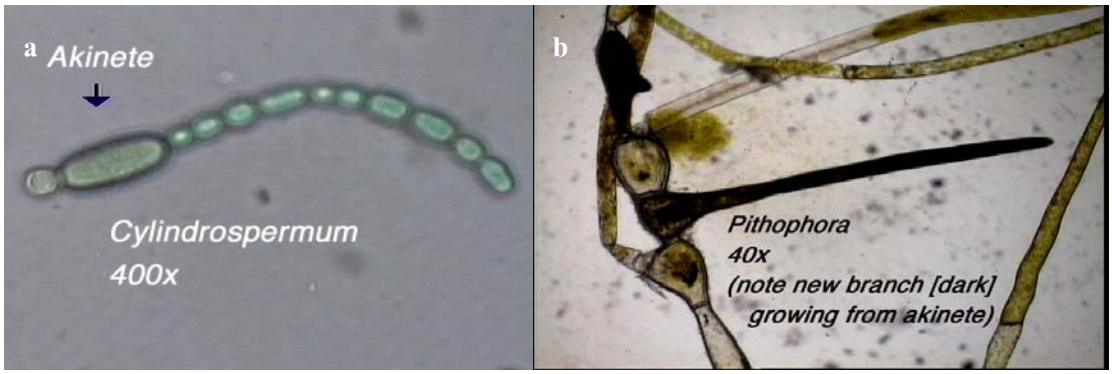

Figure 1.5 Akinete cell of some cyanobacteria; (a) *Cylindrospermum* sp. (www, 2006) and (b) *Pithophora* sp. (www, 2006).

1.1.4 Hormogonia

Hormoginia are short (some 5-25 cells) chains of cells formed and released from the trichome (Fig. 1.6). Hormogonial cells may or may not be different in size and shape from vegetative cells. Detachment may involve the differentiation of a necridic cell separating the vegetative trichome from the hormoginium. They serve a function in the dispersal of the organism. Dispersal is aided by the expression of phenotypic traits, which may vary according to strains, like gliding motility, development of gas vesicles, or changes in surface hydrophobicity. Hormogonia eventually settle and dedifferentiate into a typical vegetative organism. Hormogonium formation may be triggered by environmental factors such as phosphate repletion or changes in light quality (www, 2000).

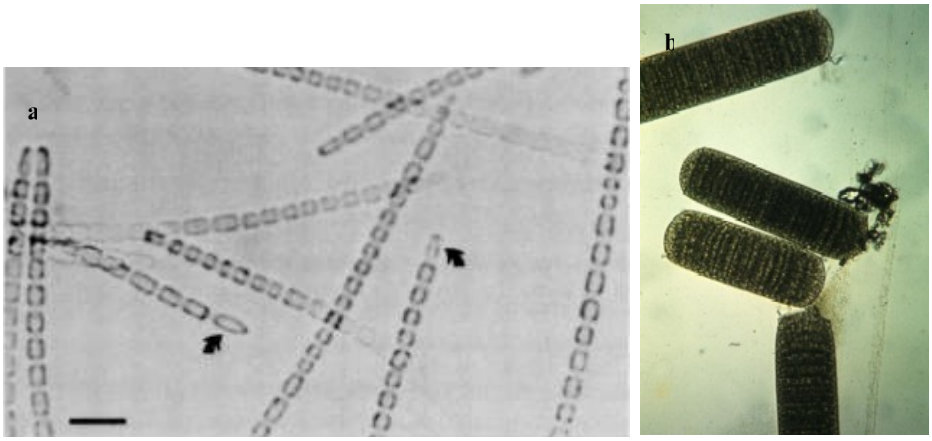

Figure 1.6 Hormogonia a; hormogonium of filamentous cyanobacteria (tapered end cells are indicated by arrows (Campbell and Meeks, 1989) and b; hormogonium of *Oscillatoria* sp. (www, 2006).

1.1.5 Necridic cells (necridia)

They occur in truly multicellular cyanobacteria Necridic cells undergo a suicidal process, which begins with the loss of turgor and leakage of some cellular contents and continues with shrinkage and the separation of the cross-walls (septa) from the adjoining cells (Fig. 1.7). Eventually the necridic cells will either rupture and disintegrate or remain as small, isolated vestigial cells (a bodily part or organ that is small and degenerate or imperfectly developed in comparison to one more fully developed in an earlier stage of the individual, in a part generation or in closely related forms). Cells adjacent to the necridium will usually develop morphologies typical for terminal (apical) cells. The formation of necridia may lead to the separation of one trichome into two (proliferation) or in the detachment of hormogonia from the vegetative filament. Most of the information about necridia is observational and no studies have been performed yet to investigate the regulation of this morphogenetic mechanism (www, 2000).

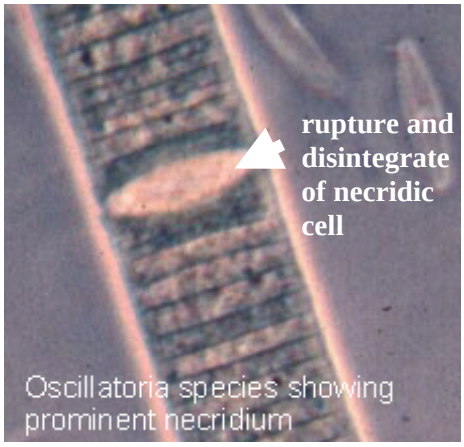

Figure 1.7 Necridium cell of *Oscillatoria* sp. (www, 2006).

1.1.6 Terminal hair

These are multicellular differentiations occurring at the tips of trichomes in some members of the genus *Calothrix* and allied cyanobacteria (botanical family Rivulariaceae) (Fig. 1.8). In response to nutrient limitation (i.e., phosphate) the terminal parts of the trichome differentiate irreversibly into thin and long rows of narrow, almost colorless, vacuolated cells (hence the term hair). The hair is a site of preferential expression of cell surface-bound phosphatase activity (www, 2000).

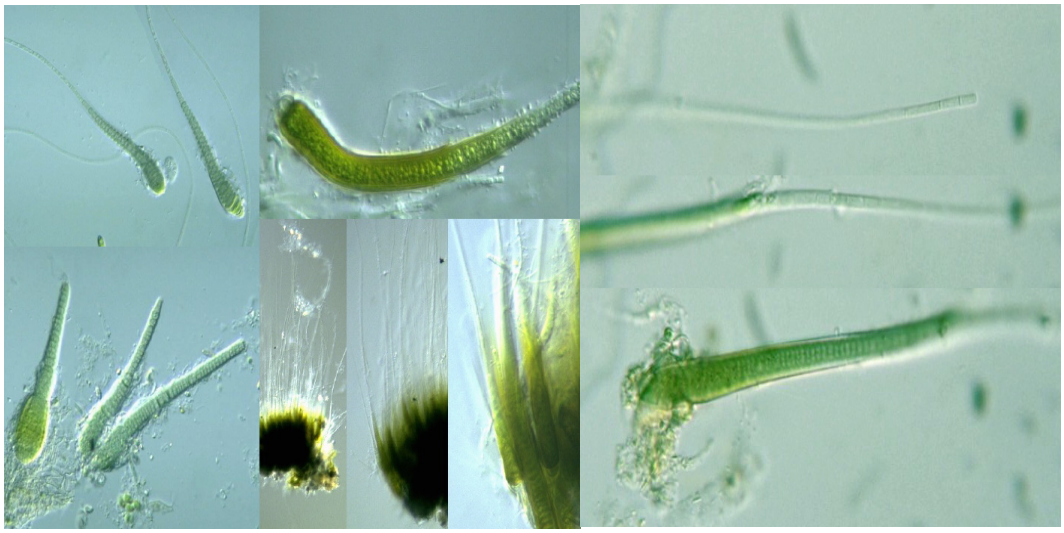

Figure 1.8 Terminal hair of Rivulariaceae (www, 2006).

1.2 Ecological Importance

The occurrence and predominance of cyanobacteria in a vast array of habitats is a result of several general characteristics and of some features characterizing certain cyanobacterial species clusters. Cyanobacteria are probably related strongly to the possession of gas vesicles and the ability to regulate buoyancy (Van Liere and Walsby, 1982). However, in eutrophic lakes, N_2 fixation capability following depletion of combined nitrogen and/or the ability to efficiently utilize a very low photon flux density is thought to enhance the success of some cyanobacteria (Gibson and Smith, 1982; Van Liere and Walsby, 1982; Mur, 1983). In oligotrophic marine or fresh waters, the N_2-fixing ability may also be of primary importance (e.g. *Trichodesmium* sp.). The unicells of *Synechococcus* may thrive in deep-mixing, oligotrophic waters because of their efficient absorption of a low photon flux (Glover *et al*., 1986). Extracellular excretions of substances such as hydroxymates, which may inhibit the growth of potential competitors, have been suggested as contributing to cyanobacterial success (Gibson and Smith, 1982). It has also been suggested that siderochrome (trihydroxymate) excretion by cyanobacteria in iron-poor waters may aid in sequestering iron (Gibson and Smith, 1982).

The temperature optimum for growth of many or most cyanobacteria is higher by at least several degrees than for most eukaryotic algae. This tendency may also play an important role in the notable summertime dominance of cyanobacteria in temperate latitudes, but this tendency is extended to even higher temperatures for some species of hot springs (up to 74°C), tropical pools, and intertidal habitats where eukaryotic phototrophs are inhibited or excluded. Cyanobacteria may also predominate at low and freezing temperatures due to the exclusion of most other

phototrophs, but in those cases the actual temperature optimum may be considerably higher (Vincent and Howard-Williams, 1986).

The ability of many cyanobacteria to tolerate high salinity (Borowitzka, 1986) results in blue-green predominance in many hypersaline marine lagoons and in land saline lakes (Bauld, 1981). Cyanobacteria may also be especially tolerant of specific substances at higher concentrations. Free sulfide is tolerated and sometimes utilized by cyanobacteria at levels much higher than those tolerated by eukaryotic algae (Padan and Cohen, 1982). Therefore, cyanobacteria act as base of food chain, moreover, the mineral still be remained after cell death.

However, several cyanobacteria produce potent toxins of two types: alkaloid neurotoxins and peptide hepatotoxins (Skulberg *et al*., 1984). Toxin blooms on aquatic ecology can provide the polluted water, give foul smell and taste to water, especially may influence several trophic levels in food chain. Primary producers are often inhibited either due to shading or by the effect of toxic substance (Demott *et al*., 1991). This ultimately influences the total energy production and oxygen content of water. These blooms are of then associated with decline of a number of secondary producers, the zooplanktons (Demott *et al*., 1991). At higher trophic levels, fishes may also be killed due to deterioration of water quality or toxicity effects. Carnivorous fishes may acquire cumulative toxicity doses while feeding on zooplanktons.

According to the ecological importance, cyanobacteria can be indicated to do harm and to do well. Cyanobacteria blooms are ubiquitous, often associated with eutrophication and appear to be on the increase normally at 21-27°C. All blooms of *Microcystis*, one of the most ubiquitous species, should be considered to be toxic and toxins are easily leached from the cells. They persist for long periods and are not

easily degraded. This organism produces vast number of peptides (microcystins), some of which are highly toxic. The most commonly occurring toxin is microcystin-LR, a cyclic heptapeptide hepatotoxin, where symptoms of exposure too, includes skin irritation, possible liver cancer as a result of chronic exposure and even death.

On the other hand, some cyanobacteria able to fix nitrogen from atmosphere and converst to ammonia that plant can use as nitrogen fertilizer. This transformation process is called nitrogen fixation which occurs only in the prokaryote microorganisms having nitrogenase enzyme.

Thus, this research was divided into two chapters such as toxin-producing cyanobacteria and N_2-fixing cyanobacteria. Since the bloom forming of toxin-producing cyanobacteria caused the harmful to human and live-stock. Thus, the cyanobacteria were removed, for example by nutrient reduction and bioaugmentation (www, 2006). On the other hand, application of N_2-fixing cyanobacteria as biofertilizer was also focused. Therefore, to monitor their persistence where were treated or applied, might need more precision in term of both quantity and quality. Since, the conventional method showed low sensitivity, low specificity and time consuming, therefore genetic molecular techniques were conducted for monitoring both cyanobacteria from sediment samples and rice field samples.

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CHAPTER II

TOXIN-PRODUCING CYANOBACTERIA

2.1 Abstract

Microcystis, known as toxic microcystin producing cyanobacteria, normally blooms in Senba Lake, Japan. Recently, this lake has been treated by ultrasonic radiation and jet circulation which were integrated with flushing with river water. This treatment was most likely sufficient for the destruction of cyanobacterial gas vacuoles. In order to confirm whether *M. viridis* still existed, a molecular genetic monitoring technique on the basis of DNA direct extraction from the sediment was applied. Three primer sets were used for polymerase chain reaction (PCR) based on a rRNA intergenic spacer analysis (RISA), the DNA dependent RNA polymerase (*rpo*C1) and a *Microcystis* sp.-specific *rpo*C1 fragment. The results from each primer were demonstrated on the basis of single strand conformation polymorphisms (SSCP). Moreover, the *Microcystis* sp.-specific *rpo*C1 fragment was further analyzed by denaturing gradient gel electrophoresis (DGGE). The DNA pattern representing *M. viridis* could not be detected in any of the sediment samples. However, the results were confirmed with another technique, terminal restriction fragment length polymorphisms (T-RFLP). Although T-RFLP patterns of 16S rDNA in sediment at 91 bp and 477 bp lengths were matched with the T-RFLP of *M. viridis* (*Hha*I and *Msp*I endonuclease digestion, respectively), the T-RFLP pattern of 75 bp length was not matched with *M. viridis* (both of *Hha*I and *Msp*I endonuclease digestion) which were

the major T-RFLP pattern of *M. viridis*. Therefore, the results most likely indicated that *M. viridis* seems to have disappeared because of the addition of the ultrasonic radiation and jet circulation to the flushing treatment.

2.2 Introduction

Cyanobacteria are found throughout the world in terrestrial, freshwater and marine habitats. However, it is the freshwater habitat that typically experiences a cyanobacteria "bloom". When conditions are good, a "clear" body of water can become very turbid with a green, blue-green or reddish-brown growth within just a few days. N, P and C are important nutrients for high growth rates and the ratios in the supplied concentrations are often decisive in selecting for cyanobacterial dominance. These organisms are capable of scavenging their environments for resources and excessive or "luxury" uptake of nutrients allow them to survive extreme nutrient deficient conditions. Cyanobacteria flourish at warming temperature (most commonly occur in late summer and early fall when water temperatures reach 21°C-27°C), neutral to alkaline conditions (pH 6-7), light intensity (about 68 μmol of photons/m^2/s) and light quality (Kaebernick *et al*., 2000), high nutrient concentrations especially where the ratios of N:P are low and adequate supply of iron is present (particularly, in case of total phosphorus greater than 10 μmol/l). Many species can regulate their buoyancy and float to the surface to form a thin "oily" looking film, or a blue-green scum several inches thick (Fig. 2.1). The film may be mistaken for a paint spill. Cyanobacteria cannot maintain this abnormally high population for long and will rapidly die and disappear after 1-2 weeks. If conditions remain favorable, another

bloom can rapidly replace the previous one. In fact, successive blooms may overlap so that it may appear as if one continuous bloom occurs for up to several months.

Figure 2.1 Blooming-cyanobacteria (www, 2006).

Since 1878, when George Francis produces first scholarly description on toxicity due to cyanobacteria, much excitement has been evoked among scientist regarding the potential lethal effects. Many reports following this investigation gradually steered the research objective of many laboratories all over the world, not only due to the worry over public health due to toxins but also because they offer beneficial research tools in applied as well as basic scientific studies (Carmichael, 1992a). However, not all cyanobacteria blooms are toxic. Even blooms caused by known toxin producers may not produce toxins, or may produce toxin at undetectable levels which cyanobacteria toxins can be lethal in relatively small amounts (Table 2.1).

Table 2.1. Cyanobacteria toxins relative to other biotoxins (Crayton, 1993)

Toxin	Organism	Common Name	Lethal Dose (LD_{50})
Botulinum toxin-a	*Clostridium botulinum*	Bacterium	0.00003
Tetanus toxin	*Clostridium tetani*	Bacterium	0.0001
Ricin	*Ricinus communis*	Castor bean plant	0.02
Diphtheria toxin	*Corynebacterium diphtheriae*	Bacterium	0.3
Koki toxin	*Phyllobates bicolor*	Poison arrow frog	2.7
Tetrodotoxin	*Sphaeroides rubripes*	Puffer fish	8
Saxitoxin	*Aphanizomenon flos-aquae*	Cyanobacterium	9
Cobra toxin	*Naja naja*	Cobra	20
Nodularin	*Nodularia spumigena*	Cyanobacterium	30-50
Microcystin-LR	*Microcystis aeruginosa*	Cyanobacterium	50
Anatoxin-a	*Anabaena flos-aquae*	Cyanobacterium	200
Microcystin-RR	*Microcystis aeruginosa*	Cyanobacterium	300-600
Strychnine	*Strychnos nux-vomica*	Plant	500
Anatoxin	*Amanita phalloides*	Fungus	600
Muscarin	*Amanita muscaria*	Fungus	1100
Phallatoxin	*Amanita phalloides*	Fungus	1800
Glenodin toxin	*Peridinium polonicum*	Dinoflagella alga	2500

*Acute LD_{50} in μg per kg bodyweight: based on intra-periotoneal injection of mice or rats

Of the known 50 genera and more than 250 species of freshwater cyanobacteria, only a handful is considered toxic (about 10% of all blooms produced toxins). Species and strains of *Anabaena, Aphanizomenon, Microcystis, Nodularia, Nostoc* and *Oscillatoria* are common toxic algae in eutrophic and hypereutrophic water bodies all over the world (a bloom can consist of one or mix of two or more genera of cyanobacteria). Other genera such as *Coelosporium, Cylindrospermum, Fischerella, Gloeotrichia, Gomphosphaeria, Hapalosiphon, Microcoleus, Schizothrix, Scytonema, Tolypothrix* and *Trichodesmium* are also known to produce toxic substances in many parts of the world (Scott, 1991; Carpenter and Carmichael, 1995). These microorganisms not only affect taste, odor and appearance of the water but also create severe problems in the management of safer aquatic environment. Most investigations of cyanobacterial toxicity have been stimulated by the occurrence of poisoning and death incidents affecting agricultural livestock, wild animals, fishes, birds etc. (Codd and Poon, 1988).

The distribution of toxic algae was also found in the reservoirs in Thailand. For example, the dominant species in the water blooms collected from two reservoirs (Kang Krachan dam and Mae Kwang dam) and a pond (a duck husbandary pond in Bangkok), were *Microcystis aeruginosa* or Pleurocapsalean filamentous cyanobacterium (Mahakhant *et al*., 1998). Toxic cyanobacteria represent a serious problem in freshwater reservoirs in northern Thailand where all these reservoirs are contaminated with toxic cyanobacteria, *M. aeruginosa* Kutz. (Peerapornpisal *et al*., 2002) as dominate in Mae Kuang Udomtara reservoir, Chaing Mai, Thailand (Peerapornpisal *et al*., 1999; Pekkoh *et al*., 2003; Vijaranakorn *et al*., 2004). Whilst in Kwan Phayao, Phayao, Thailand, *M. aeruginosa* and *M. wesenbergii* were two

dominant species in April 1999, however *Cylindrospermopsis raciborskii* was the dominant specie in September 2000 (Prommana *et al*., 2003).

2.2.1 Types and nature of toxins

Cyanobacteria produce toxins as a product of secondary metabolism that are toxic to animal and human when consume the contaminated water or food that contain toxin. The toxins that have been intensively studied to date basically belong to one of the two groups; (i) those which interfere with the functioning of neuromuscular system of the animals and often result in paralysis and very fast death are "neurotoxins" and (ii) cyanobacterial poisons, commonly known as "hepatotoxins" which damage the liver and cause animal death due to hypovolemic shock and excessive blood pooling in liver.

(A) Neurotoxin

Mass occurrences of neurotoxic cyanobacteria have been reported from North America, Europe and Australia, where they have caused animal poisonings (www, 2006). Neurotoxins are commonly produced by the species and strains of *Anabaena* (Carmichael *et al*., 1990) and *Aphanizomenon* (Mahmood and Carmichael, 1986). Certain species of *Oscillatoria* such as *O. agardhii, O. accutissima, O. formosa* and *Trichodesmium thiebautii* Gomont ex Gomont (Carpenter and Carmichael, 1995) are occasionally reported to be responsible for neurotoxicosis. Neurotoxins generally target neuromuscular system, paralyzing skeletal and respiratory muscles and cause death within minutes. The neurotoxins are called B-N-methyamino-L-alanine or BMAA.

Some of the most extreme cases of cyanobacterial poisoning have been attributed to the blooms of *Anabaena flos-aquae* (Carmichael, 1992a), the strains of which produce anatoxins (antx). The symptoms produce in mice, rat and chick were defined as six anatoxins {antx-a, -a(s), -b, -b(s), -c and –d} (Carmichael and Bent, 1981). Of these, antx-a and antx-a(s) seem unique to cyanobacteria and have been studied in detail. Antx-a (Fig. 2.2A), which mimic the effect of acetyl choline, formerly called as "very fast death factor" (VFDF) is a bicyclic secondary amine of alkaloid origin (Carmichael *et al*., 1990). Anatoxin-a is a low molecular weight alkaloid (MW = 165), a secondary amine, 2-acetyl-9-azabicyclo (4-2-1) non-2-ene (Fig. 2.2A) (Devlin *et al*., 1977). Anatoxin-a is produced by *A. flos-aquae, Anabaena* spp. *(flos-aquae-lemmermannii* group), *A. planktonica, Oscillatoria, Aphanizomenon* and *Cylindrospermum*. Homoanatoxin-a (MW = 179) is an anatoxin-a homologue isolated from an *O. formosa (Phormidium formosum)* strain. It has a propionyl group at C-2 instead of the acetyl group in anatoxin-a (Skulberg *et al*., 1992). The LD_{50} (lethal dose resulting in 50 percent deaths) of anatoxin-a and homoanatoxin-a are 200 - 250 µg/kg body wt. (Devlin *et al*., 1977; Carmichael *et al*., 1990; Skulberg *et al*., 1992). It has been isolated from *A. flos-aquae* NRC44-1 and is a potent post-synaptic neuromuscular blocker (Carmichael *et al*., 1979, Aronstam and Witcop, 1981) with a significant LD_{50} (i.p. mouse) of 200 µg/kg body wt.). Due to the high degree of toxicity, it is speculated that the animals need to ingest only a few millilitres of toxic surface water bloom to receive a lethal dose (Carmichael and Gorham, 1980).

Antx-a(s) (Fig. 2.2B), which is an anticholinesterase, a guanidine methy phosphate ester (MW = 252) has been isolated from *A. flos-aquae* NRC525-17 (Carmichael and Gorham, 1980) and is pharmacologically and chemically distinct

from antx-a. It has more recently been identified in blooms and isolated strains of *A. lemmermannii* (Henriksen *et al*., 1997; Onodera *et al*., 1997). In test animals antx-a(s) produced hypersalivation, lacrymation (discharge of tears), chromodacryorrhoea and urinary defection that differentiate it from antx-a. Antx-a(s) has a median lethal dose (LD_{50}, i.p. mouse) of 20 μg/kg body wt. with a survival time of 30-60 minutes. Of the other members of this group, only antx-b and antx-d have gross neuromuscular activity (Carmichael and Bent, 1981).

Structurally unique saxitoxin and neosaxitoxin (Fig. 2.2C), produced by the strains of *Ap. flos-aquae* NH-1 and NH-5 (Mahmood and Carmichael, 1986) are generally termed as "aphantoxins". Strains of *A. circinalis* and *Lyngbya wollei* are also known to produce these toxins (Carmichael, 1995). They are better known as red tide "paralytic shellfish poison" (PSP), which block nerve cell sodium channels, originally found in dinoflagellates. They are even more lethal (LD_{50}, i.p. mouse 10 μg/kg body wt.) than antx. They occurrence of these very peculiar compounds in both prokaryotes and eukaryotres suggests their evolutionary trend (Shimizu, 1996).

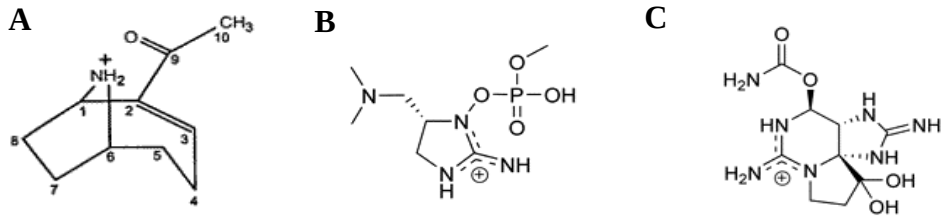

Figure 2.2 Structure of neurotoxins: A; anatoxin-a (www, 2006), B; anatoxin-a(s) (www, 2006) and C; saxitoxin (www, 2006).

Other neurotoxic cyanobacteria, in marine *Trichodesmium* blooms from the Virgin Islands, a neurotoxic factor has been reported which was not anatoxin-a or anatoxin-a(S) but remains to be characterized (Hawser *et al*., 1991).

(B) Hepatotoxins

Hepatotoxins constitute a family of at least 53 related cyclic or ring peptides (Carmichael, 1994). Those consisting of seven amino acids are microcystins (MCs), the name derived from the most common producer *Microcystis aeruginosa* (Carmichael, 1992a). Others consisting of five amino acids are pentapeptide "nodularin", originally isolated from *Nodularia spumigena* (Sivonen *et al*., 1989a). The cyclic peptides are comparatively large natural products, molecular weight (MW) ≥800-1,100, although small compared with many other cell oligopeptides and polypeptides (proteins) (MW >10,000). Both groups exhibit similar biological activity but differ in the number and type of the amino acids that makeup their cyclic skeleton. They contain either five (nodularins) or seven (microcystins) amino acids, with the two terminal amino acids of the linear peptide being condensed (joined) to form a cyclic compound. They are water soluble and, except perhaps for a few somewhat more hydrophobic microcystins, are unable to penetrate directly the lipid membranes of animal, plant and bacterial cells. Therefore, to elicit their toxic effect, uptake into cells occurs through membrane transporters which otherwise carry essential biochemicals or nutrients. The target organ range in mammals is largely to the liver. In aquatic environments, these toxins usually remain contained within the cyanobacterial cells and are only released in substantial amounts on cell lysis. Along with their high chemical stability and their water solubility, this containment has important implications for their environmental persistence and exposure to humans in surface water bodies. The first report that hepatotoxins are involved in the liver toxicosis was by Bishop and co-workers (1959) who isolated the "fast death factor" from *M. aeruginosa* NRC-1 (SS-17). The toxin was later termed microcystin (MCs) and

subsequent isolations were made from other species and strains of *Microcystis* and the blooms of *M. aeruginosa* in Canada, USA., South Africa, Australia, USSR, Finland, Norway, England, Sweden, Germany, Japan, Bangladesh, India etc. (Carmichael, 1992a and Thakur, 1996). Later on, different degree of toxicity exhibited by *Microcystis* isolates and their hydrolysates confirmed the presence of many MC variants that differ mainly in their amino acid composition. However, a single strain was found capable of producing more than one MCs (Carmichael, 1994). Moreover, Microcystins can be produced by *Anabaena, Anabaenopsis* species, *Nostoc, Oscillatoria* and from terrestrial *Hapalosiphon* genera (Carmichael, 1996). Microcystins (Fig. 2.3A) are characterized as monocyclic heptapeptide having the general structure cyclo-(D-Ala^{1}–X^{2}–D-MeAsp^{3}–Z^{4}–Adda^{5}–D-Glu^{6}–Mdha^{7}) where X and Z are variable L-amino acids, D-MeAsp is D-erythro-β-methylaspatic acid, Mdha is N-methyldehydro-alanine and Adda is 3–amino-9-methoxy-10–phenyl-2, 3, 8-trimethyl-deca-4,6-dienoic. Adda is the most remarkable structural feature of these toxins and is important in the toxicity of these compounds (Krishnamurthy *et al*., 1986). The molecular weight of MCs varies from 909 to 1067 depending upon the variable amino acids present such as MCLR (leucine-arginine), MCYR (tyrosine-arginine), MCYA (tyrosine-alanine) and MCYM (tyrosine-methionine) etc. Although of less common occurrence, nodularin (Fig. 2.3B) has been found to produce by *N. spumigena* in brackish water habitats (Sivonen *et al*., 1989a). The pentapeptide hepatotoxin is cyclo-(D-MeAsp^{1}-L-arginine^{2}-Adda^{3}-D-glutamate^{4}-Mdhb^{5}), in which Mdhb is 2-(methylamino)-2-dehydrobutyric acid (Carmichael *et al*., 1990). A few naturally occurring variations of nodularins have been found: two demethylated variants, one with D-Asp^{1} instead of D-MeAsp^{1}, the other with DMAdda^{3} instead of

Adda[3]; and the non-toxic nodularin which has the 6Z-stereoisomer of Adda[3] (Namikoshi *et al*., 1994). The equivalent 6Z-Adda[3] stereoisomer of microcystins is also non-toxic.

Cylindrospermopsin (Fig. 2.3C), a unique alkaloid hepatotoxin has been isolated from *Cylindrospermopsis raciborskii* (Hawkins *et al*., 1985, 1997), *Umezakia natans* (Harada *et al*., 1994) and *Ap. ovalisporum* (Banker *et al*., 1997). In pure form, cylindrospermopsin mainly affects the liver, although crude extracts of *C. raciborskii* injected or given orally to mice also induce pathological symptoms in the kidneys, spleen, thymus and heart. Pure cylindrospermopsin has an LD_{50} in mice (i.p.) of 2.1 mg/kg body wt. at 24 h and 0.2 mg/kg body wt. at 5-6 days (Ohtani *et al*., 1992). Recently, new structural variants of cylindrospermopsin have been isolated from an Australian strain of *C. raciborskii,* with one being identified as demethoxy-cylindrospermopsin (Chiswell *et al*., 1999). The toxin processes a sulfate ester and hydroxylmethyluracil moiety and unlike MC and nodularin, it showed congestion in kidney and heart along with hepatic necrosis.

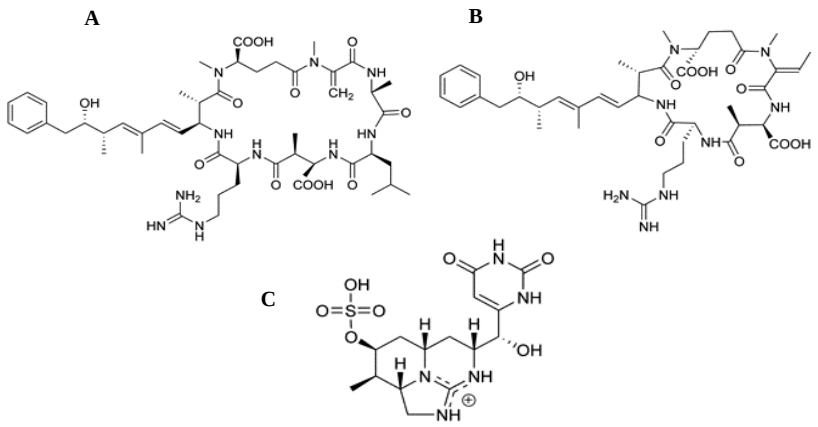

Figure 2.3 Structure of hepatotoxins: A; microcystin-LR, B; nodularin and C; cylindrospermopsin (www, 2006).

The primary toxicosis that results from ingesting cyanobaterial toxin contaminated water in general include illness, gastroentritis, dermatological and respiratory problems and allergic reaction (Carmichael, 1982, 1992a). The gross pathological and clinical effects that have been observed in the laboratory with test organisms and their LD_{50} values are listed in Table 2.2.

Table 2.2. Clinical symtoms produced by cyanobacterial toxins and their median lethal dose (LD_{50}) (Carmichael, 1994, Harada *et al*., 1994 and www, 2001).

Toxin-producing cyanobacteria	Types of toxins	Symptoms	LD_{50}* (μg/kg body wt.)	Occurrence in freshwater
Neurotoxin				
Anabaena flos-aquae	Anatoxin-a	muscular fasciculation, decreased movement, collapse, cyanosis, convulsion, death	200	not common
Anabaena flos-aquae	Anatoxin-a(s)	hypersalivation, mucoid nasal, tremors, diarrhea, cyanosis death	20	not common
Aphanizomenon floa-aquae, A.circinalis	Saxitoxin and Neosaxitoxin	irregular breathing, spasm, gasping, loss of coordination; tremors, death	10	not common

Table 2.2. (continued)

Toxin-producing cyanobacteria	Types of toxins	Symptoms	LD_{50}* (μg/kg body wt.)	Occurrence in freshwater
Hepatotoxins				
Microcystis aeruginosa, M. viridis, M. wesenbergii , Nostoc sp., *Oscillatoria sp.*	Microcystins	slow movement, increase in liver weight, hypovolemic shock, intrahepatic haemorrhage, death	50-100	common
Nodularia spumigena	Nodularin	-do-	30-50	not common
Cylindrospermopsis raciborskii, Umezakia natans	Cylindro-spermopsin	liver swelling, hepatic necrosis, congestion in kidney and heart, death	ND	not common

*Toxin was injected intraperitoneally (i.p.) of mice or rats

ND = Not determined

2.2.2 Mode of action

In general neurotoxins especially antx-a bring about the effect through acetylcholine and its related enzyme acetylcholinesterase. Effects of neurotoxins through electrochemical ion gradient interruption have also been reported (Carmichael, 1994). Electrochemical ion gradients, especially Na^+, K^+ or Ca^{++} across the cell membrane provide the basis for the propagation of the impulse. Stx and neo-stx are competitive inhibitors of voltage gated Na^+ channel and prevent the influx of Na^+ into the axon. It thus disrupts the communication between neurons and muscle cells by preventing acetylcholine to be released at the neuromuscular junction (Carmichael, 1994). These neurotoxins are selective in binding the Na^+ channel only and do not affect the flow of K^+ and the resting potential of the membrane (Gorham and Carmichael, 1988). The binding of acetylcholine with the acetylcholine receptor protein triggers a conformational change in the receptor that allows the signal propagation.

Antx-a(s) exerts its physiological effects by inhibiting the enzyme acetylcholinesterase (Hyde and Carmichael, 1991). The enzyme is secreted by the muscle cell and is located in the synaptic cleft. It is a serinesterase with a nucleophilic serine residue as the active site. The unique feature of the antx-a (s) lies in its structural fit with the enzyme acetylcholinesterase. The bimolecular reaction occurs initially with the formation of enzyme-atnx-a (s)-complex that results in the phosphorelation of the enzyme. The enzyme becomes unable to degrade acetylcholine which remains continuously available to stimulate and overstimulate muscle cell (Carmichael, 1994).

Alike neurotoxins a number of possible ways of action of hepatotoxins have been reported (Carmichael, 1992a, 1994). In all the cases the effects is confined to liver. Pharmacodynamic studies suggest that the liver organotropism is associated with a selective transport system present only in the hepatocytes namely the multispecific transport for bile acids (Runnegar *et al*., 1991). When ingested orally, the toxin is preferentially absorbed across the ilium and its transported to the hepatocytes via bile acid carries (Eriksson *et al*., 1990) and results in hepatic necrosis (Ito *et al*., 1997). In the liver, both hepatocytes and endothelial cells are destroyed with extensive fragmentation and vesciculation of the cell membrane (Gorham and Carmichael, 1988). Due to the disruption, the cells forming sinusoidal capillaries of the liver separate and blood carried by the vessels seep and accumulate into the liver cells causing haemorrhagic shock (Carmichael, 1992a, 1994).

Naseem *et al*. (1990) proposed an action mechanism through MCLR induced prostaglandin synthesis. MCLR like other toxins of the inflammatory cascade is likely to activate phospharylase-A2 to release arachidonic acid, a fatty acid precursor of prostaglandin and other immunomediators, which utimately cause inflammation and membrane damage by activating protease enzymes.

According to Hayakawa and Kohama (1995) the MYLR inhibits the ATP-dependent interaction between myosin and actin of the skeletal muscles. The toxin exerts its effect by directly activating or inhibiting the contractile elements after passage through the cell membrane and interacts with the ATPase active sites located within heads of myosin. The unique characteristic of this binding lies in the reversible nature of the effects of okadaic acid and MC.

The most widely accepted mode of action of the MC is via the interaction with the enzymes protein phosphatases PP1 and 2A (Toivola *et al*., 1994). The mammalian toxicity of microcystins and nodularins is mediated through their strong binding to key cellular enzymes called protein phosphatases. In solution, microcystins and nodularins adopt a chemical "shape" that is similar, especially in the Adda-glutamate part of the cyanotoxin molecule (Rudolph-Böhner *et al*., 1994; Annila *et al*., 1996). This region is crucial for interaction with the protein phosphatase protein molecule, and hence it is crucial for the toxicity of these cyanotoxins (Barford and Keller, 1994; Goldberg *et al*., 1995). Microcystins show an additional characteristic of forming a covalent bond between the Mdha residue and the protein phosphatase molecule. The enzymes in concert with protein kinases regulate organelle rearrangement, microfilament reorganization and appearance of surface protrusions in hepatocytes (Carmichael, 1994). In isolated hepatocytes, MCs are known to induce overall phosphorylation of the cytosolic and cytoskeletal proteins (Eriksson *et al*., 1990) which ultimately disrupt the cytoskeletal organization and result in rapid dissociation of the liver morphology (Toivola *et al*., 1994). Similarly, in reticulocytes, the toxin inhibited iron-uptake by blocking the internalization of transferrin by the receptor mediated endocytosis of ferrotransferrin (Runnergar and Falconer, 1986).

Protein kinases and protein phosphatases play a major part in regulating cell division. Protein kinases promote movement of cells through the cell division cycle whereas protein phosphatases play role in checking the cell division by arresting the activity of regulators. Inactivation of protein phosphatases by hepatotoxins disturb the normal balance, resulting in cell proliferation and cancer production. MCYSRs are reported to be the prominent tumor promotors both *in vitro* and *in vivo* (Toivola *et al*.,

1994). Of the two enzymes, PP1 has a major role in the maintenance of cytoskeletal network, the apparent inhibition of which produces liver toxicosis. Inhibition of the other enzymes, PP2A results in tumor promotion (Toivola *et al*., 1994). However, linear microcystins and nodularin are more than 100 times less toxic than the equivalent cyclic compounds. The linear microcystins are thought to be microcystin precursors and/or bacterial breakdown products (Choi *et al*., 1993; Rinehart *et al*., 1994; Bourne *et al*., 1996).

2.2.3 Occurrence of cyanotoxins

It is important to note that mass occurrences of toxic cyanobacteria are not always associated with human activities causing pollution or "cultural eutrophication". For example, massive blooms of toxic cyanobacteria have been reported in Australian reservoirs with pristine or near-pristine catchments (watersheds), and toxic benthic cyanobacteria have killed cattle drinking from oligotrophic, high-alpine waters in Switzerland.

Cyanobacterial populations may be dominated by a single species or be composed of a variety of species, some of which may not be toxic. Even within a single-species bloom there may be a mixture of toxic and non-toxic strains. A strain is a specific genetic subgroup within a particular species, and each species may encompass tens or hundreds of strains, each with slightly different traits. Some strains are much more toxic than others, sometimes by more than three orders of magnitude. This can mean that one highly toxic strain, even when occurring in minor amounts amongst larger numbers of non-toxic strains, may render a bloom sample toxic (Sivonen *et al*., 1989a, 1989b; Bolch *et al*., 1997; Vezie *et al*., 1998).

Microcystis sp., commonly *M. aeruginosa,* is linked most frequently to hepatotoxic blooms world-wide. *M. viridis* and *M. botrys* strains also have been shown to produce microcystins. Microcystin-producing *Anabaena* sp. has been reported from Canada, Denmark, Finland, France and Norway. A recent study from Egypt revealed that 25 percent of 75 *Anabaena* and *Nostoc* strains isolated from soil, rice fields and water bodies contained microcystins. *Planktothrix agardhii* and *P. rubescens* (previously called *Oscillatoria agardhii* and *O. rubescens)* are common microcystin producers in the Northern Hemisphere; toxic strains of these have been isolated from blooms in Denmark, Finland and Norway. In addition, these species were frequently shown to be dominant in microcystin containing blooms in China, in Germany and in Sweden. In Swiss alpine lakes, *Oscillatoria limosa,* which is benthic (i.e., it grows attached to sediments and rocks), is a microcystin producer. In spite of the widespread occurrence of cyanobacterial blooms in Australia, *Planktothrix* blooms are rare there. This may be due to the higher temperature and tendency for elevated clay-derived turbidity in Australian water bodies. *N. rivulare* blooms in Texas, USA have caused poisoning of domestic and wild animals (Davidson, 1959) and, more recently, two unidentified *Nostoc* strains were shown to produce microcystins.

2.2.4 Seasonal variations in bloom toxin concentration

The timing and duration of the bloom season of cyanobacteria depends largely on the climatic conditions of the region. In temperate zones, mass occurrences of cyanobacteria are most prominent during the late summer and early autumn and may last 2-4 months. In regions with more Mediterranean or subtropical climates, the bloom season may start earlier and persist longer. In shallow lakes, particularly in

north-western Europe, populations of *P. agardhii (O. agardhii)* may prevail perennially for many years. In deeper, thermally stratified lakes and reservoirs with moderate nutrient pollution, *P. rubescens (O. rubescens)* may form blooms at the interface between the warmer upper and colder deeper layers of water during summer, but maintain high, evenly distributed density throughout the entire water body during winter. Both *Planktothrix* species may contain high amounts of microcystins. Blooms of cyanobacteria, especially *P. agardhii,* have been found in winter under ice in Scandinavian and German lakes and can thus be an all year round problem.

Although toxic cyanobacteria occur in a large number of lakes, reservoirs and rivers in the world, quantitative reports on seasonal variation of cyanobacterial species composition and toxin concentration are rare. Only a few studies on seasonal, spatial and diel (day to night) variations in lakes have been published. Carmichael and Gorham (1981) showed a high degree of spatial variation of bloom toxicity that was due mostly to variations in the relative amounts of toxic *M. aeruginosa* throughout the lake, rather than to substantial variations in cell toxin content. Other measurements of toxin concentrations in lakes have revealed similar trends; samples taken at the same time from different parts of the lake may show wide divergence in cyanotoxin content (Ekman-Ekebom *et al*., 1992; Kotak *et al*., 1995; Vezie *et al*., 1998). A study in Alberta, Canada, showed considerable variation in toxin concentrations among the three lakes studied, both within and between years, even though the lakes were located within the same climatic region (Kotak *et al*., 1995).

In any year or season, individual water bodies have their own populations of cyanobacteria and algae, the dominance of which is dependent not only on the weather, but on the specific geochemical conditions of the lake. If there are no major changes

in these conditions, toxic blooms are likely to recur annually in those lakes that have a history of toxic blooms (Wicks and Thiel, 1990; Ekman-Ekebom *et al*., 1992). Certain species, including the highly toxic *P. agardhii* and *P. rubescens*, are known to produce maximum mass occurrences deep in the water column and which may be overlooked by surface monitoring of waters. Such situations may also cause problems for water treatment (Lindholm and Meriluoto, 1991).

Studies over prolonged periods usually show that toxin concentration per gram dry weight may vary substantially over a time scale of weeks to months, but rarely from day to day as is sometimes reported. The maximum toxin concentration per gram dry weight is usually reported in summer or autumn, when cyanobacterial biomass dominates dry matter (Wicks and Thiel, 1990; Watanabe *et al*., 1992; Park *et al*., 1993; Kotak *et al*., 1995; Maršálek *et al*., 1995; Vezie *et al*., 1998). However, the time of toxin concentration maximum and biomass maximum are not necessarily coincident. Thus, there can be significant variation in the amount of toxin per mass of cyanobacteria over time, independently of changes in the size of the cyanobacterial population. The explanations for this are twofold. Firstly, there may be a waxing and waning of species or strains of quite different toxin quotas (i.e. toxin content per cell). Secondly, the toxin quotas may change up to five-fold in response to changes in environmental conditions. A study by Kotak *et al*. (1995) found substantially higher concentrations of microcystin in blooms during the day than at night, whereas a study from Australia found no variation in microcystin content when samples were incubated during 24 hours at different depths in a reservoir (Jones and Falconer, 1994). Both findings need to be explored further.

High regional, seasonal, spatial, temporal and diel variations of toxin concentrations indicate that predicting or modeling the occurrence of toxin concentrations requires a comprehensive understanding of population (strain) development in different types of aquatic ecosystems, as well as of the variability of their toxin quotas. Data bases for such predictive models have yet to be compiled.

2.2.5 Biosynthesis

Biosynthesis of several cyanotoxins has been studied by feeding labelled precursors to a cyanobacterial culture and following their incorporation into the carbon skeleton of the toxins. Shimizu *et al*. (1984) used an *Ap. flos-aquae* strain to study biosynthesis of saxitoxin analogues. The condensation of an acetate unit, or its derivative, to the amino group bearing an a-carbon of arginine or an equivalent, and a subsequent loss of the carboxyl carbon and imidazole ring formation on the adjacent carbonyl carbon.

Anatoxin-a analogous to that of tropanes. Anatoxin-a was proposed to be formed from ornithine/arginine via putrescine, which is oxidised to pyrroline, a precursor of anatoxin-a.

The origin of carbons in microcystin (Moore *et al*., 1991) and in nodularin (Choi *et al*., 1993; Rinehart *et al*., 1994) have been studied by following the incorporation of labelled precursors into the toxins by NMR. Carbons C1-C8 of Adda in nodularin are acetate derived and the remaining carbons presumably originate from phenylalanine. Methyl groups in carbons 2, 4, 6, 8, and the O-methyl group in the Adda unit, originated from methionine. The D-Glu and L-Arg carbons C4-C5 were acetate derived, with C1-C2 being from glutamate. Methyldehydrobutyrine was

possibly formed from threonine, its methyl group coming from methionine. The b-methylaspartic acid was found to originate from condensation of pyruvic acid (C3-C4) and acetyl-CoA (C1-C2) (Rinehart *et al*., 1994). The studies on the carbon skeleton of nodularin, with some minor differences, agree with work on microcystin-LR by Moore *et al*. (1991). In their study, L-Leu and D-Ala units in microcystin had acetate incorporation. The dehydroamino acid in microcystin has been proposed to be formed from serine rather than from threonine (Rinehart *et al*., 1994). Rinehart's group found linear nodularin, which was shown by culture experiments to be a precursor of cyclic nodularin. Three additional linear peptides were isolated from a bloom sample, one of them was possibly a precursor of cyclic microcystin-LR and the others possibly degradation products (Rinehart *et al*., 1994).

2.2.6 Impact on aquatic biota

Direct cyanobacterial poisoning of animals can occur by two routes: through consumption of cyanobacterial cells from the water, or indirectly through consumption of other animals that have themselves fed on cyanobacteria and accumulated cyanotoxins. Cyanotoxins are known to bioaccumulate in common aquatic vertebrates and invertebrates, including fish, mussels and zooplankton. Consequently, there is considerable potential for toxic effects to be magnified in aquatic food chains. Such toxicity biomagnification is well known for anthropogenic pollutants such as heavy metals and pesticides. There is no reason to suspect that the situation would be any different with natural cyanotoxins.

It is difficult to ascribe the deaths of natural populations of aquatic animals, especially fish, unequivocally to cyanotoxin poisoning. One of the main reasons for

this is because the collapse of a large cyanobacterial bloom can lead to very low concentrations of oxygen in the water column as a consequence of bacterial metabolism; consequent fish deaths may be due to the anoxia.

2.2.6.1 Effects on aquatic bacteria

The influence of cyanobacterial toxins on bacteria is not fully understood and the scientific literature gives a number of contradictory statements. It is quite possible that cyanotoxins impact on some species of aquatic bacteria and not others. Certainly, microcystins are not toxic to all bacteria because several species are known to degrade quite high concentrations of these toxins. It is even possible that the slow release of cyanotoxins from the cell surface or from senescent cells may stimulate associations of particular bacterial types which may even act as symbionts.

Attempts have been made to use bacterial toxicity tests (based on inhibition of bacterial phosphorescence) to screen for the presence of cyanotoxins, especially microcystins. However it appears that the inhibition of bacterial phosphorescence is not related to the commonly known cyanotoxins. It has been suggested that the negative effect may be related to the presence of unidentified LPS endotoxins in the cell wall of the cyanobacterial cells.

2.2.6.2 Effects on zooplankton

There is dramatic variation among zooplankton species in their response to toxic (and even non-toxic) cyanobacteria. For example, DeMott *et al*. (1991) showed that the four species of zooplankton differed in their sensitivity to hepatotoxins by almost two orders of magnitude, but toxicity was observed only at very high

concentrations that are scarcely encountered in natural water bodies (48 h LD_{50} ranging from 450 to 21,400 µg of microcystin/l). Snell (1980) found that there was a genotype-dependent response of the rotifer *Asplanchna girodi* to toxic *A. flos-aquae* and *Lyngbya* sp. Hietala *et al*. (1997) observed a variation in susceptibility of more than three orders of magnitude in the acute toxicity of *M. aeruginosa* to 10 clones of *D. pulex*. Both DeMott *et al*. (1991) and Laurén-Määttä *et al*. (1997) suggested that clone and species differences between zooplankton susceptibilities to toxic cyanobacteria may lead to selection pressures in favour of resistant strains or species in water bodies where toxic cyanobacteria occur frequently.

Benndorf and Henning (1989) found that the toxicity of a field population of *Microcystis* was increased by the feeding activity of *D. galeata* over a period of a few months. A possible explanation for this phenomenon is offered by DeMott *et al*. (1991) who demonstrated that a number of zooplankton species will avoid grazing on toxic cyanobacteria, but continue to graze on non-toxic species. Similar results have also been shown for grazing by the phytoplanktivorous fish *Tilapia* and silver carp. Thus, grazing pressure from zooplankton and some fish may lead to the selective enrichment of toxic cyanobacterial strains over time.

It is likely that under natural conditions in water bodies, certain species and strains of zooplankton may be affected by cyanotoxins, whereas others will be unaffected. As such, cyanotoxins may influence the zooplankton community structure, especially during times when cyanobacteria are dominant within the phytoplankton.

2.2.6.3 Effects on fish

If fish are dosed with cyanotoxins by i.p. injections or by force-feeding, they develop similar symptoms of intoxication as laboratory mammals. The question relevant for field exposure is whether cyanotoxins enter healthy fish. For example, Tencalla and teams (1994) showed that gastrointestinal uptake by gavage (force-feeding) caused massive hepatic necrosis followed by fish deaths, whereas immersion of adults and juveniles in contaminated water did not cause toxic effects. Other reported evidence suggests that immersion in toxic cyanobacteria or cyanotoxins may be harmful to fish. Differences in sensitivity may be pronounced between species: goldfish were found to be nearly 30 times less susceptible to i.p. microcystin than mice (Sugaya *et al*., 1990). Release of toxic compounds from mass developments of cyanobacteria was considered to be the cause of fish kills by Penaloza *et al*. (1990). Histopathological investigations offish deaths during cyanobacterial blooms in the UK, indicated that the cause of death was mostly due to damage of the gills, digestive tract and liver (Rodger *et al*., 1994). The gill damage was probably caused by the high pH induced by cyanobacterial photosynthesis activity prior to the bloom collapse, together with the higher level of ammonia arising from the decomposition of the cyanobacteria. However, gill damage may have enhanced microcystin uptake and thus led to liver necrosis. Damage to gills by dissolved microcystin-LR has been shown experimentally in *Tilapia* and trout (Garcia, 1989; Gaete *et al*., 1994; Bury *et al*., 1996).

Other pathological symptoms ascribed to toxic cyanobacterial blooms include damage to the liver, heart, kidney, gills, skin and spleen (Garcia, 1989; Råbergh *et al*., 1991). Garcia (1989) and Rodger *et al*. (1994) carried out experiments on trout, while

Råbergh *et al*. (1991) experimented on carp. The latter study highlighted degenerative changes in kidney tubules and glomeruli. The effect of microcystins on European carp, *Cyprinus carpio,* under natural field conditions in Australia has been described by Carbis *et al*. (1997) as atrophy of hepatocytes, gills with pinpoint necrosis, epithelial ballooning, folded lamellar tips, exfoliation of the lamellar epithelium, elevated asparate aminotransferase activity and serum bilirubin concentrations. Laboratory studies indicate that dissolved microcystins may affect fish embryos (Oberemm *et al*., 1997) and behaviour of fish (Baganz *et al*., 1998).

The most definitive effect of microcystin on fish concerns Atlantic Salmon reared in net pens in coastal waters of British Columbia and Washington State, USA. As yet unidentified microcystin-producing organisms produce a progressive degeneration of the liver in salmon smolts placed into open-water net pens (Anderson *et al*., 1993). The disease, referred to as Net Pen Liver Disease (NPLD), has resulted in significant economic losses for the mariculture industry.

2.3 Review of Literatures

2.3.1 Microcystins

It is still unknown why some cyanobacteria produce microcystin. Cyanobacteria produce toxins as a product of secondary metabolism which are toxic to animal and human when consume the contaminated water or food that contain toxin. However, current research is focused on whether microcystin is a primary metabilote, participating in as yet unknown essential metabolic processes inside the cell or a secondary metabolite. Orr and Jones (1998) were the first to postulate that microcystin

is a primary metabolite. They studied the relationship between the specific cell division rate and the specific microcystin production rate in batch cultures of *M. aeruginosa* (MASH01-A19) under fixed light and nitrogen-limited conditions. They found a significant relationship between the specific cell division rate and the specific microcystin production rate. Based on the re-evaluation of batch culture data presented by others concluded 'there is a direct linear correlation between cell division and microcystin production rates in all microcystin-producing cyanobacteria regardless of the environmental factor that is limiting cell division' (Orr and Jones, 1998). Stanier *et al.* (1987) and Carmichael (1992b) stated that 'synthesis of bacterial secondary metabolites is triggered as growth ceases' and on the basis of this older definition. Orr and Jones (1998) concluded that microcystin is not a secondary metabolite as it displays many of the attributes of essential intracellular nitrogenous compounds. A coupling of microcystin production to cell division does not mean that microcystin is constitutively produced, as cell division is regulated by internal stimuli contrary to constitutuively produced metabolites. Similar, Lyck (2004) reported that cell produced microcystin at rates approximately those needed to replace losses to daughter cells during division and that microcystin was produced in a similar way to protein and chlorophyll, indicating a constitutive microcystin production.

2.3.1.1 Structure and variation

MCs are small monocyclic peptides composed of seven amino acids (Fig. 2.4) and posses the generalized structure, cyclo (-D-Ala-X-D-MeAsp-Y-Adda-D-Glu-Mdha-), where D-Ala and D-Glu are alanine and glutamic acid (in the D configuration), respectively, D-MeAsp is D-erythro-β-methylaspartic acid and Mdha is

N-methyldehydroalanine (Botes *et al*., 1982, 1984). Adda is unique to cyanobacteria and has the structural formula (2S,3S,8S,9S)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid (Botes *et al*., 1984; Rinehart *et al*., 1988). At present, more than 70 structural variants of MCs have been identified (Zurawell *et al*., 2005). In addition, substitutions of variable L-amino acids at position 2 and 4 give rise at least 21 known primary MC analogues (Botes *et al*., 1985). For example, MCLR has leucine (L) and arginine (R) at these positions and is one of the most common forms (Carmichael, 1992a; Kotak *et al*., 1993; Sivonen and Jones, 1999). Alterations in other constituent amino acids result in numerous additional analogues. For example, demethylation of D-MeAsp and/or Mdha at positions 3 and 7, respectively, yields 15 demethylated variants of primary analogues. In addition, alterations including methylesterification of D-Glu (6) or substitution (modification) of Mdha (7), with/without demethylation of D-MeAsp (3), yield another 25. Finally, modifications in Adda (5), including geometric isomerization at the C-7 position and demethylation or substitution of the methoxyl group with an acetoxyl group at the C-9 position, with/without variation in amino acids 1, 3 and/or 7, give rise to additional 12 analogues. These variants are produced nonribosomally by a multifunctional enzyme complex using a thio-template mechanism (Arment and Carmichael, 1996; Dittmann *et al*., 1997).

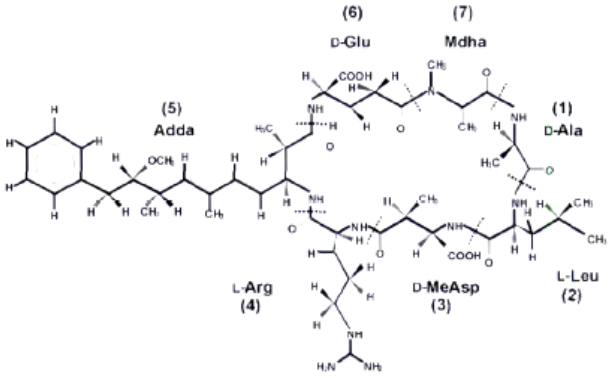

Figure 2.4 Chemical structure of microcystin-LR: Position (1) is D-alanine; (2) is L-Leucine; (3) is D-erythro-β-methylaspartic acid; (4) is L-Arginine; (5) is Adda; (6) is D-glutamic acid and (7) is N-methyldehydroalanine (Zurawell *et al*., 2005).

2.3.1.2 Microcystin synthetase gene cluster

A mixed polyketide synthase (PKS)/nonribosomal peptide synthetase (NRPS) called microcystin synthetase, are suggested to be origin for MCs (Moore *et al*., 1991; Dittmann *et al*., 1997; Arment and Carmichael, 1996) (Fig. 2.5). Both groups of metabolites are biosynthesized by large, multifunctional protein complexes that are organized into coordinate clusters of enzymatic sites termed modules, in which each module is responsible for one cycle of polyketide or polypeptide chain elongation (Cane *et al*., 1998; Marahiel *et al*., 1997; von Dohren *et al*., 1999). A large 55 kb gene cluster from *M. aeruginosa* PCC7806 responsible for the biosynthesis of microcystin-LR. Sequence analysis of the *mcy* region revealed a bidirectional operonic structure (Fig. 2.6). The large of the two putative operons (*mcy*D-J) encodes the PKS-/NRPS modules catalyzing the formation of the pentaketide-derived β-amino acid Adda and its linkage to D-glutamate, while the smaller (*mcy*A-C) encodes the NRPS modules for

the extension of this dipeptidyl intermediate to the heptapeptidyl step and subsequent peptide cyclization (Tillett *et al*., 2000).

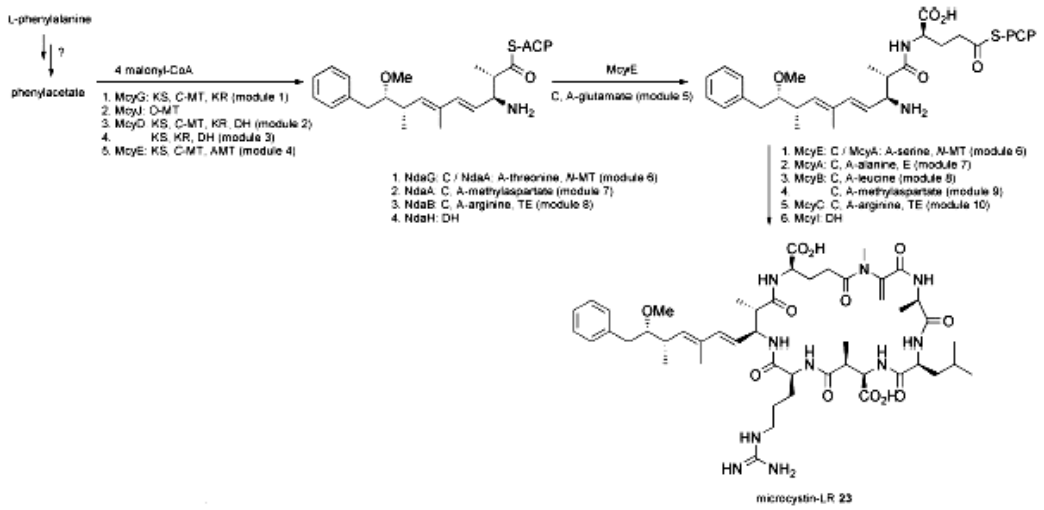

Figure 2.5 Proposed biosynthesis of the hepatotoxin microcystin-LR in *M. aeruginasa* via a common, putative intermediate. KS, β-ketoacyl synthase; MT, methyltransferase; KR, β-ketoacyl reductase; DH, dehydratase; AMT, aminotransferase; C, condensation domain; A, adenylation domain; TE, thioesterase (Moore, 2005).

2.3.1.2.1 Structural organization of the *mcy*D-J region

The *mcy*D-J gene cluster contains seven ORFs all transcribed in opposite direction to the putative *mcy*ABC operon (Fig. 2.6). The first ORF (11,721 bp) in this region (*mcy*D) is located 733 bp upstream of *mcy*A and encodes a large 435,714 Da polypeptide with high similarity to known type I PKS (Cane *et al*., 1998). Alignment of McyD with PKS domains identified two type I modules, each consisting of a β-ketoacyl synthase (KS), an acyltransferase (AT), β-ketoacyl reductase (KR), a dehydratase (DH) and an acyl carrier protein (ACP). On the basis of conserved

sequence motifs, both AT domains of McyD appear to accept malonyl-CoA. In addition, the amino-terminal module contains a putative C-methyltransferase (CM) domain of a type recently found in the yersiniabactin biosynthetic cluster (Gehring *et al*., 1996) (Fig. 2.7).

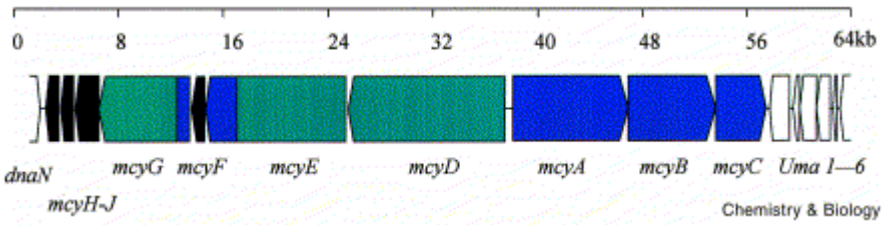

Figure 2.6 Organization of the gene cluster for microcystin biosynthesis (Tillett *et al*., 2000).

The ATG start codon and putative ribosome binding site (RBS) of the second ORF, *mcy*E, is located 167 bp downstream of the TAA stop codon of *mcy*D. This large 10,464 bp ORF encodes a 392,703 Da polypeptide product of mixed PKS and NRPS function. The amino-terminal region of McyE contains a PKS module consisting of a KS, AT and ACP domain, linked to a CM domain (as found in McyD) and a putative aminotransferase (AMT) domain. The most logical role of this module in microsystin biosynthesis would be to supply the amino group to Adda. Adjacent to the PKS module of McyE is an NRPS module composed of two condensation domains, an adenylation domain and thiolation domain (Fig. 2.8). The first condensation domain is predicted to catalyze the formation of the peptide bond between Adda and the α-amino group of D-glutamate. The adenylation domains were analyzed for substrate specificity employing a recently derived algorithum (Challis *et al*., 2000; Stachelhaus *et al*., 1999). While the second McyE condensation domain

appears to be involved in the peptide bond formation between D-glutamate and N-methyl-dehydroalanine, the mechanism of this unusual reaction remains unclear.

Located 32 bp downstream of the TAG stop codon of *mcy*E is a small ORF (*mcy*F) of 756 bp encoding a 28,192 Da polypeptide. Although the role of McyF in microcystin biosynthesis has yet to be confirmed, it appears likely to be involved in either the supply of D-glutamate or D-MeAsp, or the peptidyl epimerization of L-glutamate.

The putative RBS and ATG codon of the fourth ORF (*mcy*G) is located 132 bp downstream of the TGA stop codon of *mcy*F. This large ORF (7,896 bp) encodes a 294,266 Da polypeptide of mixed NRPS and PKS function. The amino-terminal region of McyG contains an NRPS adenylation domain that clusters with the acyl-CoA synthetases, insect luciferases and aryl-carrier protein synthetases of NRPS systems. The carboxy-terminal PKS module of McyG consists of malonyl-specific KS, AT, CM, DH, KR and ACP domains (Fig. 2.7).

The second ATG codon, located 224 bp downstream of the TAA stop codon of *mcy*G, was selected as the initiation codon for McyH. This 1,617 bp ORF encodes a putative 67,100 Da transmembrane protein belonging to the ABC transporter ATP binding family. No obvious biosynthetic function can be assigned to McyH, however, it is worth speculating that McyH may play role in the thylakoid localization of microcystin previously observed in *M. aeruginosa* (Shi *et al*., 1995).

Located 39 bp downstream of the TAA stop codon of *mcy*H is the ATG start codon of the sixth ORF, *mcy*I. This 1,014 bp ORF encodes a 36,838 Da polypeptide. While no definite function can be assigned to McyI, its location within the *mcy* gene cluster suggests it may have a role in securing the required serine precursor of N-

methy-dehydroalanine, or in the synthesis of N-methy-dehydroalanine following serine activation.

The final ORF in the cluster (*mcy*J) is located 176 bp downstream of the TAA stop codon of *mcy*I. The lack of a potential RBS upstream of the first ATG in this 837 bp ORF suggests that the alternative start codon TTG, located 9 bp downstream, is used to initiate translation.

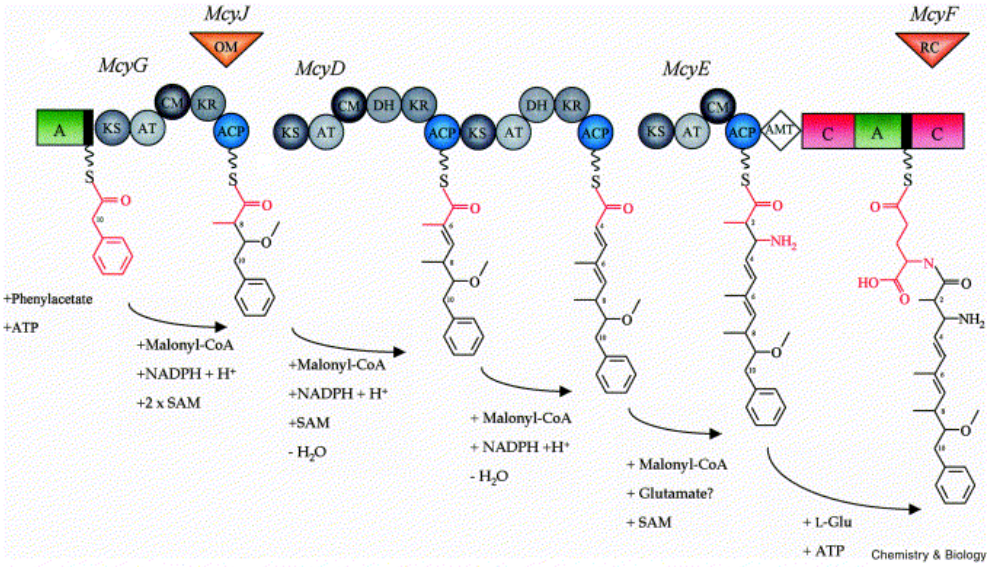

Figure 2.7 Model for the formation of Adda and predicted domain structure of McyG, McyD and McyE. Each circle and rectangle represents, respectively, a PKS or NRPS enzymatic domain. The putative aminotransferase domain is represented by a diamond. The activity of the tailoring ORFs, McyJ and McyF, are shown as inverted triangles. KS, β-ketoacyl synthase; AT, acyltrasferase; ACP, acyl carrier protein; KR, β-ketoacyl reductase; DH, dehydratase; CM, C-methyltransferase; OM, O-methyltransferase; A, aminoacyl adenylation; C, condensation; AMT, aminotransferase; RC, racemase. The NRPS thiolation motif is shown in black. Reaction order shows transfer and condensation of Adda to D-glutamate (Tillett *et al.*, 2000).

2.3.1.2.2 Structural organization of the *mcy*ABC and *uma*1-6 regions

The small of these two putative *mcy* operons, *mcy*ABC, contains three large ORFs. Translation of the first ORF, *mcy*A (8,388 bp), appears to be initiated from the second ATG codon as the first ATG codon lacks a suitable RBS. The gene encodes an NRPS of 315,717 Da, containing two adenylation and thiolation domains, respectively, a condensation domain, an N-methyltransferase (NMT) domain and an epimerization domain (Fig. 2.8). Binding pocket analysis of the first adenylation domain of McyA revealed clustering of this domain with a group of domains known or suspected to activate L-serine. The presence of an NMT domain allows the amino acid specificity of the first activation domain of McyA to be assigned, as microcystin possess only one N-methylated residue, N-methyl-dehydroalanine (Rinehart *et al*., 1988). The first thiolation domain, presumably involved in the transport of the N-methyl-seryl residue and the condensation domain of McyA, are not related to known domains involved in the condensation of N-methylated amino acids (von Dohren *et al*., 1999). The second adenylation domain of McyA, presumably activating L-alanine, shows a slightly reduced spacing of the pocket-lining residues. The sequence of the second thiolation domain of McyA, if compared to available sequences of peptidyl carrier domains, groups with the large cluster of carriers having D-amino acids attached, or those adjacent to epimerization (Ep) domains.

The ATG start codon and putative RBS of the second ORF, *mcy*B, is located 15 bp downstream of the TAA stop codon of *mcy*A. This 6,318 bp ORF encodes a peptide synthetase of 242,334 Da containing two modules, each possessing adenylation, thiolation and condensation domains (Fig. 2.8). The amino-terminal domain has been functionally identified by sequence alignment with known condensation domains as catalyzing peptide bond formation between L- and D-aminoacyl residues (von Dohren *et al*., 1999). This is in agreement with the expected acceptance of the carboxy-terminal D-alanyl-peptidyl intermediate from McyA. From the structure of microcystin, the second activation domain would be expected to activate L-leucine and is in agreement with the predicted activation domain substrate pocket specificity. The second condensation domain McyB involved in peptide bond formation between leucyl and glutamyl or glutaminyl residues. The predicted binding pocket of the adjacent adenylation domain maps to a group of domains specific for aspartic acid or asparagine.

The third ORF, *mcy*C, is located 4 bp upstream of the TGA stop codon of *mcy*B, starting with an ATG codon 7 bp downstream of a putative RBS. This 3,876 bp ORF encodes a 147,781 Da peptide synthetase with a carboxy-terminal thioesterase (TE) domain (Fig. 2.8). The final activation domain is expected to activate predominately L-arginine. Analysis of the region downstream of *mcy*C revealed the presence of six ORFs transcribed in the opposing direction to the *mcy*ABC gene cluster. Although no function can be assigned to *uma*1-6, it appears unlikely that these ORFs are involved in microcystin synthesis as they are present in both toxic and nontoxic *Microcystis* strains (Tillett *et al*., 2001). Speculatively, this ORF may have played a role in the acquisition of this biosynthetic cluster by *M. aeruginisa* PCC7806.

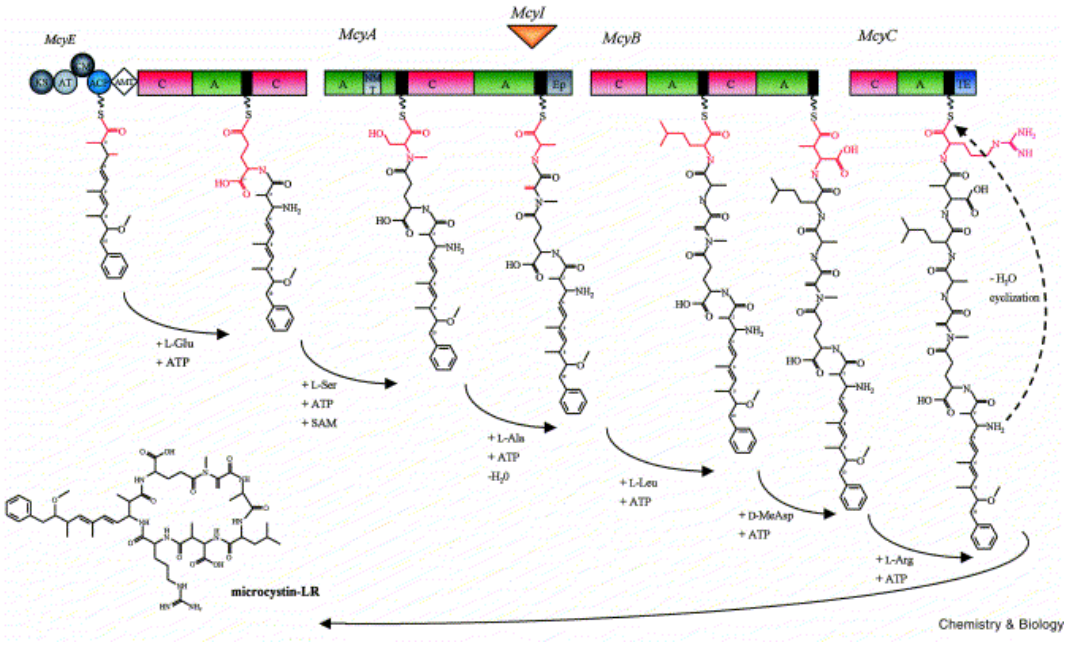

Figure 2.8 Biosynthetic model for microcystin-LR and predicted domain structure of McyE, McyA, McyB and McyC. Each circle and rectangle represents, respectively, a PKS pr NRPS enzymatic domain. The aminotransferase domain is represented by a diamond. The activity of the putative tailoring ORF, McyI, is shown as an inverted triangle. KS, β-ketoacyl synthase; AT, acyltransferase; CM, C-methyltransferase; ACP, acyl carrier protein; A, aminoacyl adenylation; C, condensation; NM, N-methyltransferase; Ep, epimerization; TE, thioesterase; AMT, aminotransferase. The NRPS thiolation motif is shown in black. Aminoacyl activation and condensation order is predicted: L-Z-Adda, L-glutamate, L-methyserine, D-alanine, L-leucine, D-methyl-aspartate, L-arginine, cyclization (Tillett *et al*., 2000).

2.3.2 MC production and variability

Mechanisms for production of toxins and their significance to cyanobacteria are still debated. MC production is regulated at three levels (Fig. 2.9).

1) Genetic level. This is the crucial factor determining the toxicity of a particular strain.

2) Cellular level. Toxin production in toxin strains is regulated by environmental factors. However, the range of influence seldom exceeds 10-fold (Sivonen and Jones, 1999).

3) Population level. Toxicity is related to the proportion of toxic and nontoxic strains in cyanobacterial communities. Since toxin concentrations vary significantly among strains (from zero to over several thousand micrograms per gram dry weight), toxin production in cyanobacterial blooms can vary more than 1,000-fold.

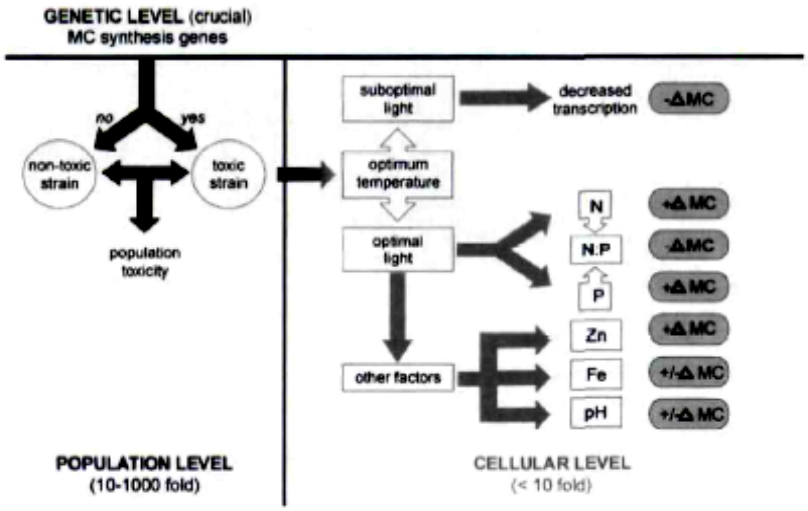

Figure 2.9 Summary of factors at the genetic, cellular and population level that affect MC production. Numbers in brackets refer to the magnitude of toxin production response (Zurawell *et al*., 2005).

2.3.2.1 Genetic basis of MC production

Clearly, a cyanobacterium must process genes for MC synthesis before factors related to production can be considered (Fig. 2.9). Thio-template nonribosomal

peptide synthesis is a mechanism for the production of secondary metabolites like MCs in microorganisms. A family of enzymes named nonribosomal peptide synthetases (NRPSs) catalyzes the formation of peptides. NRPSs assemble into large multienzyme complexes with modular structure, with each module responsible for the activation, thiolation, modification and condensation of one amino acid substrate (Arment and Carmichael, 1996; Kleinkauf and von Dohren, 1996; Marahiel *et al*., 1997). Sequence analysis shows that the *mcy* region has 10 ORFs (*mcy*A to *mcy*J) that are divided into two operons (*mcy*A to *mcy*C and *mcy*D to *mcy*J) by a promoter region (Fig. 2.10) (Tillett *et al*., 2000; Kaebernick and Neilan, 2001). Figure 2.10 also illustrated biosynthetic model for microcystin-LR (Zurawell *et al*., 2005). However, these MC synthetase genes not only exist in all toxic strains of *Microcystis*, but also in MC-producing strains of *Anabaena*, *Planktothrix* and *Nostoc*, as well as some nontoxic strains, indicating a high degree of gene conservation or a cosmopolitan distribution of hepatotoxic strains (Meißner *et al*., 1996; Neilan *et al*., 1999; Christiansen *et al*., 2003). Since some strains possess biosynthesis genes but not the corresponding products, the expression of these genes may be regulated by environmental factors (Dittmann *et al*., 2001).

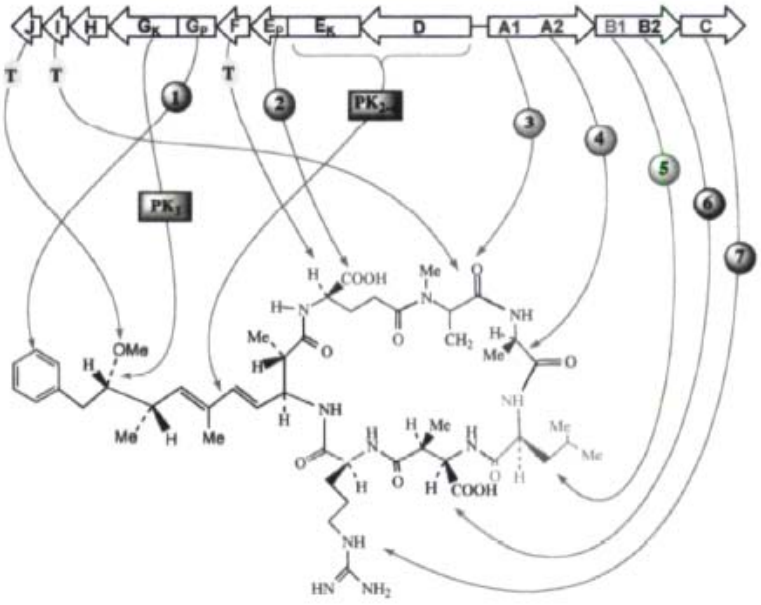

Figure 2.10 Proposed biosynthetic model for microcystin-LR showing the organization of the gene clusters *mcy*A-J and microcystin. Numbered circles indicate the order of amino acids incorporated into the growing peptide chain synthesized by NRPS genes (*mcy*A, B, C, E_p, G_p). Within *mcy*A and *mcy*B are two modules, A1/A2 and B1/B2, respectively. Numbered rectangles show the order of polyketide synthesis in the formation of Adda (*mcy*G_k, E_k, D). "T" indicates additional ORFs of putative microcystin tailoring function (Kaebernick and Neilan, 2001).

2.3.2.2 Factors regulating MC production (laboratory studies)

Among factors associated with MC production in toxic species (primary *M. aeruginosa*) under experimental conditions, the growth phase of cyanobacterial cultures has been most strongly linked to toxicity because toxin concentrations per cell are reported to peak during late exponential phase (van der Westhuizen and Eloff,

1985). Effects of water temperature and light intensity on toxin production appear to be species/strain specific. For instance, Gorham (1964) determined the optimum temperature for toxin production for *M. aeruginosa* strain NRC-1 to be 25°C and toxin content did not vary with light intensity. Cultures of *M. aeruginosa* strain UV-006 grown at pH 9.5 yielded maximum toxicity (LD_{50} ~142 mg/kg) at similar water temperatures (20 to 24 °C), but depended on light intensity (optimum 145 $\mu E/m^2/s$) (van der Westhuizen and Eloff, 1985).

Under light-limiting conditions, MC production increases with increasing light intensity, but when light is not limiting, increasing intensity has no effect or inhibits toxin production (Zurawell *et al*., 2005). Since the ratio of peptide toxin to total protein remained constant above intensities of 40 $\mu E/m^2/s$, the authors suggested that *M. aeruginosa* cells should have maximal toxin content at the lake surface and content should decrease with depth as light attenuates. Thus, between 1 and 3 m depth, light intensity declined from 40 to 0 $\mu E/m^2/s$, and toxin content dropped from 5 to 2.5 ng MC/μg dry weight (Utkilen and Gjolme, 1992).

Differences in light and temperature optima may exist not only among species, but among toxin variants. Rapala and co-workers (1997) found that two *Anabaena* strains produced the most MC at 25°C, but the composition of MC variants differed with temperature: More MCLR was produced at lower temperatures, whereas more MCRR was produced at higher temperatures. Rapala and Sivonen (1998) suggested that MCLR production is regulated primarily by light, whereas MCRR production is regulated primarily by temperature. Utkilen and Gjolme (1992) found minor effects of light quality on toxin production in continuous cultures of *M. aeruginosa*; red and green light enhanced toxin production by about 50% compared to white light. Another

study indicated that light quality and intensity influence MC production. Red light induced higher transcript levels of the two genes (*mcy* B and *mcy* D) responsible for toxin synthesis in *M. aeruginosa* PCC7806 (Kaebernick *et al*., 2000).

Toxin cyanobacteria strains appear to have higher N and P requirements than nontoxic strains, possibly due to the extra energy and materials required for toxin synthesis (Vezie *et al*., 2002). For example, Lee *et al*. (2000) found that the MC content of *M. aeruginosa* cells was positively related to the N concentration of the culture medium at a fixed P concentration (0.2 mg/l), with the highest MC content (2.7 μg/g dry weight) at about 2 mg/l N. However, when N concentration was fixed at 1 mg/l, cellular MC content was negatively (though weakly) related to P concentration. Cellular MC content was highest (1.9 μg/g dry weight) at a N:P of 100:1 (Lee *et al*., 2000). Hence heterocystous and non-heterocystous cyanobacteria differ in toxin production in response to ambient N concentrations (Rapala *et al*., 1997).

Conversely, P concentrations appear to be linked with toxin production of both heterocystous and non-heterocystous species. Therefore, total MC content increased (in particular the more toxic MCLR variant) with increasing P limitation. Thus, they concluded N and P have no influence on toxin content of *M. aeruginosa* and reiterated that these changes should be expressed as toxin content per unit protein content, not per unit dry weight (Utkilen and Gjolme, 1995). Only Zn and Fe affected toxin yield and indeed, both were found to be essential for growth of batch cultures. When a 0.01 to 0.25 μM Zn solution was added to trace-metal-depended media, the growth rate of *M. aeruginosa* PCC7806 increased to 1.5 times that of standard conditions, and MC production increased by 30%. However, higher Zn concentrations (10 μM) killed the cells and stopped toxin production. Although Zn concentration was positively related

to toxin production, Fe concentration was negatively related. Cells produced 20 to 40% more toxin (dry weight basis) at low Fe concentration ≤ 2.5 μM (Lukac and Aegerter, 1993). This level of regulation is more significant in natural waters.

2.3.2.3 Factors regulating MC production (field studies)

The MC concentration in natural phytoplankton communities can be highly dynamic on both temporal and spatial scales. In blooms dominated by one or two toxic strains, toxicity can be related to the biomass of the main producer, though this influence generally affects toxin concentrations by less than 10-fold. These results indicate that for blooms mostly dominated by one or two toxic species, and estimate of toxin concentration based on the biomass of dominant strain is acceptable (Kotak *et al*., 1995, 2000; Oh *et al*., 2001). Therefore, in water bodies with this "mosaic" pattern in the phytoplankton community, samples containing high proportions of toxic cyanobacteria could have high toxin concentrations. Conversely, when toxin cyanobacteria are not dominant, the toxins they produce will be "diluted" by the biomass from nontoxic phytoplankton (Chorus *et al*., 2001). For this reason, Kotak *et al*. (1995) recommended expressing toxin concentration per unit biomass of toxin-producing species (e.g., μg toxin/g *M. aeruginosa*). Environmental factors play an important role in regulation toxin production in natural water bodies. Solar radiation, biomass-normalized primary production, oxygen saturation, temperature, and pH were positively correlated with MC concentrations, whereas Chl-a and P concentrations were weakly negatively correlated.

In temperature eutrophic lakes, toxin dynamics in natural phytoplankton assemblages may be related to changes in the concentration and ratios of N and P. In

lakes in central Alberta, Canada, total P (TP) was the strongest correlate with both *M. aeruginosa* biomass (r = 0.57) and cellular MCLR concentration (expressed as μg MCLR/g *M. aeruginosa*) (r = 0.53) (Kotak *et al*., 1995). In these lakes, nonlinear, negative relationships existed between inorganic N (nitrate + nitrite and ammonium) concentrations and either *M. aeruginosa* biomass or MCLR concentration. This was evident as maximum toxin concentrations occurred during periods when inorganic N concentrations were at their lowest (Kotak *et al*., 2000). Accordingly, toxin production in natural waters is influenced by three major factors, which differ in the magnitude of toxin response (Fig. 2.9): (i) phytoplankton dynamics (the relative abundance or biomass of toxin-producing species), (ii) variable presence of distinct toxin- and non-toxin-producing cyanobacterial strains; and (iii) effects of environmental variables on toxin production (Carmichael and Gorham, 1981; Kotak *et al*., 1995, 2000; Chorus *et al*., 2001).

2.3.3 Biology and molecular biology of *Microcystis* sp.

For investigation of *Microcystis* sp., strains isolated from the same bloom sample are constitutely microcystin producing or nonproducing (Long *et al*., 2001; Orr and Jones, 1998; Vezie *et al*., 1998). Toxic cyanobacterium strain cannot be distinguished from nontoxic strains without isolation and testing for toxin production (Rouhiainen *et al*., 1995). Understanding of the community composition and dynamics of microcystin-producing and non-microcystin producing *Microcystis* strain in the field is very limited, due to a lack of suitable identification methods. The genus *Microcystis* includes toxic and bloom-forming morphotypes, which are usually arranged into species based on morphological features. Lopez-Rodes and Costas

(1997) used immunofluorescence assays using polyclonal and preadsorbed antibodies, as well as FITC-labeled lectins were used to characterize three morphospecies of *Microcystis* (*M. viridis, M. wesenbergii* and *M. aeruginosa*) from natural populations (several lakes, reservoirs in Denmark and Spain) and laboratory clones. The results showed that *M. viridis* and *M. aeruginosa* from Danish lakes appeared to be closely related species, whereas *M. wesenbergii* emerged as a different species.

Traditional characterization of *Microcystis* based on morphological features is very difficult and the differentiation that can be attained below genus level is limited. There are several morphotypes in the genus and each is equivalent to a morphospecies (Otsuka *et al*., 1999). For instance, Komarek (1991) distinguished six morphospecies in Japanese waters: *M. viridis* (A. Brown) Lemmermann, *M. wesenbergii* (Komarek) Komarek in Kondratieva, *M. aeruginosa* (Kutzing), *M. novacekii* (Komarek) Compere, *M. ichthyoblabe* Kutzing and *M. flos-aquae* (Wittrock) Kirchner. Watanabe (1996) reported that *M. viridis* and *M. aeruginosa* produce microcystins, *while M. wesenbergii* (Japanese strains) and *M. novacekii* produce no microcystin *and M. ichthyoblabe* includes toxic and non-toxic strains. Even the taxonomy of *Microcystis* has been done traditionally by classical phenotypic criteria such as microbial morphologies and cell arrangement of colonies (Komarek and Anagnostidis, 1986; Komarek, 1958). However, the morphological characteristics of cyanobacteria are easily altered from the environmental conditions of the habitat and cultural conditions in a laboratory. The researchers have attempted to characterize the genetic properties of toxic and bloom-forming cyanobacteria that would be useful for the identification and classification of diversity within this group. Thus, molecular biological methods are more reliable tools for recognition of *Microcystis* strains and their properties.

Kondo and co-workers (2000) determined DNA base composition and DNA-DNA hybridization among the cyanobacterial genus *Microcystis* by using nine axenic *Microcystis* strains, including the three morphological species *of M. aeruginosa, M. viridis* and *M. wesenbergii*. These *Microcystis* species showed a similar DNA base composition (42.1-42.8 mol% G+C) and demonstrated more than 70% DNA relatedness, confirming their synonym based on bacterial criteria. DNA fingerprinting was developed for detect *Microcystis* such as random amplified polymorphic DNA (RAPD) (Bowditch *et al*., 1993; Williams *et al.*, 1990) and arbitrarily primed PCR (AP-PCR) (Welsh and McClelland, 1990). In RAPD and AP-PCR, a single oligonucleotide primer with arbitrary sequence is used for random amplification of DNA by PCR. Therefore, polymorphisms can be easily analyzed by small amounts of template DNA and no DNA sequence information is required. This is advantageous as a taxonomic method for cyanobacteria because growth of some cyanobacteria is poor and the genomic DNA is not easily obtained. RAPD analysis involves one stage of the PCR reaction and amplification products on gel electrophoresis are detected by ethidium bromide (Williams *et al*., 1990). Whereas, AP-PCR involves two stages of amplification reaction (low stringency and higher stringency annealing stages) in PCR and radioactive labelling is used for the detection of amplification products (Welsh and McClelland, 1990). Thus, RAPD seems to be simpler and more convenient than AP-PCR. Nishihara and colleagues (1997) used RAPD analysis for discrimination of five species of *Microcystis* cyanobacteria that have been classified on the basis of morphology, toxicity and allozyme genotype (Watanabe, 1996). The results indicated that three species of *Microcystis* with single and unique allozyme genotype to each species, i.e., *M. novacekii, M. viridis* and *M. wesenbergii* were shown to have their

genetic homogeneity by the RAPD analysis as well as allozyme genotype. On the other hand, some genetic variations were observed among the strains of *M. aeruginosa* and *M. ichtyoblabe* according to analyses of RAPD and allozyme divergency. Further taxonomic revisions are still need for this group.

Neilan (1996) reported about the genetic relatedness between the genera of bloom-forming cyanobacteria is supported by the RAPD, 16S-23S ITS and phycocyanin PCR-RFLP profiles. At the lowest taxonomic level the RAPD procedure readily differentiates among all strains of cyanobacteria tested regardless of geographical origins. The limitation of this technique is the current need for strains to be maintained in axenic culture and has not been shown to be suitable for direct analysis of environmental isolates (Van Coppenhole *et al*., 1993; Neilan, 1995). Less discriminatory power is afforded by PCR-RFLP analysis of the ribosomal spacer and the phycocyanin operon. These methods were, however, more robust and useful for the direct analysis of cyanobacteria isolated from complex natural populations. Within this category it is recommended that the 16S-23S ITS be used for initial confirmation of genus identification and successive restriction digest to this locus and that of the phycocyanin gene be applied for delineation to the strain level. Furthermore, the sequencing of complete genes, such as 16S rRNA gene, is the most involved procedure but provides the most accurate method for determining relatedness and inferring evolution within this group of prokaryotes. Neilan *et al*. (1997) performed a phylogenetic analysis of toxic and non-toxic *Microcystis* strains based on 16S rDNA sequences and reported that 16S rDNA was useful for delineating toxic and non-toxic strains of *Microcystis*. Otsuka *et al*. (1998) found, however, that five morphospecies of *Microcystis* were so closely related in terms of 16S rDNA sequence that they may

be integrated into one species and concluded that the 16S rDNA sequence is insufficiently variable for phylogenetic analysis of these organisms at the species level. DNA sequences of the 16S to 23S internal transcribed spacer region (16S-23S ITS) are known to be more variable and exhibit significant differences in sequence and length (Barry *et al.*, 1991; Navarro *et al.*, 1992). As Otsuka and co-workers (1999) found that 16S-23S rDNA internal transcribed spacer sequences of 47 strains of genus *Microcystis* can be divided into three clusters. The first cluster included toxic and non-toxic strains, the second only toxic ones and the third only non-toxic ones. The tree topologies were not necessarily correlated with morphospecies distinction or phycobilin pigment composition and one genotype may have more than one morphotype. The *rpo*C1 gene analysis had been shown to be more discriminatory than using the 16S rRNA analysis, especially for the chlorophyll-containing prokaryotes (Palenik and Haselkorn 1992). *rpo*C1 sequences can be easily and specifically PCR amplified from cyanobacteria and used to elucidate phylogenetic relationships between distantly related cyanobacterial groups, especially when the third codon position is eliminated from the data set (Palenik, 1992; Palenik and Swift, 1996). Janse *et al*. (2004) interested in the genetic differentiation of *Microcystis* colonies based on rRNA internal transcribed spacer (ITS) sequences provides an adequate basis of recognition of microcystin producers. They were grouped by rRNA ITS denaturing gradient gel electrophoresis (DGGE) typing. The results show that microcystin-producing and non-microcystin producing colonies ended up in different class.

Furthermore, many several researches have used molecular genetic based on microcystin synthetase gene to detect *Microcystis*. Tillett and co-workers (2001) showed the relationship between toxigenicity and phylogeny within the cyanobacterial

genes *Microcystis*. PCR primers for the N-methyltransferase (NMT) domain of the *mcy*A were designed. No consistent relationship was found between the NMT genotype, PC-IGS (phycocyanin intergenic spacer) sequence, 16S rRNA sequence, geographical region of isolation or morphological species identification. Otsuka *et al*. (1999) has reported a similar lack of correspondence between the 16S-23S rRNA intergenic spacer sequence and morphospecies designation or toxicity in *Microcystis* spp. The patchy distribution of toxic and nontoxic organisms within various PC-IGS (Tillett *et al*., 2001) and 16S-23S rRNA ITG (Otsuka *et al*., 1999) groups of *Microcystis* could arise if either (i) *Microcystis* was originally able to produce microcystin, with presently nontoxic strains being defective mutants or (ii) *Microcystis* was originally nontoxic, with toxicity being acquired by lateral genetic transfer from some other organism. Genetic characterization of a region of the adenylation domain in module *mcy*B1 resulted in identification of two groups of genetic variants in closely related *Microcystis* strains (Mikalsen *et al*., 2003), 11 strains containing different variants of the *mcy*ABC gene cluster and 7 strains lacking the genes were found. There is no concordance between the phylogenies generated with *mcy*B1, 16S rDNA and DNA fingerprinting. Moreover, horizontal gene transfer can explain the distribution and variation within the *mcy*ABC operon. Kurmayer and co-workers (2002) also analyzed the genotype of microcystin production of *Microcystis* spp. from Lake Wannsee (Berlin, Germany) based on *mcy*B gene. The results concluded that the gene product found for most of the microcystin-producing colonies in the lake is rather unspecific and the diversity of microcystin variants in the lake results from activation of various amino acids during microcystin biosynthesis in the same genotype.

However, identification of the most potent microcystin producer in a lake has not been possible due to a lack of quantitative methods. Quantitative real-time PCR was applied to identify the microcystin-producing genera and to determine the copy number of microcystin synthetase gene E (*mcy*E) in Lake Tuusulanjarvi and Lake Hiidenvesi in Finland (Vaitomaa *et al*., 2003). The main microcystin producer in Lake Tuusulanjarvi was *Microcystis* spp., since average *Microcystis mcy*E copy numbers were >30 times more abundant than those of *Anabaena*. Lake Hiidenvesi seemed to contain both nontoxic and toxic *Anabaena* as well as toxic *Microcystis* strains. Kurmayer and Kutzenberger (2003) developed the real-time PCR for quantification the two genes region such as the intergenic spacer region within the phycocyanin (PC) operon to quantify the total population and the *mcy*B gene, which is indicative of microcystin synthesis by the *Taq* nuclease assay (TNA). The results indicated that the mean proportion of microcystin genotypes is stable from winter to summer and that *Microcystis* cell numbers could be used to infer the mean proportion of *mcy* genotype in Lake Wannsee.

2.3.4 Stability and degradation of microcystins

MCs are endotoxins and thus remain within viable cyanobacterial cells throughout growth. However, as cyanobacterial populations proceed through a stationary phase leading to death, autolysis causes membranes to become susceptible to microbial attack (Berg *et al.*, 1987). For example, Sugiura and co-workers (1992, 1993) demonstrated the lytic capabilities of the bacterium *Pseudomonas* and the microfagellate *Monas guttula* on viable *M. aeruginosa* cells. Cells become permeable, allowing soluble intracellular compounds, including MCs, to diffuse out into the

surrounding environment. Alternatively, the use of chemicals to control cyanobacterial blooms or as flocculants in water treatment processes may cause cell lysis and an increase in extracellular toxin concentration (Jones and Orr, 1994). Until recently, the environmental fate of dissolved MCs in natural waters was poorly understood. Sediment sorption, photolysis, and, most important, microbial degradation, are the main routes of natural detoxication of MCs in fresh waters (Harada and Tsuji, 1998). These is also evidence that some aquatic organisms, such as macrophytes, mollusks, crustacea, and fish, can accumulate and detoxify MCs in their tissues via the Phase II detoxication pathway (Pflugmacher *et al*., 2001; Pietsch *et al*., 2001).

Natural sediments may adsorb MCs (13 to 24 μg/ml toxin in sediment; Rapala, *et al*., 1994), particularly hydrophilic variants like MCRR (Harada and Tsuji, 1998). Based on their observation that > 81% of MCLR is removed from solution by clay minerals, Morris *et al*. (2000) suggested that the Adda moiety makes MCs susceptible to scavenging by fine-grained particles such as suspended clay minerals of the smectite group. Considering clay minerals can remain suspended for long periods and may protect otherwise labile adsorbed organic compounds, serious implications exist with respect to the biogeochemical fate (i.e., toxin transport, degradation, food-web availability) of MCs in natural environments (Morris *et al*., 2000).

Ultraviolet (UV) light can induce significant MC decomposition at wavelengths near the absorption maxima of the toxins (Tsuji *et al*., 1995). Photolytic effects depend on UV radiation levels, and low levels induce random isomerization of the Adda moiety (Tsuji *et al*., 1995). The isomerization process is reversible and products decompose under UV light, suggesting that the photolytic degradation of MCLR by UV radiation proceed via isomerization products (Kaya and Sano, 1998). Since

nontoxic products are formed, water treatments involving UV irradiation may be feasible for removing MCs from raw water (Tsuji *et al*., 1995). Consequently, UV irradiation in the presence of a titanium dioxide catlyst has been employed to degrade MCs (Shephard *et al*., 1998, 2002; Lawton *et al.*, 1999; Feitz and Waite, 2003).

Pigments and humic substances (HS) in natural waters could enhance photolytic degradation. Tsuji *et al*. (1994) reported that MCLR and its nontoxic geometric isomer, [6(Z)- $Adda^5$] MCLR, were stable when exposed to fluorescent light and experienced only slight isomerization under natural sunlight throughout a 26-d period. However, the presence of photosynthetic pigments (particularly water-soluble phycocyanins) significantly accelerated isomerization and decomposition of MCLR and [6(Z)- $Adda^5$] MCLR under natural sunlight, but not fluorescent light. Hence, they concluded that although isomerization and decomposition rates of MCs are dependent on both pigment type and concentration, decomposition might predominate at higher pigment concentrations (Tsuji *et al*., 1994). Welker and Steinberg (1999) demonstrated indirect photolytic degradation of several MCs in the presence of HS and natural sunlight and estimated the half-life of MCRR to be about 10 h. Since HS are present in most natural waters in concentrations of several milligrams per liter, the authors concluded such a process could contribute to the elimination of MCs from natural environments. Treatment with HS, combined with coagulation-flocculation with alum, could hasten photolysis by transforming the conjugated double bond in the Adda moiety into a less toxic isomer (Welker and Steinberg, 2000).

In the presence of natural microbial populations, biodegradation can rapidly reduce extracellular toxin concentrations. Variation in microbial degradation rates among lakes probably stems from the bloom history of the lake and environmental

factors that affect microbial metabolic rates (e.g., temperature). Lakes with a history of cyanobacterial blooms may have bacterial communities that are capable of exploiting the toxin as a food source and rapidly (within 8 d) degrading it (Christoffersen *et al.*, 2002). Berg *et al.* (1987) estimated that 90% of the toxin released from decomposing bloom material was degraded by microorganisms over a 26-d period. In addition, Jones and Orr (1994) determined the in situ degradation of MCLR following algicide treatment of an *M. aeruginosa* bloom on Lake Centenary, Australia. Degradation was biphasic, consisting of an initial rapid phase (3 d) during which 90 to 95% of the extracellular toxin decomposed, followed by a second, slower, phase. Toxin concentration and toxicity were reduced significantly after incubation of purified MLCR with heterotrophic microbes from wastewater treatment plant effluent; the half-life of purified MCLR ranged from 0.2 to 3.6 d (Lam *et al*., 1995). Loss of toxicity likely resulted from biotransformation (modifications to the heptapeptide ring and Adda) of the toxin (Lam *et al.*, 1995).

Bourne *et al*. (1996) described the biotransformation of MCLR by the bacterium *Sphingomonas* and proposed the involvement of at least three enzymes. In the first step, a metal- activated protease catalyses the hydrolytic cleavage of the Adda-Arg peptide bond, which opens the cyclic ring to yield acyclo-MCLR (NH_2-Adda-D-Glu-Mdha-D-Ala-L-Leu-D-MeAsp-L-Arg-OH) and produces a concomitant 160-fold reduction in toxicity from the parent compound. Next, a serine protease catalyzes the cleavage of the Ala-Leu peptide bond to yield a nontoxic tetrapeptide (NH_2-Adda-D-Glu-Mdha-D-Ala-OH). Finally, another metal-activated protease catalyzes the cleavage of other bonds (yet to be described), yielding undetected peptide fragments and amino acids (Bourne *et al*., 1996). Takenaka and Watanabe (1997) tested several

species of bacteria isolated from a Japanese lake and found a strain of *Pseudomonas aeruginosa* that could degrade MCLR through the activity of an alkaline protease. Park *et al*. (2001) isolated a strain of *Sphingomonas* from a hyper reutrophic lake in Japan that could degrade MCRR, MCYR, and MCLR. They detected two intermediates during the degradation process, which have structure similar to the two products reported by Bourne *et al*. (1996) and disappear within 6 d. Biotransformation is regarded as an important detoxication route for MCs following collapse of cyanobacterial blooms.

Based on diverse microbial species and degradation abilities in the natural environment, Welker and co-workers (2001) suggested that MC degrading ability is common in surface- water bacteria and can be induced by a wide range of dissolved organic compounds. Manage and co-workers (2000) isolated a freshwater gliding bacterium, *Alcaligenes denitrificans* from a hypereutrophic pond in Japan. This bacterium caused cell lysis and death of some cyanobacterial species, but showed no algicidal effects on the species of chlorophyceae tested. *M. aeruginosa, M. viridis* and *M. wesenbergii* were susceptible to the bacterial attack and the growth-inhibiting effect of the bacterium was significant on *M. aeruginosa*, particularly when the alga was in the exponential growth phase. The results suggested that *A. denitrificans* plays an important role in influencing the growth of *Microcystis* spp. and contributes to the death of *Microcystis* spp. in freshwater environments. Bourne and co-workers (2001) further proposed that MC degradation genes are involved in normal cell metabolism. They successfully cloned and sequenced the genes *mlr*A, B, and C, encoding three peptidases in *Sphingomonas.* They determined that these MC- degrading peptidases

are involved in cell-wall peptidoglycan cycling, while another gene cluster (*mlr*D) encodes a transport protein.

In addition to bacteria, several aquatic plants and animals can also degrade MCs. This detoxication pathway is mediated by glutathione S-transferase. It involves conjugation of the MC with glutathione (GSH) and then degradation to a gamma-glutamylcysteine and a cysteine conjugate (Pflugmacher *et al.*, 1999, 2001). In the common reed, *Phragmites australis*, the conjugation of MCLR to GSH occurs in chloroplasts and the cytosol, and the final conjugate is transported to vacuoles and excreted by the cell (Plugmacher, 2002).

Chemical also can inhibit the growth of *Microcystis* spp. such as $NaHCO_3$, $KHCO_3$, NaCl and KCl (Parker *et al.*, 1997). The results indicated that the growth of *Microcystis* spp. was inhibited by potassium while no effect in sodium. The cause of the potassium to *Microcystis* spp. is still unknown, but several trend explanations involve potassium inhibit of sodium related phenomena. Sodium is required for various aspects of cyanobacterial metabolism, including the uptake of bicarbonate, the protection of photosystem II and the maintenance of the intracellular pH.

2.3.5 Algal bloom control in Lake Senba

A novel strategy for *in situ* water bloom control was applied and evaluated in Lake Senba (Nakano *et al.*, 2001). Lake Senba is a small recreational lake in Mito City, Japan, where many people go for relaxation. Recently, the lake has been plagued by water blooms that are dominated by *Microcystis* and this has damaged the natural scenery of the water (Fig. 2.11). This lake has two seasons for *Microcystis*, the

blooming season (BS) from May to October and the non-blooming season (NS) from November to April (Fig. 2.12).

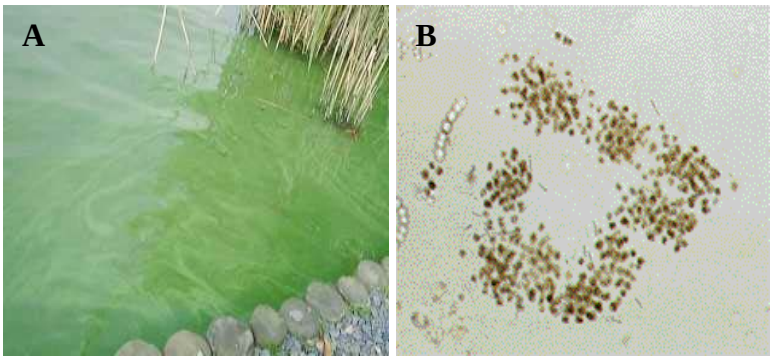

Figure 2.11 (A) The bloom forming of cyanobacteria in Lake Senba and (B) *Microcystis* sp. isolated from Lake Senba.

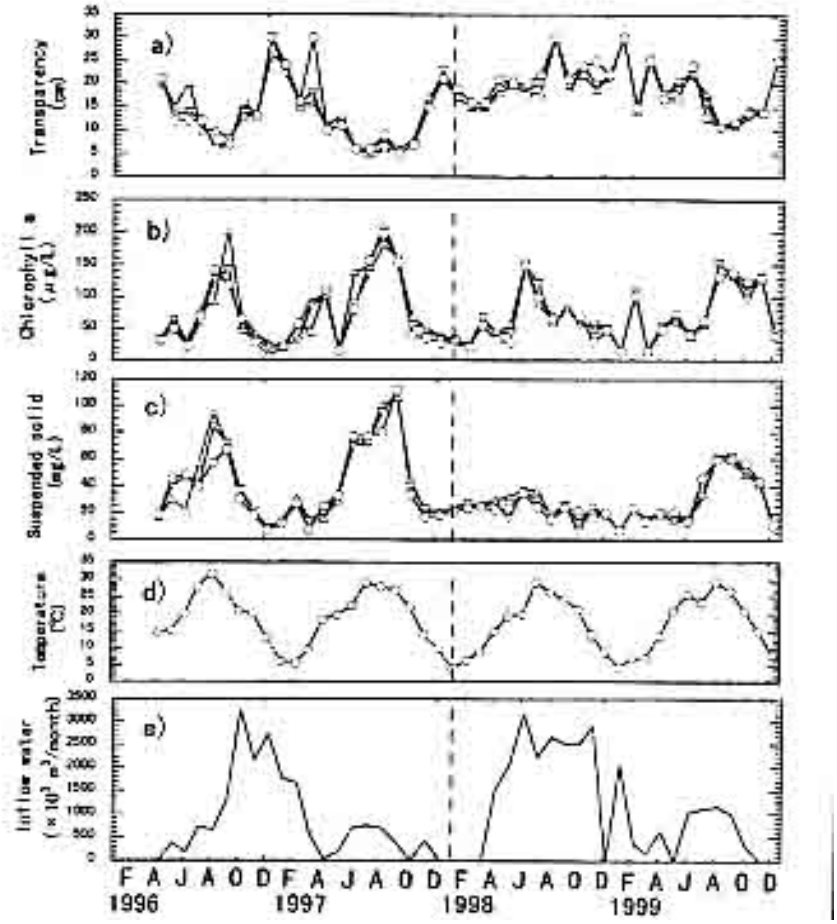

Figure 2.12 The dynamic population of bloom-forming *Microcystis* during 1996-1999 (Nakano *et al*., 2001).

Thus, there was increasing pressure from Mito citizens for drastic measures to control these blooms. The municipal government of Mito initially used flushing of the lake with river water for water bloom control. However, the flushing rate was not sufficient because of the limitation of available river water. Therefore, ultrasonic radiation system (USRS) and water jet circulation were integrated with the existing flushing process (Nakano *et al*., 2001). Three seconds of ultrasonic radiation was sufficient for the destruction of gas vacuoles and the buoyant ability of cyanobacteria to find optimum levels of illumination in the water column. Damage was also inflicted on the photosynthetic machinery. Consequently, the damage would delay growth recovery and slow the growth rate (Fogg *et al*., 1973). Therefore, the washout of sonicated cyanobacteria with a slower growth rate would be possible at the existing flushing rate, and water bloom occurrence would be controlled despite eutrophic lake conditions. A unit of the USRS basically consisted of an ultrasonic radiation module and water jet circulation module. This unit was housed in a swan structure which added aesthetic sense to the appearance in the lake as shown in Figure 2.13A. The water jet circulation module consisted of an intake duct (A), an ejector inside the intake duct (D) and a drive liquid suction pump (G). Since air can be introduced into the discharged water, oxygen supply to the lake bottom can also be done effectively. As shown in Figure 2.13B, when the driving liquid is supplied into the jet pump through a narrow slit at a flow rate of Q_1, the discharge flow rate becomes $Q_1+Q_2+Q_3$. The water jet circulation system realized continuous treatment by pumping in the water through a small zone of intense sonication in the ultrasonic irradiation module and releasing the treated water as a jet stream to provide mixing. Additionally, strategic positioning of the USRS containing the water jet circulation module might also be

effective for preventing stagnant regions which favor algal growth. Ten units of the USRS were installed throughout the lake.

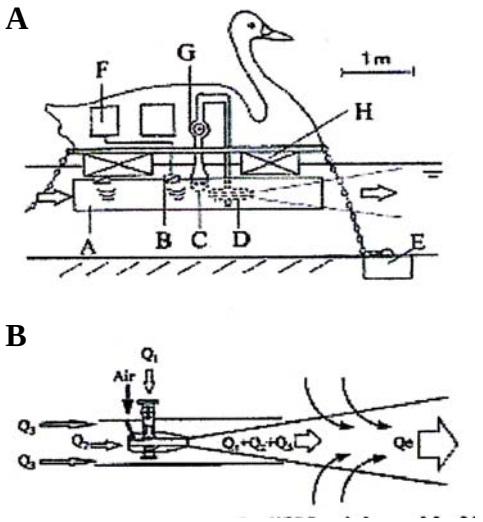

Figure 2.13 Schematic diagram of a USRS unit housed inside the swan structure and the water jet circulator integrated into USRS. (A) The USRS unit (symbols; A, ultrasonic radiation duct; B, ultrasonic transducer; C, inlet of driving liquid; D, jet pump; E, anchor; F, ultrasonic generator; G, drive liquid suction pump and H, float. (B) Water jet circulator and its mechanism to make fluid currents.

The performance of the integrated treatment system was evaluated by monitoring the water and sediment quality of the lake for two years. The results showed that the water quality was improved such as for chemical oxygen demand (COD) and phosphorus, and the operation of an ultrasonic irradiation system might reduce floating cyanobacteria; therefore, a higher transparency per chlorophyll a ratio was observed during the blooming season. Although the changes in the sediment quality were monitored at different distances from the ultrasonic radiation system, it is

not yet clear whether the cyanobacteria which have lost their buoyancy ability by this method will accumulate on the lake bottom or not.

Therefore, this research aims to confirm whether there is a persistence of *Microcystis* in the lake sediment after treatment with the integrated system on the basis of conventional enumeration and molecular genetic techniques.

2.4 Research Objective

To confirm the persistence of *Microcystis* in the lake sediment after treatment with the integrated system on the basis of conventional enumeration and molecular genetic techniques.

2.5 Materials and Methods

2.5.1 Cyanobacterial strains and culture condition

Nostoc linckia was obtained from the Tsukuba Algal Collections, National Science Museum, Tsukuba, Japan. *Microcystis viridis*, *M. aeruginosa* and *Phormidium* sp. were previously isolated from Lake Senba, Mito, Japan. *Anabaena* sp., *Hapalosiphon* sp. DASH05101 and *Scytonema* sp. were obtained from the Department of Soil Science and Conservation, Faculty of Agriculture, Chiangmai University, Thailand.

M. viridis, *M. aeruginosa* and *Phormidium* sp. were grown in MA medium (Ichimura 1979), whereas *N. linckia*, *Anabaena* sp., *Hapalosiphon* sp. DASH05101 and *Scytonema* sp. were cultured in BG11 medium (Richmond, 1986) under

continuous aeration at 25±1°C with a 12h/12h light/dark cycle with an average light irradiance of 400 μE/m^2/s for 3-4 weeks.

2.5.2 Sampling and DNA extraction

The samples of sediment from Lake Senba were collected during 1999 to 2000. The lake has a surface area of 33 ha and a water volume of 365,000 m^3. It is a very shallow lake, with a mean depth of 1.0 m, and the study site was depicted in Fig. 2.14. The sample collection was conducted during both blooming season (BS) from May to October and the non-blooming season (NS) from November to April.

DNA direct extraction from the sediment samples was developed in this study. One gram dry weight of sediment sample was washed in 0.1 M phosphate buffer (pH 7.0). After centrifugation, the pellet was resuspended with lysozyme solution and incubated at 37ºC for 30 min. The aliquot was added with 1 ml of 10% (w/v) SDS and incubated at 50ºC for 30 min. Then 20 μl of proteinase-K solution (20 mg/ml) was applied in the sample before being incubated at 65ºC for 1 h. The mixture, after the addition of 1 ml of 0.2 N NaOH, was shaken at room temperature for 15 min. Three cycles of freezing in liquid nitrogen and thawing at 70ºC were carried out. An equal volume of water-saturated phenol solution was added and the phases were mixed by intermittent vortexing and then separated by centrifugation at 8,500×g for 10 min. The nucleic acid in aqueous phase was precipitated with 2 volumes of isopropanol at -20ºC, washed with 70% (v/v) ethanol, dried and dissolved in TE buffer containing 1/10 RNase A, and then incubated at 55ºC for 10 min and stored at 4ºC for further analysis.

Crude DNA from the sediment was purified by application through a MicroSpin Sephacryl S-300 column (Edgcomb *et al.*, 1999) twice, before being

applied onto 1% (w/v) agarose gel electrophoresis at 80V for 2 h. The purified DNA was extracted from the agarose gel by using a Quantum Prep Freeze N Squeeze Spin Column and applied through a Sephacryl S-300 Microspin column again.

The DNA extraction method for cyanobacterial culture was followed by Teaumroong *et al.* (2002).

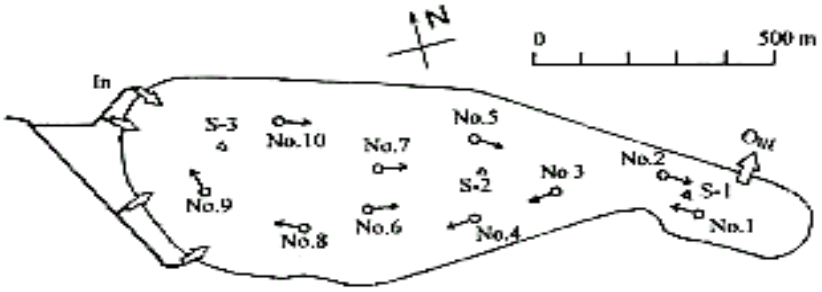

Figure 2.14 Sampling sites and the location of the ten USRS units arranged in Lake Senba, symbols: O; USRS, Δ; sampling sites. Arrows show the direction of the water jet current generated by USRS (Nakano *et al*., 2001).

2.5.3 PCR analysis

The DNA primers used were rRNA intergenic spacer analysis (RISA) (Borneman and Triplett 1997), *rpo*C1 (Wilson *et al.*, 2000) and microcystin synthesis gene *(mcyB)* (Tiquia *et al.*, 2002). The sequences of primers were summarized in Table 2.3.

The reactions were run in a Thermal cycler (GeneAmp®PCR System 9700, Perkin Elmer). The PCR was done in a 50 µl reaction mixture using 2.5 U of *Taq* polymerase (Promega, USA), the buffer supplied by Promega, 1.5 mM $MgCl_2$, 0.2 mM dNTPs and 500 nM (each) primer. For RISA, all reagents were combined and reaction was carried out in accordance with Borneman and Triplett's (1997) condition. For *rpo*C1, the mixture was run according to Wilson *et al*. (2000). For *mcy*B, the reactions

were heated at 94°C for 5 min, then PCR was performed with 35 cycles at 94°C for 30 sec, 60°C for 60 sec and 72°C for 120 sec, followed by elongation at 72°C for 10 min. The results were detected on 1% agarose gel electrophoresis staining with 10 mg/ml of ethidium bromide.

Table 2.3. Summary of primers used in this study

Gene region and Primers	Sequences	References
RISA		
1406F	5'-TGYACACACCGCCCGT-3'	Borneman and
23SR	5'-GGGTTBCCCCATTCRG-3'	Triplett
		(1997)
***rpo*C1**		
*rpo*C1-1	5'-GAGCTCYAWNACCATCCAYTCNGG-3'	Wilson *et al*.
*rpo*C1-T	5'-GGTACCNAAYGGNSARRTNGTTGG-3'	(2000)
***Microcystis* sp. specific *rpo*C1 fragment**		
MV1-f	5'-GATGGGAATAGCGAGACTAAAGCC-3'	This study
MV1-f-GC-clamp[a] (DGGE)	5'-GC-clamp-GATGGGAATAGCGAGACTAAAGCC-3'	
MV1-r	5'-AAGCTCCAAGAATCTTTAGGAGGA-3'	
***myc*B**		
$tox2^+$	5'-AGGAACAAGTTGCACAGAATCCGCA-3'	Kaebernick, *et*
$tox2^-$	5'-ACTAATCCCTATCTAAACACAGTAACTCA-3'	*al*. (2000)

Table 2.3. (continued)

Gene region and Primers	Sequences	References
16S rDNA		
27f	5'-AGAGTTTGATCCTGGCTCAG-3'	Martin-Laurent *et al.* (2001)
1392r	5'-ACGGGCGGTGTGTACA-3'	Amann *et al.* (1995)

[a]GC-clamp : 5'-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGG-3'

2.5.4 Designed *Microcystis* sp.-specific *rpo*C1 fragment

The *rpoC*1 PCR product of *M. viridis* was sequenced directly by a DNA sequencer (Applied Biosystems, USA) and aligned with amino acid of the other cyanobacteria obtained from the GenBank database to design the specific primer. The specific primers used were MV1-f and MV1-r (Table 2.3). The PCR product was used in a final PCR with MV1-f and MV1-r to give a 419 bp. Fifty µl PCR mixture contained 10-50 ng of sediment DNA, 500 nM (each) primer, 200 µM dNTPs, 1.5 mM $MgCl_2$ and 2.5 U *Taq* polymerase (Promega, USA) in reaction buffer (20 mM Tris-HCl [pH 8.0], 100 mM KCl, 0.1 mM EDTA, 1 mM DTT, 50% glycerol, 0.5% Tween®20 and 0.5% Nonidet®-P40). The thermal-cycling conditions were run with one cycle at 95°C for 3 min, then 35 cycles at 92°C for 60 sec, 45°C for 60 sec and 72°C for 60 sec, followed by elongation at 72°C for 10 min and the results were detected on 1% (w/v) agarose gel.

2.5.5 Analysis of PCR product by SSCP

The PCR products were denatured by heating at 95ºC for 5 min. The DNA was loaded with a loading buffer on 6% polyacrylamide-Tris-borate-EDTA gel containing 7 M urea for the SSCP technique with silver staining. Small pieces of selected SSCP bands were punched from the gel. The PCR products of the cutouts were reamplified based on 16S rDNA by PCR (Table 2.3). Before being sequenced, the PCR products were purified with a QIAquick Spin PCR Purification kit (Qiagen, Germany). The purified PCR products were sequenced directly by using an ABI model 310 automated DNA sequencer (Applied Biosystems) and a BigDye terminator cycle sequencing kit (Applied Biosystems). All the sequences were compared with similar sequences of reference organisms by a BLAST search.

2.5.6 Analysis of PCR product by DGGE

The MV1-f primer which is specific for *M. viridis* contains at its 5' end a 40-base GC clamp (Table 2.3) to stabilize the melting behavior of the DNA fragment. The MV1-r primer was used as a reverse primer (Table 2.3). The PCR was performed with a Thermal cycler (Applied Biosystems, USA). The PCR mixture contained 10-50 ng of genomic DNA of the bacterial isolates or 1 μl of DNA preparations from sediment samples, 500 nM of each primer, 800 μM dNTPs, 2.5 mM $MgCl_2$, 0.1% (v/v) BSA and a PCR buffer (Promega, USA). The sample was first incubated at 95°C for 10 min to denature the DNA, then 5 U of *Taq* DNA polymerase (Promega, USA) was added. PCR conditions were 35 cycles at 92°C for 120 sec, 45°C for 60 sec and 72°C for 120 sec, followed by elongation at 72°C for 10 min.

The PCR product obtained from the genomic DNA of pure culture and the extracted DNA from the sediment were used for separation in a denaturing gradient gel. 300 μl of the PCR product were pooled, precipitated and resuspended in 30 μl of TE buffer. Before loading to the DGGE gel, the PCR products were incubated at 95°C for 5 min and gradually cooled to 4°C to avoid non-complementary annealing of DNA. Gels for DGGE were 6% polyacrylamide gel (6%-acrylamide and N, N-methylenebisacrylamide solution (37.5:1, v/v), 40% (v/v) formamide, 7 M urea and 1X TAE) containing a linear gradient of the denaturant concentration ranging from 25% to 60%. The denaturing gradient gel was run for 300 min at 60°C and 250V by the Dcode system (Bio-Rad, USA). After completion of electrophoresis, the gels were stained in an ethidium bromide solution (0.5 μg/ml) and documented on Gel documentation and analysis (Ultra Violet Product, USA).

2.5.7 Analysis of PCR product by T-RFLP

For the T-RFLP analysis, primer 27f end-labeled with 6-FAM (5-[6]-carboxy-fluorescien, Operon Technology, Alameda, CA, USA) and unlabeled 1392r primers were used to an amplified extracted DNA sample from the samples of the sediment (Table 2.3). The PCR mixture contains 10-50 ng of genomic DNA of the bacterial isolates or 1 μl of DNA preparations from the sediment samples, 500 nM of each primer, 800 μM dNTPs, 2.5 mM $MgCl_2$, 0.1% BSA and PCR buffer (Promega, USA). The sample was first incubated at 94°C for 3 min to denature the DNA, then 5 U of *Taq* DNA polymerase (Promega, USA) was added. PCR conditions were 35 cycles at 94°C for 30 sec, 59°C for 15 sec and 72°C for 60 sec, followed by elongation at 72°C for 15 min. The PCR products were purified using a Qiaquick® PCR purification kit

(Qiagen). The purified PCR products (10-50 ng) were digested with 1 U of the *Msp*I and *Hha*I restriction endonuclease (Promega, USA) for 1 h at 37°C. The digested samples were analyzed on an ABI PRISM™ 310 Genetic Analyzer (Applied Biosystems, USA). The sizes of fragments were compared with internal standards and determined by the GeneScan software (Applied Biosystems, USA).

2.6 Results and Discussion

2.6.1 PCR analyses

The cyanobacteria from the sediment were enumerated by the plate count method. These results suggested that the cyanobacterial population in the sediment from the blooming season was higher than the non-blooming season, about 360 cells /g dry weight of sediment and 150 cells /g dry weight of sediment, respectively. Whereas unicellular cell population in the blooming season sediment was lower than the non-blooming season sediment, about 90 cells /g dry weight of sediment and 120 cells /g dry weight of sediment, respectively. The results indicated that the unicellular cyanobacteria might precipitate in the sediment during the non-blooming season. The unicellular cells showed a colony type in the *Synechocystis*-group, expected to be a *Microcystis* cluster. The cells are spherical to oval and vary from 3 to 8 μm in diameter; some cells occur singly or in pairs with light refractile gas vacuoles (data not shown) that are characteristic of a natural population of *Microcystis* (Stanier *et al.* 1971). The results from a conventional plate count were further confirmed using the molecular genetic techniques. The microbial community DNA from the sediment at

Lake Senba in each season was directly extracted, and the yield was about 0.3 μg /g dry weight of sediment.

Since the detection and analysis of the cyanobacterial morphology from the sediment samples performed by the conventional plate count method was inconclusive, the analysis had to be combined with molecular approaches. The rRNA intergenic spacer analysis (RISA) fingerprinting was used because it is easy to perform, allows the rapid examination of the composition of complex bacterial communities, and can also be performed without the use of specific and expensive equipment. In this case, the rRNA internal spacer analysis was represented because this spacer was more variable in size than the 16S rRNA gene (Martinez *et al.*, 1999). Enzymatic amplification of rRNA intergenic spacer and *rpo*C1 were performed on DNA extracted from the winter, spring and summer sediment samples.

Application of the RISA analysis in the PCR from the samples of the sediment and reference strains yielded multiple distinct DNA products ranging in size from approximately 400 to 1,500 bp (Fig. 2.15). In the case of the *rpo*C1 analysis, *Scytonema* sp. showed two band products with sizes about 650 and 900 bp. *N. linckia* showed four band products with sizes about 400, 650, 900 and 1,400 bp. *M. aeruginosa* showed three band products with sizes about 400, 650 and 900 bp. *Phormidium* sp. showed a one band product with a size about 700 bp. *Hapalosiphon* sp. DASH05101 showed two band products with sizes about 650 and 1,400 bp, and *M. viridis* showed four band products with sizes about 500, 600, 800 and 1,300 bp. However, the bands of sediment samples on 1 % agarose gel electrophoresis could not be clearly compared with the reference strains even though they yielded distinct DNA products ranging in size from approximately 250 to 1,500 bp (Fig. 2.16).

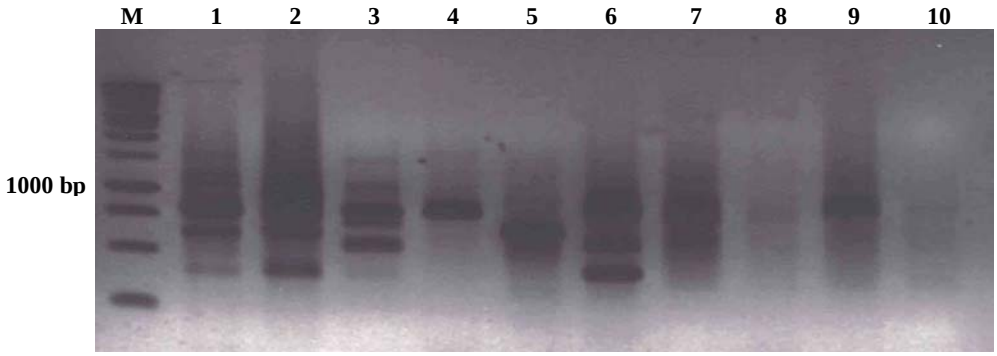

Figure 2.15 RISA analysis patterns on 1% agarose gel. Lane M; 1 kb ladder marker, lane 1; *Hapalosiphon* sp. DASH05101, lane 2; *Scytonema* sp., lane 3; *N. linckia*, lane 4; *Phormidium* sp., lane 5; *M. viridis*, lane 6; *M. aeruginosa*, lane 7; sediment from winter+*M. aeruginosa*, lane 8; sediment from winter lane 9; sediment from spring and lane 10; sediment from summer.

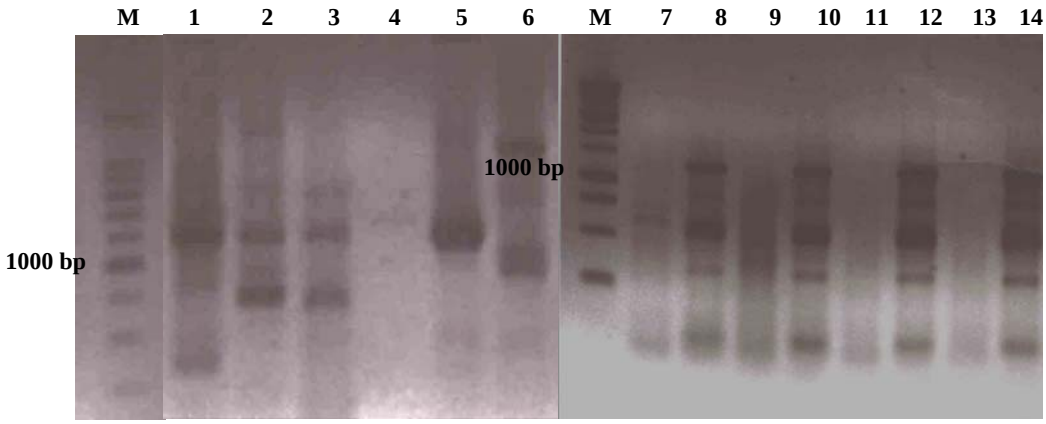

Figure 2.16 *rpo*C1 analysis patterns on 1% agarose gel. Lane M; 1 kb ladder marker, lane1; *Scytonema* sp., lane 2; *N. linckia*, lane 3; *M. aeruginosa*, lane 4; *Phormidium* sp., lane 5; *Hapalosiphon* sp. DASH05101, lane 6; *M. viridis*, lane 7; sediment from spring, lane 8; sediment from spring+*M. viridis*, lane 9; sediment from summer, lane 10; sediment from summer+*M. viridis*, lane 11; sediment from winter, lane 12; sediment from winter+*M. viridis*, lane 13; sediment from winter+*M. aeruginosa* and lane 14; sediment from winter+*M. viridis*.

The results on single strand conformation polymorphism (SSCP) had better resolution than agarose gel electrophoresis and showed different community structures in Lake Senba in each season. A possible reason for the formation of more than one product from a pure culture is that the universal primers amplified more than one operon. It is widely recognized that several bacterial species contain more than one 16S rRNA gene in their genomes. Another reason for detecting more than one fragment from pure culture by PCR-SSCP was the formation of metastable conformers, i. e., where the same molecule folds into more than one conformation with different electrophoresis mobilities (Clapp, 1999). Thus the PCR products were again distinguished on the basis of the single strand conformation polymorphism technique (SSCP). Four signal bands of the PCR product based on the rRNA intergenic spacer were similar with the band product of *M. aeruginosa* with sizes about 500, 650, 720 and 1,000 bp (Fig. 2.17). Those band products were sequenced and compared with the database in the GenBank. The results were not identical correlation with *M. aeruginosa* (data not shown). In contrast, the RISA analyses were different from the *rpo*C1-PCR analysis because there were no signal bands at the same size when compared with the reference strains (lanes 1 to 5 in Fig. 2.18). Nevertheless, the results indicated that the sediment samples from the spring season showed higher conformation than the sediment samples from the summer and winter seasons, respectively. Phylogenetic trees devided with *rpo*C1 data match those derived with 16S rRNA data. When the third codon position is included, *rpo*C1 shows greater divergence between two related strains and is thus better able to resolve genus-species-level questions than 16S rRNA. In general, the use of RNA polymerase gene sequences as a phylogenetic tool is increasing in other taxonomic groups as well

(Klenk and Zilling, 1994). Wilson *et al*. (2000) also used *rpo*C1 to design species-specific PCR of *Cylindrospermopsis raciborskii* and concluded that a region of *rpo*C1 gene unique to *C. raciborskii* able to develop for the specific identification *C. raciborskii* from both purified genomic DNA and environmental samples. Thus, PCR primers designed from conserved regions of the cyanobacterial *rpo*C1 gene were used to analyze the *Microcystis* sp. in the sediment samples. In a previous study, these primers were used in PCRs for strain-level identification of a number of taxonomic groups to study the diversity of the cyanobacterial genus *Synechcoccus* (Toledo and Palenik 1997). In addition, the primers have been used to examine the phylogenetic relationship of prochlorophytes to each other and to the green chloroplasts (Rippka and Herdman 1992) and to analyze the *C. raciborskii* isolates (Wilson *et al.*, 2000). Thus, the *rpo*C1 gene might differentiate strains of *Microcystis* sp. isolated from the sediment of Lake Senba. However, larger community structures were found in the summer and spring seasons because the conditions such as transparency, temperature and suspended solids in Lake Senba were suitable for growth.

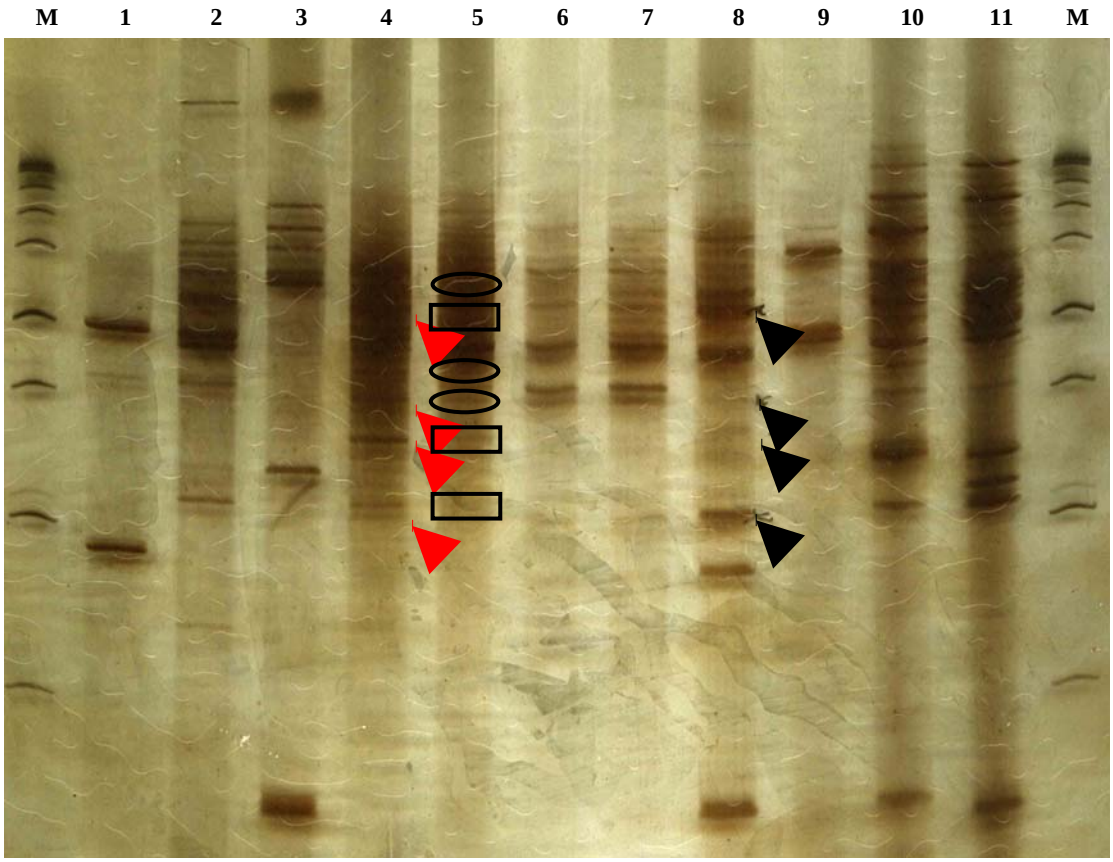

Figure 2.17 rRNA intergenic spacer patterns on SSCP from sediment samples (RISA). Lane M; 1 kb ladder marker, lanes 1 to 4 and lanes 9 to 11; reference strains following by; *Hapalosiphon* sp. DASH05101, *Scytonema* sp., *M. viridis, M. aeruginosa, Anabaena* sp., *Phormidium* sp. and *N. linckia*, respectively, lane 5; sediment from winter + *M. aeruginosa* (the combination with lane 4 and 6 were shown in boxes and oval circle, respectively), lanes 6; sediment from winter, 7; sediment from spring and lane 8; sediment from summer.

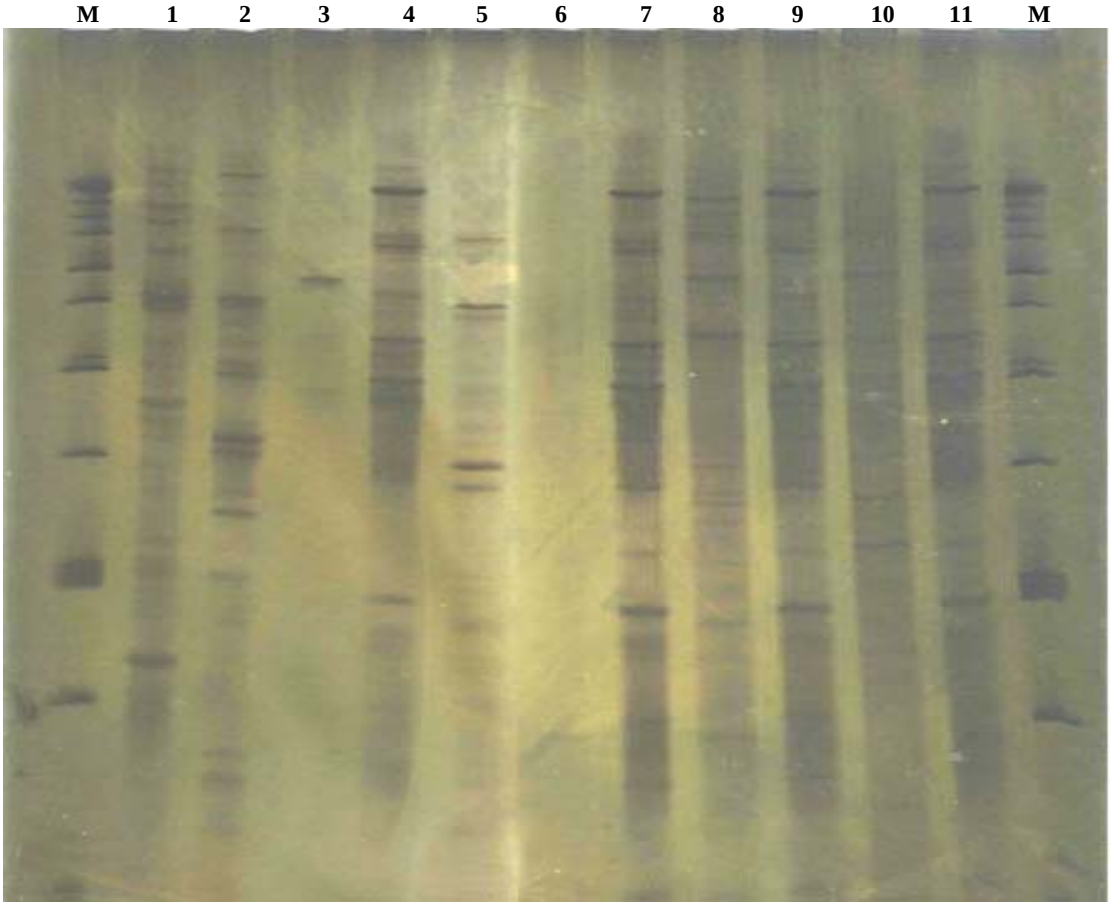

Figure 2.18 *rpo*C1 analysis patterns on SSCP from sediment samples. Lane M; 1 kb ladder marker, lanes 1 to 5; reference strains, as *Scytonema* sp., *N. linckia*, *Phormidium* sp., *M. viridis* and *M. aeruginosa*, respectively, lane 6; sediment from winter, lane 7; sediment from winter + *M. viridis*, lane 8; sediment from spring, lane 9; sediment from spring + *M. viridis*, lane 10; sediment from summer and lane 11; sediment from summer + *M. viridis*.

Use of the RISA and *rpo*C1 as a primer could not clearly distinguish *Microcystis* sp. from the other microorganisms in the sediment samples. Therefore, a specific primer was designed for detecting *Microcystis* sp. The PCR product based on

*rpo*C1 was sequenced and aligned with amino acid of the other cyanobacteria. There was sufficient difference between the *rpo*C1 amino acid alignment of *Microcystis* sp. and the other cyanobacteria at the positions 256 to 333. Primers MV1-f and MV1-r were used to amplify a 419 bp diagnostic PCR product from the *rpo*C1 gene of *Microcystis* sp. Then extracted DNA was amplified and the results were illustrated in Fig. 2.19. Although DNA from every reference strain could be amplified with the specific primer, the major band of *Microcystis* sp. appeared at about 400 bp (lanes 3 and 6, Fig. 2.19). Furthermore, the *Microcystis* sp. specific *rpo*C1 fragment was also used to analyze DNA from the sediment samples. The results showed no signal band that correlated with *Microcystis* sp. (data not shown).

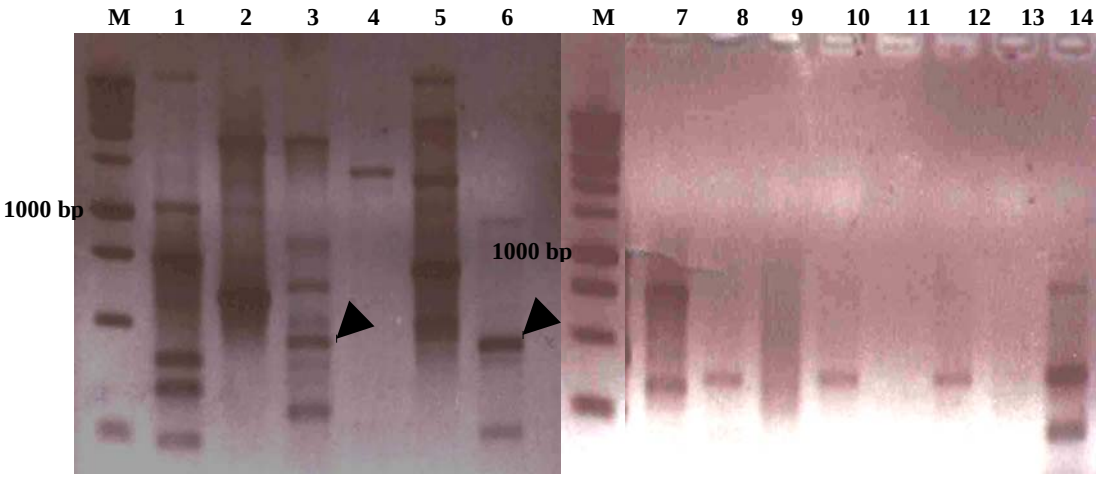

Figure 2.19 *Microcystis* sp.-specific *rpo*C1 fragment analysis from sediment samples. Lane M; 1 kb ladder marker, lanes 1 to 6; reference strains, *Scytonema* sp., *N. linckia*, *M. aeruginosa*, *Phormidium* sp., *Hapalosiphon* sp. DASH05101 and *M. viridis*, respectively, lane 7; sediment from spring, lanes 8; sediment from spring + *M. viridis*, lane 9; sediment from summer, lane 10; sediment from summer + *M. viridis*, lane 11; sediment from winter, lane 12; sediment from winter + *M. viridis*, lane 13; sediment from winter and lane 14; sediment from winter + *M. aeruginosa.*

To confirm the results of the PCR analyses, *mcy*B was also performed with DNA from the sediment samples of both the blooming and non-blooming seasons. There were 3 bands of PCR products from *M. viridis* in size about 400, 750 and 1,000 bp, on the other hand, no signal bands on all sediment samples were found (Fig. 2.20). Microcystin is produced nonribosomally via a multifunctional enzyme complex, consisting of both peptide synthetase and polyketide module codes for the *mcy* gene cluster (Teske *et al.*, 1996). *mcy*B, the number of microcystin synthetase gene clusters was observed in the sediment samples, and were also not found in the PCR products in this case. The morphology of *Microcystis* also related to a *mcy*B genotype, for example, the *Microcystis* population of Lake Wannsee (Germany) consists mainly of the morphospecies *M. aeruginosa*, *M. flos-aquae* and *M. ichthyoblabe*. Those morphospecies also differ significantly in the percentage of microcystin genotype; i. e. 73% of colonies assigned to *M. aeruginosa* contain *mcy*B, while only 16% of colonies assigned to *M. ichthyoblabe* contain it (Kurmayer *et al.*, 2003). *M. aeruginosa* has frequently been reported to form large and firm colonies, while the colonies of *M. ichthyoblabe* are typically small and fragile (Watanabe, 1996). Kurmayer and Kutzenberger (2003) reported that the large colonies of *Microcystis* (>100 μM) had a high *mcy*B proportion. However, *Microcystis* normally has a size about 3-8 μM (Rippka *et al*., 1979) as in this study, thus the results mentioned above tended to represent the disappearance of *Microcystis* sp. in the Lake Senba sediment.

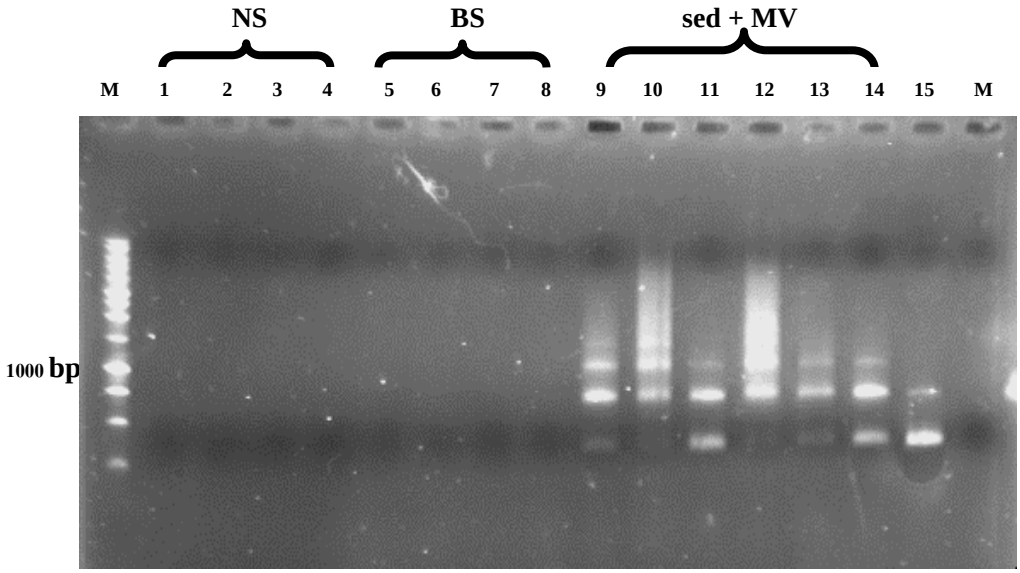

Figure 2.20 *mcy*B analysis from sediment samples. Lane M; 1 kb ladder marker, lane 1 to 4; NS, lane 5 to lane 8; BS, lane 9 to 14; sed+MV, lane 15; MV and lane 16; NC. (NS; sediment from non-blooming season, BS; sediment from blooming season, sed +MV; sediment sample + *M. viridis*, MV; *M. viridis* and NC; negative control).

2.6.2 DGGE analyses of PCR products based on *Microcystis* sp.-specific *rpo*C1 fragment

To enhance the resolution of the detection system, the DGGE technique was employed. Figure 2.21 showed a DGGE analysis pattern of the PCR products obtained after amplification based on a *Microcystis* sp.specific *rpo*C1 fragment of four DNA aliquots from sediment in the non-blooming season (lanes 2 to 5), four DNA aliquots from sediment in the blooming season (lanes 6 to 9) and one from the purified *M. viridis* (lane 1). There were 6 fragments found from the *M. viridis* pure culture. Several fragments appeared in three lanes (lanes 3 to 5) of the sediment from the non-blooming season; however, the fragments were disappesred the equal-size with *M.*

viridis. Each band in the electrophoresis pattern presumably originates from one bacterial species present in the original material. In contrast, each lane of sediment from the blooming season showed no fragments. The complex patterns of the DGGE fragment were derived from the bacterial populations and their nucleic acid. Thus the results indicated that there was a larger community of bacteria from the sediment samples in the non-blooming season than from the sediment in the blooming season. The finding of radiations of highly similar *Microcystis* sp.-specific *rpo*C1 fragment sequences contained in the DGGE bands also has an interesting implication for the population dynamics and ecological functions of sediment samples. The appearance and disappearance of a DGGE band reflects the increase and decrease of the corresponding bacterial population, respectively. Intensities of different DGGE bands derived from different bacterial species, do not allow quantitative conclusions about the abundance of the different bacteria, because of a possible unknown PCR basis in the amplification of different templates (Teske *et al*., 1996).

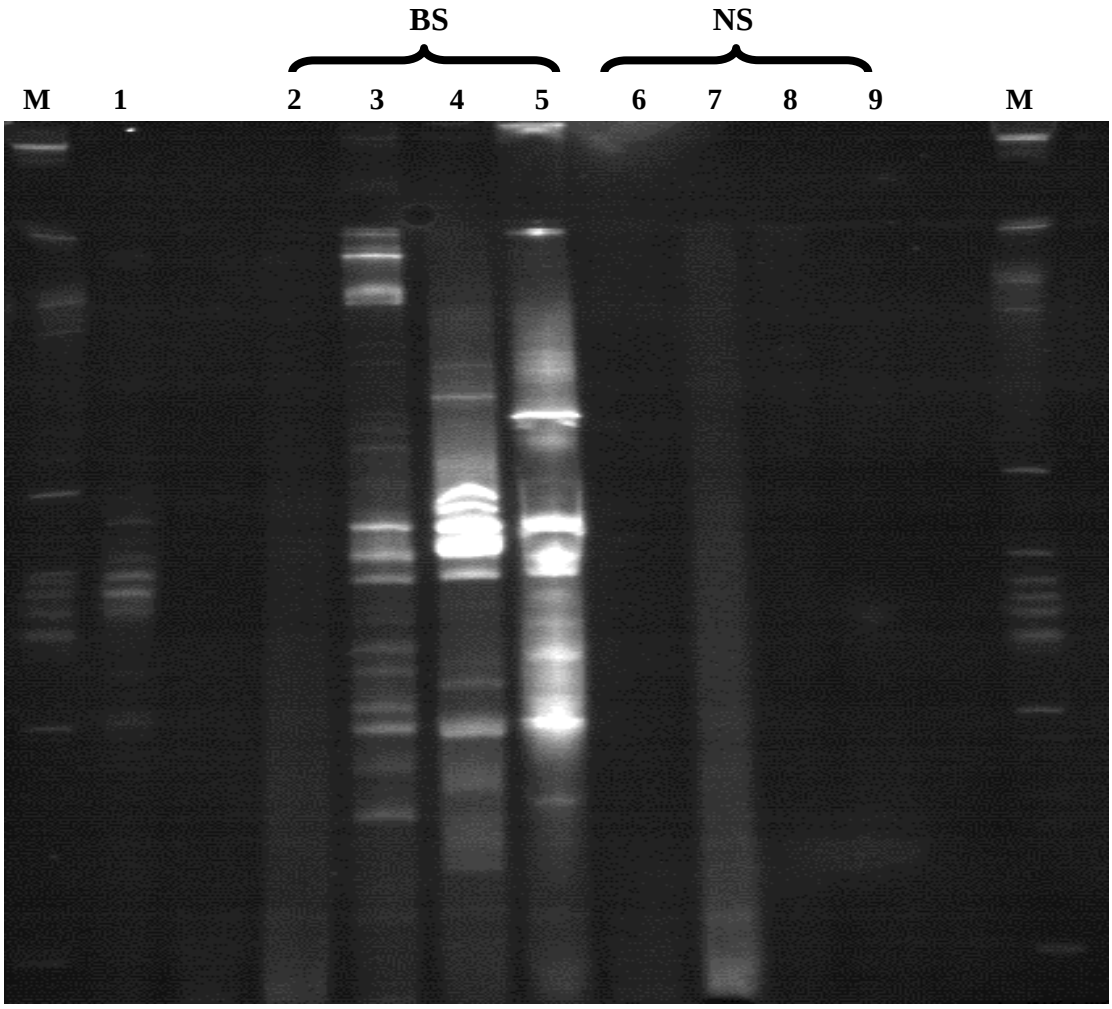

Figure 2.21 Ethidium bromide-stained DGGE pattern of PCR product derived from *Microcystis* sp.-specific *rpo*C1 fragment. Lane M; 1 kb ladder marker, lane 1; MV, lanes 2 to 5; NS, lanes 6 to 9; BS and lane M. (MV; *M. viridis*, NS; sediment from non-blooming season, BS; sediment from blooming season, lane 2 to lane 9 represent the collection time; January to August).

2.6.3 T-RFLP analyse of PCR products based on 16S rDNA

The diversity of 16S rDNA in two different bacterial sediment samples was examined with a T-RFLP analysis. The T-RFLP analysis detected relatively few peaks in the sediment samples which were digested with both restriction enzymes *Hha*I and *Msp*I. The T-RFLP pattern of the sediment samples showed at least three major T-RFLP patterns of 84 bp, 91 bp, and 208 bp lengths based on restriction enzymes *Hha*I (Fig. 2.22a). The T-RFLP pattern of 91 bp length matched with the T-RFLP of *M. viridis.* While the T-RFLP pattern obtained from the sediment samples which were digested with *Msp*I, the results indicated that only one major T-RFs 477 bp lengths and matched with T-RFLP of *M. viridis* (Fig. 2.22b). Eventhough the T-RFLP technique based on 16S rDNA showed the 91 bp length and 477 bp length were matched with *M. viridis* by digestion of *Hha*I and *Msp*I, respectively, the T-RFLP with *Hha*I endonuclease digestion patterns of *M. viridis* were 35 bp, 75 bp, 91 bp, 119 bp and 373 bp lengths. While the T-RFLP with *Msp*1 endonuclease digestion patterns of *M. viridis* were 35 bp, 75 bp, 119 bp and 477 bp lengths, the T-RFLP pattern of the sediment samples that seem to be *M. viridis* should have a peak size of 75 bp length. Therefore, the results indicated that *M. viridis* might not remain in the Lake Senba sediment. Furthermore, the T-RFLP patterns also indicated more diversity of microorganisms in the non-blooming season than the blooming season. Corresponding with the T-RFLP pattern, T-RFLP fingerprints of the 16S rRNA gene from sediment samples were surprisingly similar in each season. While significant differences in the T-RFLP-based diversity indices (Shannon diversity index and equitability index) were not observed, the T-RFLP patterns showed quite a larger community of a bacterial population during the non-blooming season than during the blooming season. When

Microcystis sp. blooms cover the surface area of the Lake and decrease the light intensity and O_2 for microorganisms, the community of microorganisms decreases in the blooming season. The results also showed no excised band product similar to *Microcystis* sp. Nevertheless, supposing a constant PCR amplification bias for (or against) a specific bacterial *Microcystis* sp.-specific *rpo*C1 fragment, T-RFLP patterns clearly demonstrated that there was no major band which indicated specificity with the *M. viridis* fragment; therefore, the results suggested that *M. viridis* might not remain in the Lake Senba sediment after algal bloom was controlled by the addition of ultrasonic radiation and jet circulation to the flushing treatment.

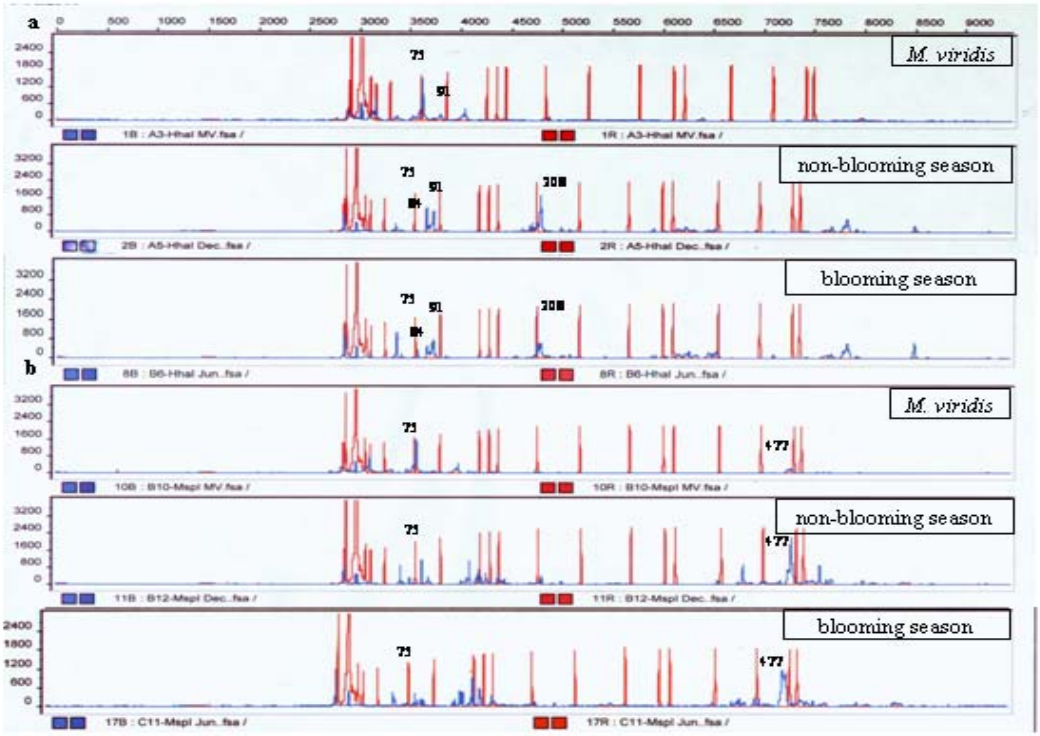

Figure 2.22 T-RFLP analysis of 16S rDNA in sediment samples: a; T-RFLP patterns with the *Hha*I endonuclease digestion and b; T-RFLP patterns with the *Msp*I endonuclease digestion. (red; size standard GeneScan-500 pattern and blue; T-RFLP pattern of sediment samples).

2.7 Conclusions

Lake Senba is a small recreational lake in Mito City, Japan, where many people go for relaxation. Recently, the lake has been plagued by water blooms that are dominated by *Micocystis*. *Microcystis*, known as toxic microcystin producing cyanobacteria, normally bloom in summer and drops during winter in this lake. There is increasing pressure from the citizens of Mito City for drastic measures to control these bloom. The integration of ultrasonic radiation and jet circulation to flushing was used for treated the Senba Lake. The ultrasonic radiation was sufficient for the destruction of gas vacuole and buoyant ability of cyanobacteria. It also inflicted damage on its photosynthetic machinery. Consequently, the damage would delay growth recovery and slow growth rate. While the water jet circulation module might also be effective for preventing stagnant regions which favor algal growth. After treated Senba Lake, a molecular genetic monitoring technique on basis of DNA direct extraction from sediment was applied to confirm whether *M. viridis* still existed.

Three primer sets were employed for PCR based on rRNA intergenic spacer analysis (RISA), the DNA dependent RNA polymerase (*rpo*C1) and *Microcystis* sp.-specific *rpo*C1 fragment. The results from each primer were demonstrated as single strand conformation polymorphism (SSCP). Using RISA primer showed different results from *rpo*C1 and *Microcystis* sp.-specific *rpo*C1 fragment, while *rpo*C1 *Microcystis* sp.-specific fragment was more specific than using RISA primer.

Therefore, *Microcystis* sp.-specific *rpo*C1 fragment was further analyzed by denaturing gradient gel electrophoresis (DGGE). The DNA pattern representing *M.viridis* could not be detected in any sediment samples. However, the results were further confirmed with another technique such as terminal restriction fragment length

polymorphisms (T-RFLP). Although, T-RFLP patterns of 16S rDNA in sediment at 91 bp and 477 bp length were matched with T-RFLP of *M. viridis* (*Hha*I and *Msp*I endonuclease digestion, respectively). Nevertheless, T-RFLP pattern of 75 bp were not matched with *M. viridis* (both of *Hha*I and *Msp*I endonuclease digestion) which were the major T-RFLP pattern of *M. viridis*. Therefore, the results most likely indicated that *M. viridis* seems to be disappeared by ultrasonic radiation and jet circulation to flushing treatment (Innok *et al*., 2005).

2.8 References

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CHAPTER III

N_2-FIXING CYANOBACTERIA

3.1 Abstract

Nostoc sp. strain VICCR1-1 was isolated from rice in rotation of other crops cultivation showed the highest nitrogen fixation efficiency about 11 μmol C_2H_4/h/mg total chlorophyll a. The *Nostoc* sp. strain VICCR1-1 was induced in order to form heterocysts and akinetes on basis of nutrient modification. Absence of $CaCl_2$ played the role of heterocyst differentiation which was induced as high as 46.61%. The number of heterocyst was induced up to 62.59% when transferred the cyanobacterial cells from BG11 to $BG11_0$ (without $CaCl_2$) medium. Proteins were extracted after heterocyst induction. There were 72 KDa and 140 KDa proteins expected to be chaperonin GroEL (HSP60 family) and phycobilisome core-membrane linker protein, respectively in both the medium with and with out N-source. Besides protein in size 45 KDa (expected to be outer membrane protein, porins) was up-regulated only when grown in $BG11_0$ (without $CaCl_2$) medium. In case of akinetes induction, phosphorus and iron were found to be the critical composition in akinete differentiation, especially when lack of both elements. The number of akinete cells could be increased up to 21.17% compared with culturing in normal condition ($BG11_0$ medium). The gene expression which involved heterocysts and akinetes differentiation was observed based on *het*R (heterocyst differentiation), *sod*F and *ava*K (akinete development). The results suggested that only *het*R expression alone could not be the indicator for

heterocyst development and *sod*F and *ava*K were not detected during akinete differentiation.

The germination of akinete cell was tested under various stress conditions. Cells could well germinate under the broad range of pH from 3 to 10, at high temperature as 40ºC and high salinity as 0.5 M NaCl, eventhough grown on these conditions for 7 days. In order to prepare akinete inoculum, akinete cells were homogeneously mixed with montmorillonite clay at 4.0×10^6 cfu/g of montmorillonite clay. The akinetes could survive in the montmorillonite clay in constant number up to 1 year. The cells were still survived for 3.3×10^5 cfu/g of montmorillonite clay. To test the effect of *Nostoc* sp. strain VICCR1-1 as biofertilizer with rice, inoculum was applied in amount 2.8×10^6 cfu/m^2 in the field. After harvesting, the grain yields from chemical-N fertilizer, vegetative cells and akinete inoculum treatments were not significantly different. To monitor the persistence of *Nostoc* sp. strain VICCR1-1 after harvesting, the MPN-DGGE technique using 16S rRNA gene was employed. The results indicated that the remaining population is at 1.0×10^7, 2.5×10^5 and 1.62×10^6 cell/m^2 in treatments supplied N-fertilizer, vegetative cells and akinete inoculum, respectively.

3.2 Introduction

Nitrogen fixation by cyanobacteria was firstly reported in 1889, shortly after the significance of legume root nodule was first shown. Cyanobacteria have been classified on morphological and anatomical ground-type of cell, branched or unbranched filaments etc. and also by physiological attributes. There are three main types of cell (Fig. 3.1) such as i) vegetative, ii) heterocyst and iii) akinete cells.

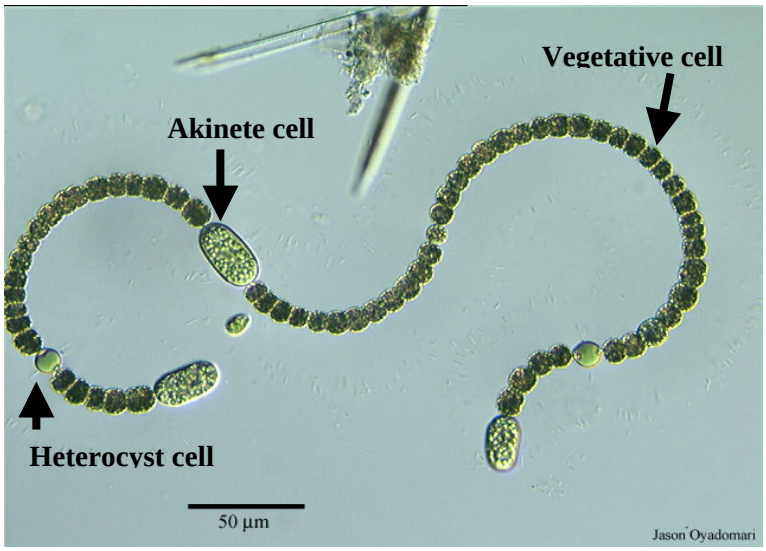

Figure 3.1 Three types of cyanobacterial cell (www, 2005).

3.2.1 Cyanobacterial heterocysts

Cyanobacteria are oxygenic phototrophs. Under conditions where N_2 is the only nitrogen source, some genera of filamentous cyanobacteria (e. g. *Anabaena, Nostoc* and *Cylindrospermum*) can convert their vegetative cells into heterocysts. Differentiation of vegetative cells into heterocysts occurs at regular intervals within each filament and is completed within 20-24 hrs. Heterocysts are special cells with the primary function of N_2 fixation. Mature heterocysts are slightly larger than vegetative cells and are enveloped by two thickened layers external to the vegetative cell wall such as laminated glycolipid layer and fibrous polysaccharide layer which are a diffusion barrier for gases. They serve to protect the nitrogenase of heterocysts from molecular oxygen. To fulfill their function in N_2 fixation, heterocysts thus contain: i) photosystem I; cyclic electron transport through photosystem I components contributes to the establishment of a proton motive force that is used for ATP synthesis by ATP synthase and ii) increased respiration rate; the increased respiration rate serves to scavenge residual O_2 for additional protection of nitrogenase. In addition, aerobic

respiration contributes to establishment of a proton motive force that is used for ATP generation by ATP synthase. In heterocysts, ATP is generated by the following two mechanisms such as electron transport phosphorylation (ETP); using the proton motive force established by cyclic electron transport through photosystem I and by electron transport through the respiratory chain. And substrate level phosphorylation (SLP); during the oxidation of organic carbon. However, to support the function of N_2 fixation, therefore heterocysts lack: i) Photosystem II; this is the oxygen-evolving photosystem that generates NADPH using electrons derived from H_2O and ii) ribulose biphosphate carboxylase; this is the key enzyme of the Calvin cycle for CO_2 fixation. Because heterocysts are not able to fix CO_2, they receive organic carbon compounds (likely sucrose) from surrounding vegetative cells in the cyanobacterial filament. The heterocysts depend on organic carbon compounds for the following reasons; i) the oxidation of organic carbon compounds provides heterocysts with NAD(P)H that they require for N_2 fixation, ammonia assimilation and aerobic respiration. ii) Heterocysts need organic carbon to generate 2-oxoglutarate for ammonia assimilation by the following reaction:

$$\text{2-oxoglutarate} + NH_3 + \text{NADPH} + H^+ \xrightarrow{\text{GDH}} \text{glutamate} + H_2O + NADP^+$$

$$\text{glutamate} + NH_3 + \text{ATP} \xrightarrow{\text{GS}} \text{glutamine} + \text{ADP} + \text{Pi}$$

$$\text{2-oxoglutarate} + \text{glutamine} + \text{NADPH} + H^+ \xrightarrow{\text{GOGAT}} 2\text{glutamate} + NADP^+$$

overall reaction: $\text{2-oxoglutarate} + 2NH_3 + \text{NADPH} + H^+ + \text{ATP} \longrightarrow \text{glutamine} + NADP^+ + \text{ADP} + \text{Pi} + H_2O$

(GDH: Glutamate dehydrogenase, GS: Glutamine synthetase, GOGAT: Glutamate synthase)

The heterocysts supply combined source of nitrogen (glutamine from ammonia assimilation) to the surrounding vegetative cells in the filament and, in turn, receive organic carbon compounds (likely sucrose). Microplasmodesmata join the cytoplasmic membranes of the heterocysts and its neighboring vegetative cells to facilitate this nutrient exchange (Fig. 3.2).

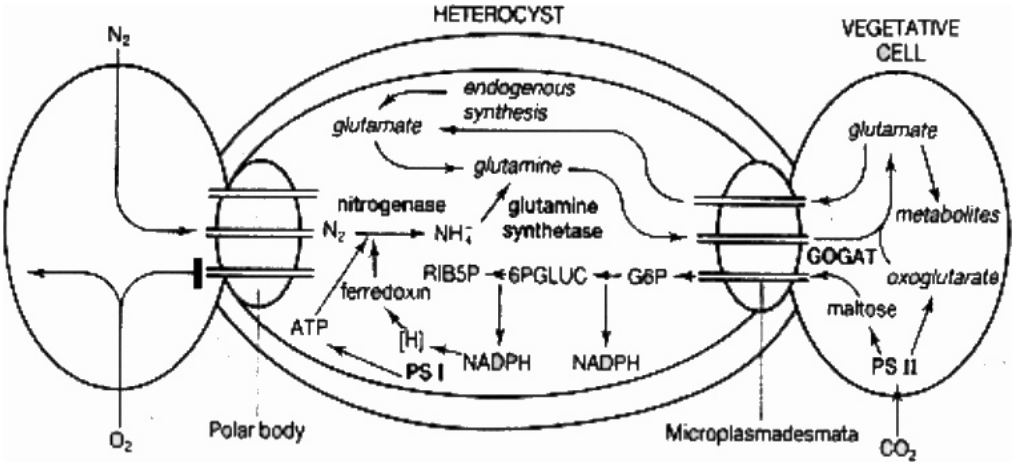

Figure 3.2 Diagrammatic representation of carbon and nitrogen exchange between the heterocyst and vegetative cells (www, 2006).

In addition to the metabolic changes discussed above, the genome of heterocysts is rearranged during differentiation. This genome rearrangement involves excision of two intervening sequences in operons encoding components required for synthesis of nitrogenase. Failure to remove these intervening sequences results in the inability for differentiation and N_2 fixation (www, 2001).

3.2.2 Nitrogen fixation

An in depth examination of the biological, biochemical and genetic aspects of biological nitrogen fixation exceeds the purview if primary interest of most soil

microbiologists. Yet a foundational understanding of some essential properties of the enzymes and the organisms involved is necessary to comprehensed the properties of the soil ecosystem that control the dynamics of nitrogen fixation in native ecosystem. Primary traits of biological nitrogen fixation controlling the yields of fixed nitrogen in native soils and the kinetics of this production to be considered herein are i) the complexity of the enzyme (nitrogenase) catalyzing the reduction of dinitrogen (N_2) to ammonium, ii) the quantities and sources of the energy required to drive the process and iii) the diversity of microbes responsible for the process.

Diazotrophs are ubiquitous in the earth's soils and exhibit a range of metabolic life styles. Represented in almost 100 prokaryotic genera, diazotrophs are distributed over most of the major phylogenetic divisions (Masepohl and Klipp, 1996). Genes involved in nitrogen fixation, *nif* genes, of evolutionary diverse diazotrophs are remarkably conserved. Nitrogen fixation appears to be a widespread activity in microorganisms and the reason many bacteria are not considered diazotrophic is because they have not yet been tested (Young, 1992). All diazotrophic utilize the same basic biochemical machinery for nitrogen fixation which is carried out by the nitrogenase enzyme system (Kim and Rees, 1994). The genes involved in nitrogen fixation have a very ancient origin, perhaps even in the ancestor of all of today's organisms. Nitrogen fixation must have first occurred around 3 billion years ago under an anaerobic atmosphere, after bacteria had developed the ability to fix carbon dioxide. It is believed that carbon dioxide fixation in due course led to nitrogen limitation, setting the scene for the evolution of nitrogen fixation. The next metabolic process to evolve was oxygenic photosynthesis, probably in cyanobacteria, which posed a

problem for nitrogenase because of its high sensitivity to oxygen, oxygen competed nitrogenase to bound with e^- from fererdoxin. (Sprent and Raven, 1992; Young, 1992).

Under optimal conditions at 30° C in the presence of saturating nitrogen, nitrogenase catalyzes the reaction:

$$8H^+ + 16MgATP + 8e^- + N_2 \longrightarrow 2NH_3 + H_2 + 16\ MgADP + 16Pi$$

Nitrogenase is a promiscuous enzyme that catalyzed the reduction of dinitrogen to ammonia, the reduction of protons to hydrogen, which appears to be an obligatory part of ammonia formation and the reduction of small unsaturated molecules such as acetylene, azide and cyanide (Kim and Rees, 1994; Oelze and Klein, 1996). The function and synthesis of nitrogenase are expensive metabolic processes requiring a considerable fraction of the cellular pool of energy and metabolites (each electron transfer is accompanied by the hydrolysis of two ATP molecules). Hence, as a cellular strategy to economize energy, nitrogenase is subject to strict control by the intracellular concentration of ammonium and other forms of combined nitrogen (Oelze and Klein, 1996; Smith and Eady, 1992) and is activated only under nitrogen deficient conditions (Fig. 3.3).

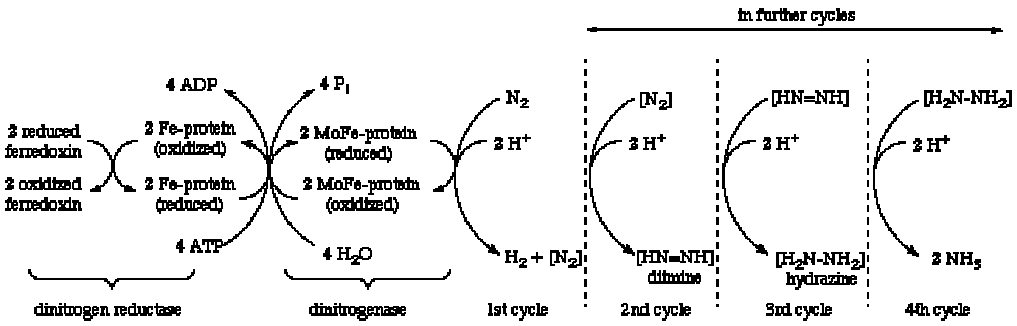

Figure 3.3 The two components system for atmospheric nitrogen fixed via nitrogenase function (www, 2006).

3.2.3 Structure of nitrogenase enzyme

The nitrogenase complex is a two component metalloenzyme composed of i) dinitrogenase reductase, the iron protein, a dimer of two identical subunits with a total molecular mass of 60 kDa which contains the sites for MgATP binding and hydrolysis and supplies the reducing power to the dinitrogenase and ii) the dinitrogenase component, a large protein, 220 to 240 kDa that contains a metal cofactor (Dean and Jacobson, 1992).

1. Fe-protein (dinitrogenase reductase enzyme)

The Fe-protein is a dimer of identical ~32 kDa subunits containing a single 4Fe:4S cluster similar to that found in small molecule weight electron carrier proteins such as ferredoxins. This cluster is redox active center of the protein which exhibits changes in numerous electromagnetic and biochemical properties upon binding either MgATP or MgADP. These changes in properties have been assigned to protein conformational changes, perhaps relevant to the enzyme mechanism. The three dimensional structure of Fe-protein of *Azotobacter vinelandii* (Fig. 3.4) as a ribbons diagram which shows the two fold symmetry of the dimer (Schlessman *et al.*, 1998). The overall peptide fold has the α/β pattern typical of many nucleotide binding protein. The 4F:4S cluster bridges the subunits with two cysteinyl ligands from each subunit and is nested in the subunit interface just beneath the surface. A direct solvent path is evident to the cluster when looking down the two-fold axis in Figure 3.4A.

Inspection of the amino acid sequence as well as the three dimensional structure reveals a number of homologies to other nucleotide binding proteins including the *ras* and G-protein superfamily of signal transduction proteins and myosin family of energy transduction proteins (Schlessman *et al*., 1998). Most notably is the

central β-sheet core surrounded by α-helices, the so-called Walker P-loop and the switch loops I and II that interact with the nucleotide γ-phosphate and Mg^{++}. Identifying these regions in the Fe-protein structure of *A. vinelandii* indicated that nucleotide binding would be parallel along the interface of the two subunits, away and below the cluster (Fig. 3.4). Most tantalizing was the observation that the cysteinyl ligands, residue 132 in each subunit, were part of the putative Switch II region. These proposed interactions with the nucleotide have to be corrected in the nucleotide-Fe-protein-MoFe-protein complex.

2. MoFe-protein (dinitrogenase enzyme)

The MoFe-protein, the other component of the functioning nitrogenase system, is an α2β2 structure with two fold symmetry between pairs of αβ dimers as shown for the MoFe-protein of *A. vinelandii* x-ray structure in Fig. 3.4B. The overall impression is that each αβ dimer appears to be an independent structural and functional unit containing a copy of each type metal center. Although the α and β subunits have only minimal sequence homology, they have remarkably similar folding patterns where each subunit has three internal folding domains. In turn, the folding topology of the internal domains are similar to each other where core of four or five parallel β-sheets are connected by helices. Thus, as a dimer, the two subunits exhibit pseudo two-fold symmetry roughly through one of the metal centers, the P-cluster (Fig. 3.4B). External to the P-cluster, along the pseudo two-fold axis and at the subunit interface is a cleft that would appear to be a potential binding site for the Fe-protein.

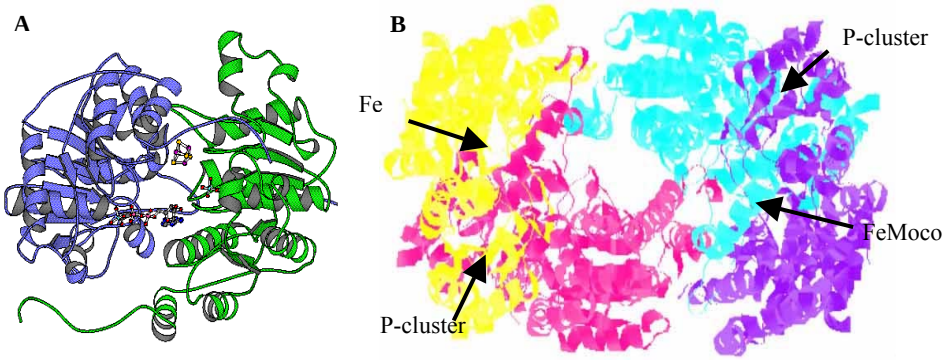

Figure 3.4 Nitrogenase structure: A; Fe-protein (www, 2006) and B; MoFe-protein (www, 2006).

Perhaps the most exciting finding in the MoFe-protein structure was the two types of iron and inorganic sulfur containing cluster, clusters that had not been previously seen in synthetic or protein based structures (Kim and Rees, 1992 and Kim *et al.*, 1993). The metal centers of the MoFe-protein can be divided into two classes based upon electromagnetic spectroscopy; P-clusters and FeMoco. The electromagnetic spectral properties of the clusters had indicated that they were unusual but had not predicted their remarkable structures and complexities. Indeed, the interpretation of the spectral studies predicted four 4Fe:4S clusters for the P-clusters rather than the two 8Fe:7S clusters that were found by x-ray diffraction. The P-cluster (Fig. 3.5) is at the α and β subnit interface, about 12-15 A° below the protein surface and has three cysteinyl ligands from each subunit (total of 6 cysteinyl ligands) (Fig. 3.5). Cys α88, β95 and one of the inorganic S atoms; these three sulfur group bridge the elements of two Fe:S clusters to make the P-cluster. The P-cluster is more structural dynamic than observed for other clusters and has different structures in the so-called P^{OX} and P^{N} oxidation states (Peter *et al.*, 1997). The primary structure

change is going from the P^N to P^{OX} state involves movement of two Fe atoms associated with the bridging inorganic sulfur. The cluster appears to peel apart leaving the bridging S more associated with the 4Fe atoms of the α subunit in P^{OX}. Two of the Fe atoms of the β subunit side of cluster have moved 1-1.5 A°. This requires several partial bonds of the bridging S atom to be broken which are then compensated by two new bonds involving a side chain hydroxyl, Ser β188 and a main chain backbone amide N of Cys α88 (Fig. 3.5).

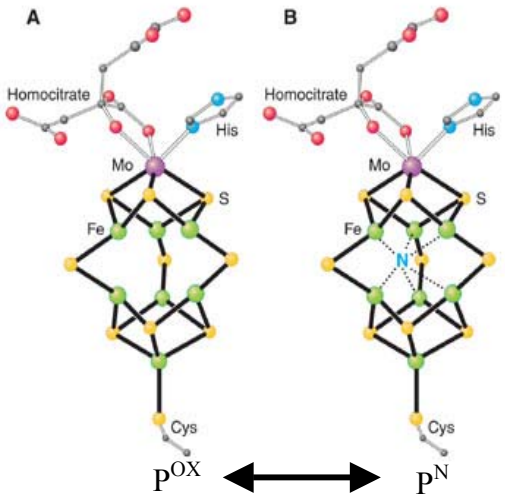

Figure 3.5 P-cluster in two oxidation states (www, 2006).

Both component proteins of nitrogenase are extremly oxygen sensitive and can be purified independently of each other. Dinitrogenase reductase, an α dimer of the *nif*H gene product (Mw ~64,000 Da), specifically donates electrons one at a time to dinitrogenase, an α2β2 tetramer of the *nif*D and *nif*K gene products (Mw >230,000 Da), with the concomitant hydrolysis of two MgATPs (Fig. 3.6). The electrons transferred to dinitrogenase are channeled to the iron-molybdenum cofactor (FeMoco), the active site where substrate reduction is believed to occur nitrogenase is a relatively

slow enzyme with a turnover time per electron of ~5/s. This may be due to the fact that each electron transfer step between dinitrogenase reductase and dinitrogenase requires the obligatory association and dissociation of the two proteins; the rate of dissociation is likely to be the rate-limiting step for the overall reaction.

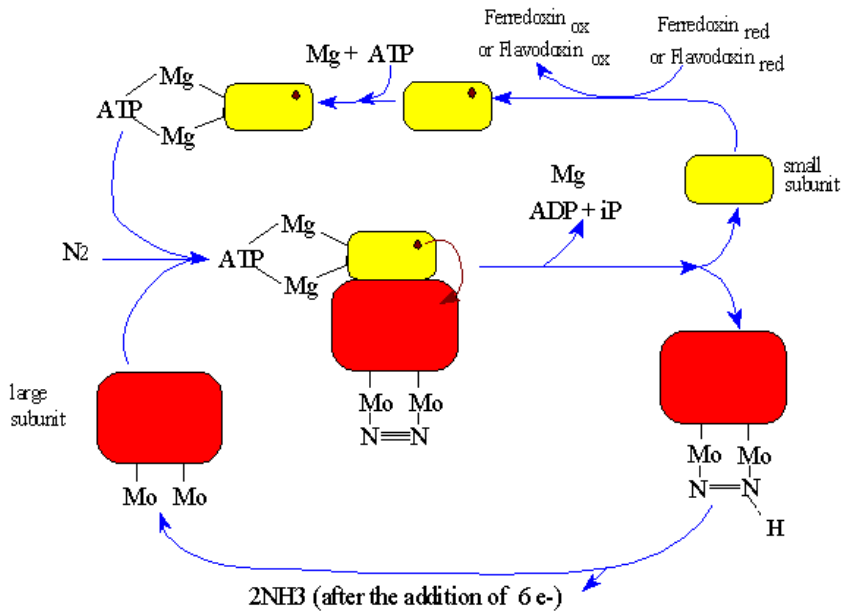

Figure 3.6 Nitrogenase activity (www, 2006).

3.2.4 Biochemical of nitrogen fixation in cyanobacteria

Cyanobacteria have the same general requirements for N_2 fixation common to all diazotrophs: a nitrogenase complex including iron and molybdenum coenzymes, ATP, a source of low potential electrons and an aerobic atmosphere. Fleming and Haselkorn (1974) identified polypeptides, newly synthesized in *Anabaena* heterocysts, that had the same molecular weight as dinitrogenase (encoded by *nif*D and *nif*K) and dinitrogenase reductase (encoded by *nif*H) subunits purified from *Azotobacter* and *Klebsiella*. Sequence comparisons show that the nitrogenase proteins of *Anabaena* are very similar to those of *Klebsiella, Azotobacter, Clostridium* and *Rhizobium*.

Additionally, *Anabaena* contains counterparts of the *nif*B and *nif*N genes whose functions in *Klebsiella* are required for MoFe-protein synthesis.

ATP production for N_2 fixation in cyanobacteria is not fully understood. *Anabaena* vegetative cells carry out oxygenic photosynthesis. They have two photosystems and an electron transport chain that transfers electrons from photosystem II (PSII) through cytochromes and plastocyanin to photosystem I (PSI), resulting in a proton gradient that produces ATP. PSII obtains electrons for re-reduction of oxidized chlorophyll from water, generating O_2 in the process. Since O_2 is inimical to N_2 fixation, PSII must be inactivated in N_2–fixing cells. ATP can also be produced by oxidative phosphorylation, a process that consumes O_2. Heterocysts have all the components of an electron transport chain from reduced pyridine nucleotide to O_2. The reduced pyridine nucleotide might be NADPH from the oxidative pentose pathway or NADH. Loss of PSII usually means loss of ability to reduce $NADP^+$ to NADPH photochemically which occurs in heterocysts. However, an alternate source of NADPH is made available by importing carbohydrate from neighboring vegetative cells.

The biochemical work on the flow of electrons to nitrogenase in heterocysts is summarized in Figure 3.7. Heterocysts have no photosystem II and thus no photosynthetic O_2^- production. Reduced ferredoxin, produced in a light-dependent fashion and ATP, produced by photophosphotylation, can be used directly for the reduction of N_2 to ammonia. A heterocyst-specific form of ferredoxin has been identified (Schrautemeier and Bohme, 1985). Heterocysts also lack a Calvin cycle, the C-source being disaccharides from vegetative cells. These are metabolized to glucose-6-phosphate, then degraded by the oxidative pentose phosphate cycle. The NADPH

thus formed reduces ferredoxin and forms a second source of reduced ferredoxin. A second ATP-source is the respiratory chain in the heterocyst. The cytochrome c oxidase from heterocysts has high cyanide sensitivity and is of the aa3 type. The tricarboxylic cycle is non-functional in heterocysts and 2-oxoglutarate cannot be further oxidized. However, it is used in the glutamate synthase reaction. Malate and 2-oxoglutarate are formed by very active malate dehydrogenase and PEP-carboxylase respectively. H_2 can be used in the respiratory chain at low rates because photosynthesis and respiratory both use the plastoquinone and cytochrome b2f complex. An uptake hydrogenase, activated by thioredoxin, can feed in electrons close to the plastoquinone sithe of thylakoids. Light-dependent nitrogenase activity is saturated at 8 W/m^2, but photosynthetic CO_2^- fixation in the vegetative cells is saturated at 32 W/m^2 (Peters *et al*., 1985).

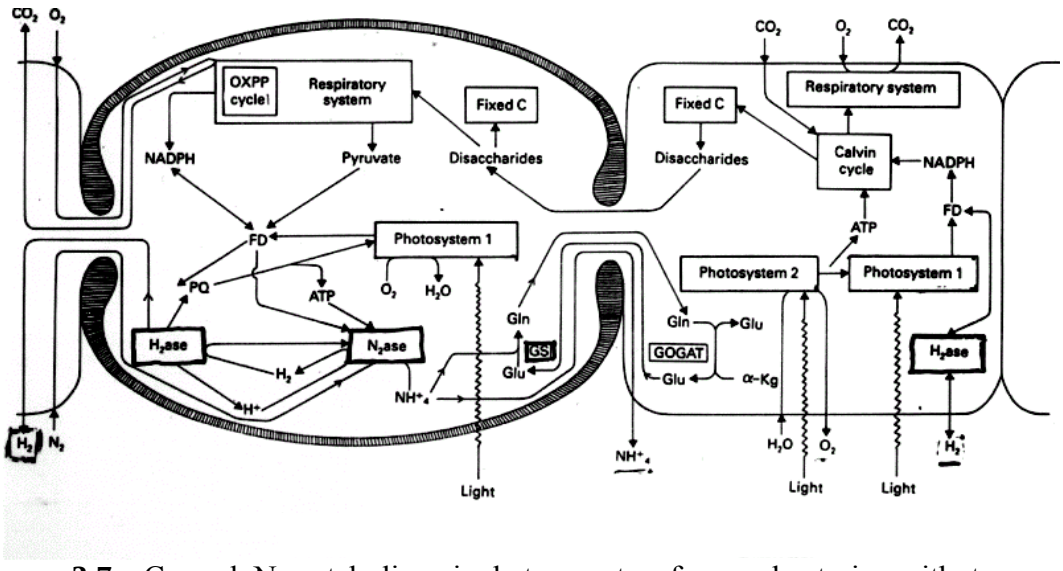

Figure 3.7 C- and N-metabolism in heterocysts of cyanobacteria, with transport processes to and from neighboring vegetative cells (www, 2006).

All the major cellular forms of cyanobacteria include species capable of fixing N_2, such as many filamentous nonheterocystous forms, *Plectonema boryanum*, can fix N_2 under anaerobic or microaerobic conditions (Rippka and Waterbury, 1977). Although this ability may be significant in nature (e.g. in particular layers of complex mats or during the night). Several unicellular species, such as *Synechococcus* could carry out photosynthesis and N_2 fixation in the same cell as well as *Gloeocapsa* and *Gloeothece* are synchronized with a light/dark cycle, phosynthesis is confined to the daytime but N_2 fixation occurs extensively at night (Mitsui *et al*., 1986). While *Anabaena,* the light was essential for N_2 fixation (accompanying CO_2 fixation and ATP production).

3.2.5 *nif* gene organization

Most of the information about cyanobacterial *nif* gene is based on studies of one strain, *Anabaena* sp. strain PCC7120. The cloned *nif* gene from this strain have been used to determine the organization of the structural genes for nitrogenase in other strains. Extended nucleotide sequence information is available only for *Anabaena* sp. strain PCC7120, where counterparts of most of the known *nif* genes of *K. pnuemoniae* have been identified. In addition, the *nif* gene region of *Anabaena* sp. strain PCC7120 contains several open reading frames similar to ones found in the *nif* gene region of *Azotobacter* spp., as well as a unique gene encoding a heterocyst-specific ferredoxin and two DNA elements that interrupt the *nif*HDK and the *nif*BSU operons. Finally, a complete physical map of the *Anabaena* sp. strain PCC7120 chromosome has been constructed. In addition to the 6.4 Mb chromosome, *Anabaena* sp. strain PCC7120 contains three megaplasmids (400, 200 and 100 kb) (Bohme and Haselkorn, 1988).

nif gene organization in *Anabaena* sp. strain PCC7120 differs from the other diazotrophs. The major *nif* region in the *Anabaena* sp. strain PCC7120 vegetative cell chromosome is demonstrated in Fig. 3.8. The orientation of the *nif*HDK operon is showed right to left as it is in all early maps of the *Klebsiella nif* gene region. The *nif*D gene is interrupted by an 11 kb element that is excised during heterocyst differentiation and the *fdx*N gene is interrupted by a 55 kb element that is similarly excised. The final arrangement of the *nif* genes in *Anabaena* sp. strain PCC7120 heterocysts is showed in the lower part of Fig. 3.8. The first operon on the right includes four reading frames: *nif*B, *fdx*N, *nif*S and *nif*U. Transcription in *Anabaena*, ferredoxin play a role in electron transfer to nitrogenase. Cyanobacterial ferredoxins are plantlike in that they contain 2Fe:2S centers. There is a unique 2Fe:2S ferredoxin, found in *Anabaena* heterocysts, which can donate electrons directly to nitrogenase *in vitro*. This ferredoxin was purified, its amino-terminal sequence determined and an oligonucleotide probe based on that sequence was used to clone the *fdx*H gene. That gene was found at the left end of the *nif* gene region (Fig. 3.8). The *fdx*H gene sequence is found in two RNA transcripts that appear, following heterocyst induction, with the same kinetics as *nif*HDK mRNA. The smaller of these about 0.6 kb encodes *fdx*H alone. The larger, 1.8 kb, starts upstream of ORF1 and includes ORF2 and *fdx*H. ORF1 encodes a 33 kDa protein with no identified counterpart on other diazotroph. ORF2 encoded a 9 kDa protein. The *nif* genes of *Klebsiella* and other diazotrophs are clustered according to biochemical function, except for *nif*B, however, *Anabaena* is the only organism in which *nif*B, *nif*S and *nif*U are transcribed together in a single operon.

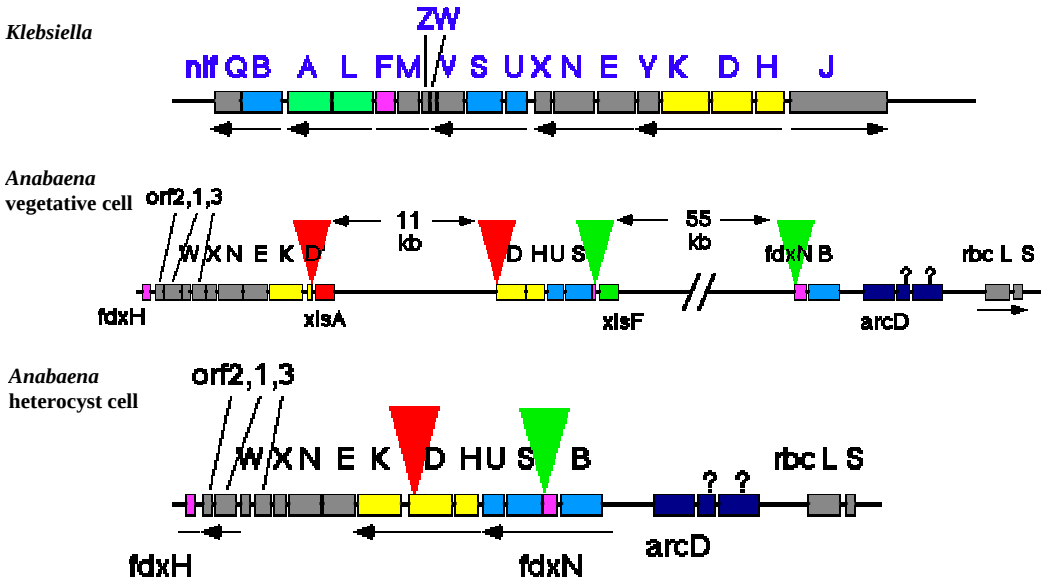

Figure 3.8 Organization of the *nif* gene region of *Klebsiella, Anabaena* vegetative cell and heterocyst cell after excision of the 11-kb and 55-kb (www, 2006).

3.2.6 Cyanobacterial *nif* gene

Nitrogen fixation is mediated by an enzyme complex, containing dinitrogenase (encoded by *nif*D and *nif*K) and dinitrogenase reductase (encoded by *nif*H), whose assembly requires many *nif* gene products (Dean and Jacobson, 1992). A large cluster of *nif* and *nif*-related genes from *nif*B to *fdx*H is highly conserved in cyanobacteria (Thiel *et al.*, 1995, 1997) including *N. punctiforme* and *Anabaena* sp. strain PCC7120 (Fig. 3.9). A major difference between the latter two clusters is the excision elements that interrupt both *fdx*N and *nif*D in *Anabaena* sp. strain PCC7120 (Golden *et al.*, 1985, 1988). In *N. punctiforme*, the *fdx*N excision element is missing and the 24 kb *nif*D element in *N. punctiforme* is almost completely different from the 11 kb *nif*D element in *Anabaena* sp. strain PCC7120. The two *nif* elememts share only the *xis*A gene that is required for site-specific recombination that removes the element during heterocyst differentiation and a small open reading frame of unknown function.

Within the *N. punctiforme* excision element, there is a bacterial retron-like gene. *Anabaena* sp. strain PCC7120 also has a gene that appears to be a retron; however, it is not located in the excision element and it is closely related phylogenetically to the gene in *N. punctiforme*. Upstream of *nif*H in *N. punctiforme* and in *N. commune* (Potts *et al.*, 1992) is a hemoglobin-like gene called cyanoglobin (*glb*N) whose function unknown. The *nif* genes of *N. punctiforme* are most closely related to those of *N. commune*, as determined by phylogenetic analysis. Upstream of the major *nif* cluster in *N. punctiforme* are *nif*P, *nifZ* and *nif*T and downstream are genes for an uptake hydrogenase, including *hup*S and *hup*L which lack an excision element that is present in the *hup*L gene of *Anabaena* sp. strain PCC7120 (Carrasco *et al.*, 1995). The genome of *Rhodopsuedomonas palustris* was sequenced by the Joint Genome Institute at about the same time as the *N. punctiforme* genome. Analysis of the *R. palustris* genome indicates an usually large number of *nif*-like genes. Like *Rhodobacter capsulatus, R. palustris* has genes that appear to encode a Mo-nitrogenase and an alternative Fe-nitrogenase. Unlike *R. capsulatus, R. palustris* also has genes that appear phylogenetically to be closely related to V-nitrogenase genes in *A. vinelandii* and *A. variabilis*. The organization of these three gene clusters is depicted in Fig. 3.9.

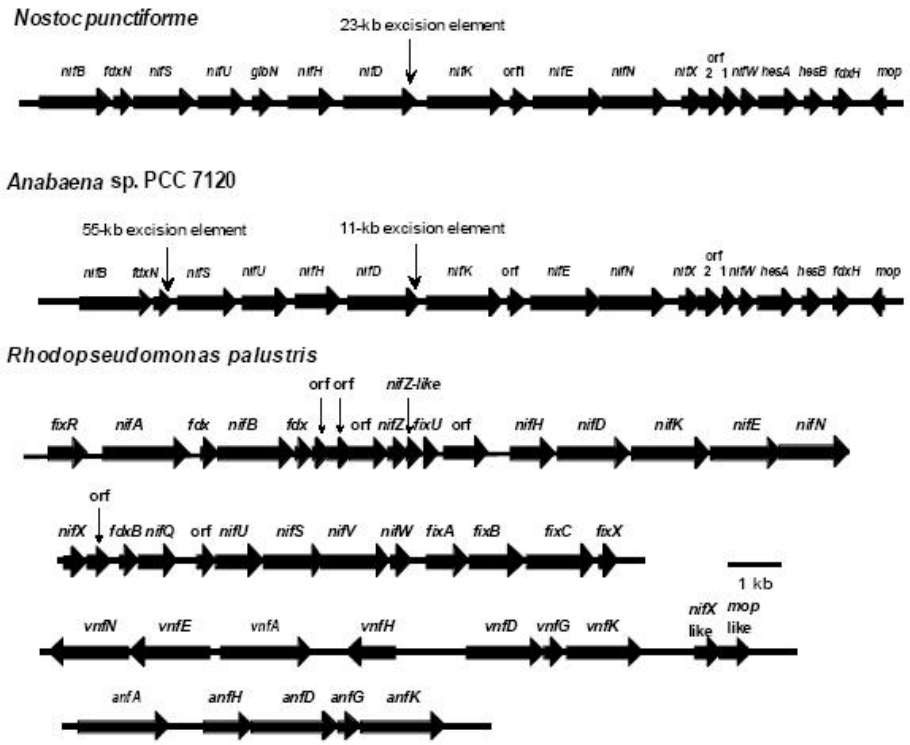

Figure 3.9 Organization of the *nif* and *nif*-related gene in three diazothrophs (Note that genes shown on two lines representing the entire *nif* cluster of *R. palustris* are contiguous in the genome) (Thiel *et al*., 2002).

3.2.7 Regulation of nitrogen fixation cyanobacteria

The knowledge of genes involved in heterocyst differentiation and nitrogen fixation has been greatly increased with the development of tools and techniques for genetic analysis of cyanobacteria. However, mechanisms of regulation of these genes are still poorly understood. The nitrogenase genes of *Anabaena* sp. strain PCC7120, the most studied of the heterocystous cyanobacteria are not expressed in vegetative cells, even under strictly anaerobic conditions and appear to be controlled primarily by developmental factors that regulate heterocyst differentiation (Elhai and Wolk, 1990). One of the events that is developmentally controlled and required for expression of

nitrogenase genes is the excision of the two large DNA elements that interrupt the nitrogenase genes (Carrasco *et al.*, 1994). The differentiation of heterocysts requires *het*R, an autoregulatory gene that is expressed very early after nitrogen starvation, particularly in cells destined to become heterocysts (Buikema and Haselkorn, 1991). The mechanism by which HetR controls heterocyst formation is still studied.

The only global regulatory protein that has been demonstrated to respond to nitrogen status in cyanobacteria is *ntc*A (*bif*A) (Frias *et al.*, 1994), which is expressed in vegetative cells and in heterocysts (Bauer and Haselkorn, 1995). An *Anabaena* sp. strain PCC7120 *ntc*A mutant fails to differentiate heterocysts and hence cannot fix N_2 (Wei *et al.*, 1994). In addition, the mutant cannot use nitrate as a nitrogen source but able to grow in ammonium. NtcA, a member of the CRP family of transcriptional regulators, binds upstream of several *Anabaena* genes, including *gln*A and *nif*H *in vitro* and may be required for transcription of the *gln*A promoter that functions in heterocysts (Wei *et al*., 1994) and for its own transcription (Ramasubramanian *et al.*, 1996).

3.3 Review of Literatures

Microbial inoculants are carrier-based preparations containing beneficial microorganisms in a viable state intended for seed or soil application and designed to improve soil fertility and help plant growth by increasing the number and biological activity of desired microorganisms in the root environment. Starting from modest laboratory preparations in the mid-1930s in the United States, rhizobial inoculants (also known as legume inoculants) have become industrial propositions in the United States, Europe, Australia and India. Following the success of legume inoculants all

over the world, carrier-based *Azotobacter* and *Azospirillum* inoculants for non-leguminous crops are becoming increasingly popular in India in recent year. Cyanobacteria also play a role in the nitrogen economy of tropical rice soils (De, 1939; Singh, 1961; Subramanyam and Sahay, 1964; Venkataraman, 1972). The nitrogen-fixing cyanobacteria can be cultured in open-air tanks and used for rice cultivation. The results obtained by using cyanobacterial inoculum in rice fields in India have shown the promising result for yield improving in rice cultivation.

Rice is the staple food for almost half of the world's population and approximately 90% of the world's rice is produced in Asia (De Datta, 1981). Most rice is produced in shallow, flooded paddy-fields in lowlands under rainfed or irrigated conditions, but other rice varieties can be grown in deep waters or in upland agriculture under rainfed conditions, deepwater rice is produced in low lying fields that are flooded to 0.5-1.0 m depth for half of the crop's growth. "Floating" rice is grown where the floodwater is up to 6 m depth; the rice plants root in the soil but are able to elongate as the floodwater rises gradually. Over 2 million hectares of rice are produced in this way in Bangladesh and flooded rice is also produced in India, Thailand and West Africa. Upland rice amounts for about 10% of the world's rice production and is grown in Asia, Africa and Latin America. The flooded rice plant ecosystem is extremely complex, physically, chemically and microbiologically (Fig. 3.10). One of the effects of flooding in uncropped rice field is a fall in O_2 content. However, in rice-cropped soil, due to aeremyma in the rice plant, O_2 is capable of moving from the leaf blade to the root cortex. This results in the oxidation of soil around the actively growing root system. Flooding of soil results in ammonium accumulation and nitrate instability. Ammoniacal nitrogen, the dominant form of

mineral nitrogen in lowland rice soil, is liable to fixation by clay, loss by volatilization, nitrification, denitrification, leaching, runoff and seepage. About 60-80% of nitrogen absorbed by crops (40-50 kg N/ha) can be attributed to the native nitrogen pool. Approximately 60% of the rice yields (2-4 t/ha) can be obtained without the application of nitrogen fertilizer. The soil nitrogen does not show decreasing trends by rice planting and harvest, indicating the existence of biological mechanisms to renew the depleted nitrogen from the soil nitrogen pool. Legumes, *Azolla*, nitrogen-fixing bacteria and cyanobacteria take part in biological fixation of nitrogen. The fixed nitrogen is mostly mineralized to NH_4^+, which is the key process of nitrogen nutrition in waterlogged soil which is subjected to environmental stress (Roger and Watanabe, 1986; De Datta, 1987)

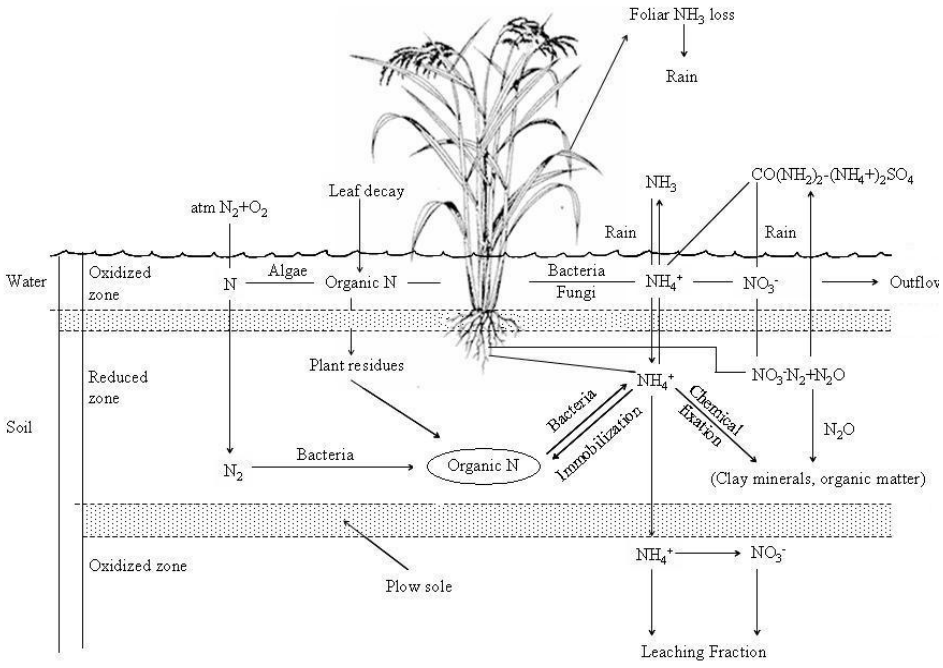

Figure 3.10 Schematic representation of nitrogen transformations in a lowland rice ecosystem (Mitsh and Gosselink, 1993).

The rice ecosystem is an ideal environment for cyanobacteria. High humidity and temperature and shade provided by the crop canopy favour the luxuriant growth of these organisms (Roger and Reynaud, 1979). With the introduction of high yielding rice varieties, the demand for inorganic fertilizers has increase several-fold. However, due to increasing fertilizer prices and poor purchasing power of the Indian farmers, particularly those with less than 2 hectares land area, fertilizer consumption is restricted. This is imply evident from the fact that average fertilizer-N consumption in Assam is 7 kg/ha as against the Punjab where it is 160 kg/ha. By the year 2000 A. D. the total fertilizer consumption is targeted at 233 to 243 lakh (one hundred thousand) tonnes, of which nitrogen would form 136-140 lakh tonnes alone. Rice covers 41.6 million hectares of land area and the fertilizer nitrogen requirement for rice alone is 60 lakhs tones of the total available 140 lakh tones fertilizer N, with the present level of consumption (150 kg N/ha). The best nitrogen-use efficiency of the applied fertilizer-N is 40% and therefore about 60% of applied nitrogen is lost either through leaching or volatilization (Kaushik, 1998). Yields of rice can be sustained in some regions at a moderate level of production of about 2 t/ha even where no fertilizers are used and this has caused scientists to speculate about the importance of biological N_2-fixation in the maintenance of soil fertility. Much research has been carried out on the role of free-living heterotrophic N_2-fixation bacteria in paddy soils. De (1939) recognized the potential importance of N_2-fixation by cyanobacteria in maintenance of soil fertility in paddy soils, and farmers have long been aware of the benefits of *Azolla*, a floating fern with symbiotic N_2-fixation cyanobacteria, which has been used to enrich the soil in parts of China and Vietnam for centuries (Lumpkin and Plucknett, 1980, 1982).

Analysis of the cyanobacteria flora from rice fields has revealed the occurrence of species of *Anabaena, Anabaenopsis, Aulosira, Cylindrospermum, Nostoc, Calothrix, Scytonema, Tolypothrix, Fischerella, Hapalosiphon, Mastigocladus, Stigonema, Westiella, Westiellopsis, Campylonema* and *Microchaete* as dominant nitrogen fixers. Besides fixing nitrogen, these cyanobacteria excrete vitamin B_{12}, auxins and ascorbic acid which may also contribute to the growth of rice plants (De, 1939; Fogg, 1939; Singh, 1961; Venkataraman and Neelakantan, 1967; Watanabe, 1967; Stewart, 1970, 1971, 1974)

3.3.1 Cyanobacteria

The cyanobacteria are photosynthetic bacteria and some of them are able to fix N_2 and these abilities, together with great adaptability to various soil types, make them ubiquitous. Cyanobacteria have been reported from a wide range of soils, thriving both on and below the surface. They are often also characteristic features of other types of sub-aerial environment and many intermittently wet ones such as rice fields. Most paddy soils have a natural population of cyanobacteria which provides a potential source of nitrogen fixation at no cost (Mishra and Pabbi, 2004). They can be divided into two major groups based on growth habit: the unicellular forms and the filamentous forms. N_2-fixing species from both groups are found in paddy fields although the predominant ones are the heterocystous filamentous forms (Table 3.1). Cyanobacteria are not restricted to permanently wet habitats as they are resistant to desiccation and hot temperatures, and can be abundant in upland soils (Roger and Reynaud, 1982). However, wet paddy soils and the overlying floodwaters provide and ideal environment for them to grow and fix N_2.

Table 3.1. The main taxa of N_2-fixing cyanobacteria found in rice soils in South-east Asia (Roger *et al.*, 1987)

Unicellular group	Unicellular strains (*Aphanothece, Gloeothece*)
Anabaena group	Heterocystous strains with a thin sheath, without branching, do not form mucilaginous colonies of definite shape (*Anabaena, Nodularia, Cylindrospermum, Anabaenopsis*)
Nostoc group	Heterocystous strains with a thick sheath, without branching, forming mucilaginous colonies of definite shape (*Nostoc*)
Aulosira group	Heterocystous strains with a thick sheath, usually without branching, do not form diffuse colonies on agar medium (*Aulosira*)
Scytonema group	Heterocystous strains with false branching, without polarity, forming velvet-like patches on agar medium (*Scytonema*)
Calothrix group	Heterocystous strains with false branching, with polarity, forming velvet-like patches on agar medium (*Calothrix, Tolypothrix, Hassalia*)
Gloeotrichia group	Heterocystous strains, with polarity, forming mucilaginous colonies of definite shape (*Gloeotrichia, Rivularia*)
Fischerella group	Heterocystous strains with true branching (*Fischerella, Westiellopsis, Stigonema*)

3.3.2 Natural distribution

Free-living cyanobacteria can grow epiphytically on aquatic and emergent plants as well as in floodwater or on the soil surface. Early surveys indicated that cyanobacteria were only present in a small proportion of rice fields. Only 5% of over 500 soil samples from Asia and Africa (Watanabe, 1959) and only some 35% of more than 2200 samples of Indian rice soils (Venkataraman, 1975) were found cyanobacterial establishment. This reported infrequent occurrence was almost certainly due to only small samples of soil being taken from each field and also to the use of unsuitable methods for detection (Roger and Reynaud, 1982). Many other studies have found cyanobacteria in all of the soils sampled (Roger *et al*., 1987). Although the relative abundance may widely vary, heterocystous genera generally account for about half of the cyanobacteria in rice fields (Whitton and Roger, 1989). In fact in deepwater rice fields studied in Bangladesh virtually all of the cyanobacteria were heterocystous forms (Whitton *et al.*, 1989).

Numbers of heterocystous cyanobacteria in rice soils expressed as colony-forming units (cfu) ranged from 10 to 10^7 cfu/g soil with a mean value of 2.5×10^5 cfu/g soil or 8.3×10^4 cfu/cm^2 in 10 surveys in which more than 280 soils were sampled (Roger *et al*., 1987). In most of these studies, a most propable number (MPN) method for counting was used in which serial dilutions are made and the population size estimated on the basis of presence or absence of growth of cyanobacteria at the different dilutions. However, a modified direct-plating method using selective media gave an average value of 3.2×10^5 cfu/cm^2 over 102 soil samples, which was roughly four times greater than the mean number found previously (Roger *et al*., 1987). On average, heterocystous cyanobacteria numbered less than 10% of the population of

eukaryotic algae and the abundance of cyanobacteria increased with the amount of available phosphorus and increased with pH over the range 4-6.5. Above pH 6.5 the numbers of cyanobacteria showed no obvious relationship with pH. These results agree with earlier observation that N_2-fixing cyanobacteria are more abundant in phosphorus-rich soils of neutral to alkaline pH (Roger and Kulasooriya, 1980).

3.3.3 Amount of N_2 fixed by cyanobacteria in rice production

An average value from 38 measurements of N_2-fixation by cyanobacteria collected from the literature was 27 kg N/ha per rice crop with a maximum of 50-80 kg N/ha (Roger and Kulasooriya, 1980). However, most of these measurements were made using the acetylene reduction assay (ARA) and are unlikely to be accurate given the problems of calibration of the assay and the well-documented diurnal fluctuations in measured rates of N_2-fixation. In a detailed study of ARA due to cyanobacteria in 190 rice fields in the Philippines, the mean activity was 126 μM C_2H_2/reduced m^2/h, roughly equivalent to 12 kg N/ha fixed over a cropping season (Fig. 3.11). ARA estimates of N_2-fixation indicated that greater amounts of N were fixed by cyanobacteria (7 kg N/ha) on the wet soils before flooding than the standing waters (2 kg N/ha) of deepwater rice fields (Rother *et al.*, 1989). A bloom of cyanobacteria usually contains about 15-25 kg N/ha (Roger *et al.*, 1986) and such blooms may exhibit high rates of N_2-fixation and can persist for several weeks (Rother *et al.*, 1989). For an algal bloom to be sufficiently large to make a significant input of fixed N it would have to be readily visible in the field (Roger *et al.*, 1986).

Of course, in the short term, the important measurement is not simply the amount of N_2 fixed but the amount acquired by the rice crop. Experiments using ^{15}N-

labelled algal cells spread on the soil surface or incorporated into the soil showed that between 36 and 51% of the added N was recovered by rice in the first season (Wilson *et al.*, 1980). Similar pot and field experiments indicated that 23-28% of the N in ^{15}N-labelled cyanobacteria incorporated into the soil was recovered in the first rice crop whilst only 14-23% of the N was recovered if the cells were left on the soil surface (Tirol *et al.*, 1982). Timing is also important; N fixed or released towards the end of the growing season will be too late to influence production of the current rice crop (Whitton and Roger, 1989).

Based on these data, one quarter of the N_2 fixed by cyanobacteria is utilized by the next rice crop. Then if 15-25 kg N/ha were fixed during each crop, this would represent a benefit of some 4-6 kg N/ha. Thus the amounts of N_2 fixed by cyanobacteria are likely to be insufficient to sustain high yields of rice but will be important in the long-term maintenance of soil fertility in paddy fields.

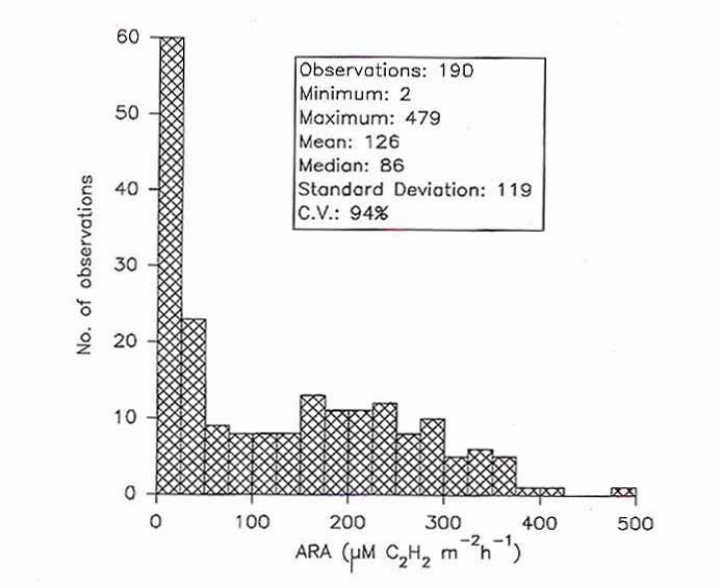

Figure 3.11 Measurements of acetylene reduction activity (ARA) in flooded rice soil (unpublished data of P.A. Roger).

3.3.4 Manipulation of indigenous populations of cyanobacteria

Given the lack of success of inoculation, an alternative strategy to improve the inputs of N_2-fixation is to enhance the growth of indigenous cyanobacteria. Low pH, low temperatures and phosphorus deficiency are all factors which are known to limit growth, but the ecology of cyanobacteria is poorly understood (Roger and Watanabe, 1986). It is apparent that addition of phosphorus fertilizers is likely to stimulate their growth and addition of lime to floodwaters will help in acid soils.

Another problem which restricts the size of populations of cyanobacteria is predation by invertebrates. Some cyanobacteria (e. g., *Aphanothece, Gloeotrichia* and *Nostoc*) are able to form mucilaginous colonies which renders them more resistant to grazing by invertebrates (Grant *et al.*, 1985), but such strains generally contain little N. A standing crop of these cyanobacteria of 10 t/ha may contain as little as 3 kg N/ha (Grant *et al.*, 1985). Pesticides, including some natural products from plants such as neem (*Azadirachta indica*), can be used to reduce grazing pressure (Reddy and Roger, 1988). The earlier reports of the sparse distribution of cyanobacteria in paddy fields have been used as a justification for an intensive research effort into technology for inoculation with cyanobacteria, or "algalization", particularly in India. A method for production of algal inoculants was developed in India, which was suitable for use by small-scale farmers (Venkataraman, 1981). An initial inoculum containing six species of cyanobacteria was provided to farmers by the "All India Coordinated Project on Algae". The farmers then multiplied this inoculum in shallow tanks in up to 15 cm of water to which some soil, phosphorus fertilizer and insecticide were added, together with some lime where necessary. The tanks were simple in design consisting either of a shallow pit lined with plastic sheet, or larger inoculum production units were made

by mounding up soil to make shallow bunds (ridges) in the field. After a few weeks a mat of cyanobacteria and algae developed, and this was allowed to dry out and flakes of the inoculum were scraped up and stored for later use. Sufficient flakes to inoculate a hectare (8-10 kg) were produced from a single tank in 2-3 months. The density of total cyanobacterial and algal propagules in these inocula varied from 2×10^6 to 9×10^7 cfu/g soil but only 2-32% of these were heterocystous cyanobacteria, the type most important for N_2-fixation (Roger *et al*., 1987). It has been suggested that use of the multi-strain starter inoculum and production of inoculum using local soil in the tanks would lead to selective growth of the strains best adapted to the local soil conditions. However, this was not borne out by the results of Roger *et al*. (1987) who found that one or two strains, most commonly a *Nostoc*, were generally dominant among the N_2-fixing cyanobacteria present in the inocula produced. Of course, the results of these workers, indicating that N_2-fixing cyanobacteria are in fact abundant in rice fields, put the necessity for inoculation in question. Nevertheless, should inoculation be deemed necessary, Roger *et al*. (1987) recommended that a better multi-strain inoculum would be produced by mixing single strain inocula at the time of inoculation. Even so, as the inoculum is produced in local soil, it is still possible that strains present in the soil may dominate so that the original inoculum strains are lost before the inoculum even reaches the field (Whitton and Roger, 1989).

Many studies have been reported on the use of dried cyanobacteria to inoculate soils as a means of aiding fertility, and the effect of adding cyanobacteria to soil on rice yield was first studied in the 1950s in Japan. The term “algalization” is now applied to the use of a defined mixture of cyanobacterial species to inoculate soil, and research on algalization is going on in all major rice producing countries. The average

of the results from all these studies have shown an increase in grain yield of 15-20% in field experiments. It has been suggested that the cyanobacteria introduced as a result of algalization can establish themselves permanently if inoculation is done consecutively for 3-4 cropping seasons (Mishra and Pabbi, 2004).

Results of inoculation experiments are not encouraging even though many of these were conducted with inocula produced in the laboratory i. e., in which local strains had not had a chance to outcompete inoculum strains prior to inoculation. An average response in yield of rice of 15% was found in experiments where inocula of cyanobacteria were applied in the field (Roger and Kulasooriya, 1980). In many experiments the effects of inoculation and N-fertilizer application on growth of rice were often additive and were attributed to production of plant-growth-stimulation compounds by the cyanobacteria and not to N_2-fixation. However, screening of 133 strains of cyanobacteria showed that 70% of the strains had an inhibitory effect on germination of rice and only 20% of the strains stimulated elongation of rice shoots (Pedurand and Reynaud, 1987). This suggests that hormonal effects of cyanobacteria are not the principal cause of improved rice growth when responses to inoculation are observed.

In many cases no benefits in yield were found after inoculation. This is perhaps not surprising as the recommended rate of inoculum application will provide on average less than 1 propagule for every 130 indigenous cyanobacteria already present (Roger *et al*., 1987). In one study the inoculated strains could not be detected even immediately after inoculation, presumably as they formed such a small proportion of the algal population (Grant *et al*., 1985). In other experiments, inoculated strains did multiply but rarely dominated the population of cyanobacteria

(Reddy and Roger, 1988). When beneficial effects on plant growth due to inoculation have been found it is likely that establishment of large populations of cyanobacteria has been possible due to the large phosphorus content of propagules in the inocula which will give the introduced cells a substantial growth advantage over indigenous strains (Roger *et al*., 1986). In any case, whether the inoculum strains succeed in becoming established or not, there is little evidence which indicates that they can fix N_2 more effectively than indigenous cyanobacteria.

3.3.5 Biofertilizer production technology

The exiting technology harnesses sunlight as the source of energy and the soil as a source of mineral nutrition for the growth of cyanobacteria. Once enough biomass is built they are allowed to dry and are collected along with soil and therefore, the soil also acts as a carrier for the organism. During the process of multiplication, superphosphate as a source of P and water are added. If there is mosquito or predator-infestation, these are controlled by insecticides like malathion, furadan or carbofuran (Venkataraman, 1981). The basic method of mass production involves a mixture of nitrogen fixing cyanobacteria in shallow trays or polythene lined pits filled with water kept in open air, using clean, sieved farm soil as a carrier material. To each pit 10 kg soil and 250 g single super phosphate is added and water is filled up to a height of 12-15 cm. Starter culture, a mixture of *Anabaena, Nostoc, Aulosira* and *Tolypothrix*, is inoculated in each multiplication unit. Malathion (5-10 ml per tank) or carbofuran (3% granules, 20 g per tank) is also added to prevent insect breeding. In hot summer months, the cyanobacteria form a thick mat over the surface after 10-12 days of growth in open sun. The contents are allowed to dry and the dried flakes are collected, packed

and used to inoculate rice fields. The basic advantage of this technology is that farmers after getting the soil based starter culture can produce the biofertilizer on their own with minimum additional inputs. An inoculum of 10-12 kg is considered sufficient to inoculate one hectare of paddy field 3-4 days after transplantation. The application of biofertilizer with existing technology led to a net saving of 25-30 kg N/ha/season. Unfortunately, the open-air algal biofertilizer production technology for production at farmers' level is not popular among the farming community, due to open air nature can be produced only in summer and sometime may contain airbone contaminants. Moreover, cyanobacteria were slow production rate and low production density, hence the cells were needed for heavy inoculum per hectare. However, some cyanobacteria was not able to compete over the indigenous flora and agro-climatic factors may not permit optimum proliferation of inoculated cyanobacterial strains like drought, etc (Kaushik, 1998; Mishra and Pabbi, 2004).

There is attempt to improve the technology by developing new economically feasible protocols for production of quality inoculum so that these organisms can be practically exploited on a large scale. This is possible only if multiplication is carried out under controlled conditions. The production technology has been substantially improved with introduction of new and cheap carrier materials that support higher cyanobacterial load with longer shelf life, thus considerably reducing the quantity of inoculum per unit area. The basic changes the technology has undergone include, i) indoor production of algal biomass under controlled conditions; ii) a suitable and cheap growth medium for faster growth of the organisms, and iii) mixing with a suitable carrier material.

Indoor production involves the growth of algae in a unit that may be a polyhouse or glasshouse (Fig. 3.12). The individual unit in the polyhouses can be of either, brick and mortar, or even polythene lined pits in the ground. The cyanobacteria are grown individually as species, by inoculating separate tanks with laboratory grown pure cultures, so as to ensure the presence of each required strain in the final product. Once fully grown, the culture is harvested, mixed with the carrier material, presoaked overnight in water and multani mitti clay (in 1:1 ratio) and sun dried. The dried material is ground and packed in suitable size polythene bags, sealed and stored for future use. The final product contains 10,000 to 100,000 units or propagules per gm of carrier material and, therefore, 500 g material is sufficient to inoculate one acre of rice growing area. A number of field trials conducted with this material have shown promising results both in terms of nitrogen saving as well as crop yield (Table 3.2).

Figure 3.12 Indoor production of cyanobacterial biofertilizer in polyhouse under semi-controlled conditions (Mishra and Pabbi, 2004).

Table 3.2. Effect of cyanobacterial biofertilizer inoculation on rice yield at a farmer's field (Mishra and Pabbi, 2004).

Name of Village (Area)	Grain yield (Quintals per hectare)		
	Uninoculated	Inoculated	% Increase
Asoda Todran (7 ha)	19.25	23.00	19.48
Asoda Shivan (4 ha)	13.66	15.22	15.81
Jakhoda (9 ha)	17.93	20.13	12.26
Under farmer's own management practices			

In India, considerable progress has been made in the development of cyanobacteria based biofertilizer technology. It has also been demonstrated that this technology can be a powerful means of enriching the soil fertility and improving rice crop yields. Furthermore, the researchers applied cyanobacterial inoculum which comforted and high efficiency to use in agricultural application. For example, Nohr (1990) produced immobilized cyanobacteria by preparation a composite which included a substantially water-insoluble particulate or fibrous support having a surface energy of at least about 19 dynes per cm to which nitrogen-fixing filamentous heterocystous cyanobacteria were attached. The support did not have a deleterious effect on the viability of the attached algae and was preferably polypropylene (meltblown) or cellulosic (wood pulp). Preparing the composite included contacting the support with cyanobacteria to permit the algae to attach to the support by means of heterocyst cells. The attached cells in a nitrogen-deficient environment fix nitrogen at a rate substantially greater than unattached cells and have agricultural application. Hiroyuki and co-workers (1996) used a low-melt-point agarose gel as an immobilization agent and polypyrrole as a conductive polymer to immobilize

cyanobacteria. In addition, at least one company was marketing a cyanobacterial fertilizer for the lawn and garden market (Schlender, 1986).

Montmorillonite clay is the novel one way to apply as carrier for cyanobacteria. This clay consists of SiO_2: 56-60%, Al_2O_3: 16-18%, Fe_2O_3: 5-7%, Na_2O: 2.4-3%, MgO: 1.5-2%, CaO: 1.9-2.1%, K_2O: 0.3-0.5% and TiO_2:1.2-1.5% (Fig. 3.13) which no suitable for cyanobacterial growth, thus cyanobacteria can be stored in this clay as long period.

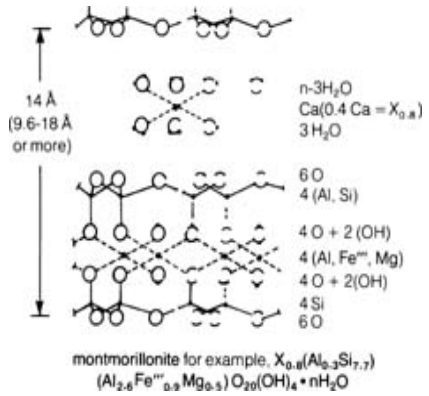

Figure 3.13 Structure of montmorillonite clay (www, 2006).

3.3.6 Cellular differentiation in heterocystous cyanobacteria

Many heterocystous cyanobacteria can undergo one or several of a variety of cellular differentiation processes that most commonly take place as adaptive responses to environmental changes. In general, these differentiation processes allow the cyanobacterium to make use of some nutritional options or to better stand unfavourable conditions, but are dispensable for the survival of the organism under other circumstances. In some cases, multiple relationships, both nutritional and regulatory, are established between the different types of cell of the filament, so that in some respects the heterocystous cyanobacteria can be regarded as simple multicellular

organisms (Herrero *et al.*, 2004). Heterocystous cyanobacteria reduce atmospheric dinitrogen to ammonia in specialized terminally differentiated cells called heterocysts. The regulation of heterocyst development is influenced by several factors including nitrogen deprivation, light quality, cell physiology and intercellular communication (Wolk *et al.*, 1994; Cai and Wolk, 1997a). When grown on dinitrogen, a one-dimensional developmental pattern of single heterocysts separated by approximately ten vegetative cells is established to form a multicellular organism composed of two interdependent cell types.

3.3.6.1 Differentiation of hormogonia

Hormogonia are short, motile filaments of small cells, generally distinguishable both in morphology and shape from the mature trichome, which function in the dispersal of the cyanobacterium in the environment. The differentiation of hormogonia takes place through a number of rapid cell division events that are not coupled to net DNA synthesis or to an increase in cell biomass. Whereas, it produces partitioning of the many copies of the chromosome that are usually present in vegetative cyanobacterial cell (Herdman and Rippka, 1988; Meeks and Elhai, 2002). The *fts*Z gene (which in *Escherichia coli* has been shown to encode a self-assembling, filament-forming protein essential for cell division) has been cloned from the hormogonium-forming cyanobacterium *Fremyella diplosiphon* (*Calothrix* sp. strain PCC7601) and characterized (Doherty and Adams, 1999). *fts*Z has been shown to increase its expression preceding the peak of cell division, after a shift to conditions that induce hormogonium formation. This obsevation suggests that, as seems to be also the case in *E. coli*, the amount of FtsZ protein could be rate-limiting for cell

division in *F. diplosiphon* (Doherty and Adams, 1999), at least during the burst of cell division that produces the hormogonium. Hormogonia represent a transient state of the cyanobacterium that, subsequently, losses motility and resumes the synthesis of macromolecules leading to the production of mature vegetative trichome.

In some strains of genera *Nostoc, Tolypothrix* and *Calothrix*, the differentiation of hormogonia may take place as a transient stage of the cell cycle (Tandeau der Marsac, 1994). In the case of symbiotic associations with other organisms, hormogonia represent the infective form of the cyanobacterium that initiates the contact with the partner, hormogonium development being influenced both positively and negatively by host-released factors during the progression of the symbiosis (Meeks *et al.*, 2002; Campbell *et al.*, 2003). Nevertheless, in many cyanobacteria the differentiation of hormogonia seem to be a dispensable event taking place in response to changes in diverse external factors, including light and nutrients, that in fact can affect the differentiation process either positively or negatively (Tandeau der Marsac, 1994). It can be envisioned that rather than in response to a specific environmental cue, the differentiation of hormogonia may respond to changes that could impact the coordination between cell growth and division. In particular, the relation of hormogonium differentiation to nitrogen availability is suit to be nonspecific and in fact hormogonium differentiation can be induced both in the presence and absence of combined nitrogen (Damerval *et al.*, 1991). However, a mutant of the global nitrogen regulator NtcA derived from *N. punctiforme* has been reported to differentiate hormogonia at lower frequency than the wild-type strain when tested in co-culture with its symbiotic partner *Anthoceros punctatus* and is unable to infect it (Wong and Meeks, 2002). In contrast, *N. punctiforme* strains with mutations in *het*R or *het*F genes

(involved in heterocyst development, see below) infect *A. punctatus* at frequencies similar to that of the wild type (Wong and Meeks, 2002).

3.3.6.2 Differentiation of akinetes

Akinetes are cells distinguishable from vegetative cells of the filament by their larger size, thicker cell wall and conspicuous granulation consisting of cyanophycin and glycogen. Akinetes are considered as propagating, or perennating, bodies exhibiting resistance to adverse conditions, mainly cold and dessication. However, similarly to *Azotobacter* cysts, akinetes are sensitive to high temperatures, in this respect differing from bacterial endospores (Sutherland *et al*., 1979). Under favourable conditions, akinetes germinate producing short filaments that emerge through ruptures of the akinete cell wall (Nichols and Adams, 1982). The amount of DNA is generally reported to be similar in akinetes and vegetative cells and while some metabolic activities such as CO_2 fixation are very low in akinetes, the rate of respiration is often high (Adams and Duggan, 1999). Also, akinetes have been shown to make at least a few proteins, so that they seem to maintain some, although low metabolic activity (Thiel and Wolk, 1983).

Similar to the situation with the development of hormogonia, no single environmental trigger has been demonstrated to promote akinete development. Under laboratory conditions, akinetes are profusedly formed at the end of the exponential growth phase. Their appearance being delayed by factors that prolong active growth of cultures, so that the most widely recognized factors influencing akinete differentiation, such as light or phosphate limitation, could act by causing energy limitation (Sutherland *et al*., 1979). Akinete germination can be induced by dilution of

stationary-phase cultures into fresh medium, in general by changes favouring active growth of cultures and it should be aided by their usually high nitrogen (cyanophycin and carbon (glycogen) reserves content. Initiation of akinete germination does not require DNA synthesis, but may be sustained by cell division events distributing between the newly formed vegetative cells the various copies of the chromosome present in the akinete. Koksharova and Wolk (2002a) reported that mutation of genes *ftn*, which would encode products containing a DnaJ motif, causes the formation of akinete-like cells in *Anabaena* sp. strain PCC7120 (also known as *Nostoc* sp. strain PCC7120), a strain not previously recognized as capable of akinete differentiation. The observation suggests that these genes could be involved in akinete differentiation through an effect on cell division.

In some cyanobacteria, in the absence of combined nitrogen akinetes are frequently formed adjacent to a heterocyst, which specifically differentiates in response to combined nitrogen deprivation and the addition of some nitrogen nutrients like nitrate or urea inhibits akinete formation (Fay *et al.*, 1984). Nevertheless, in other strain akinete first appear distant from heterocysts in the absence of combined nitrogen (Herdman, 1988). Information currently available suggests that limitation of nitrogen may be a factor that induces akinete development directly by provoking a decrease of the growth rate.

The differentiation of akinetes has evident connections to the differentiation of heterocysts. In some strains the pattern of heterocyst distribution determines akinete differentiation in the absence of combined nitrogen (Sutherland *et al.*, 1979). Additionally, some common specific structural components have been identified in the wall of both cell types that are synthesized during the differentiation processes (Wolk

et al., 1994). Mutation in *A. variabilis* strain ATCC29413 of the gene *hep*A (which in *Anabaena* sp. strain PCC7120 has been characterized as involved in formation of the heterocyst polysaccharide layer) also impairs akinete development (Wolk *et al*., 1994). In *N. ellipsosporum*, inactivation of the gene *het*R (whose mutation prevents heterocyst differentiation) has been reported to impair also akinete differentiation and the *het*R gene is expressed also in the akinete (Leganes *et al.*, 1994). It has been suggested that the differentiation of heterocysts may have evolved based on that of akinetes, which would have formerly existed (Wolk *et al*., 1994). While some genetic elements (e.g. *het*R and *hep*A) act in a supposedly common stem of the differentiation of both types of cell, then other elements (e.g. *het*P) would act later and be specific for the differentiation of heterocysts and akinete (Leganes *et al*., 1994). On the other hand, in *N. punciforme*, inactivation of *het*R has been reported to prevent heterocyst development but permit the formation of akinete-like cells (Wong and Meeks, 2002). Strain differences might respond for these apparently contrasting results. Alternatively, akinete-like cells could develop in the *N. ellipsosporum het*R mutant as cells more resistant to certain stress conditions, but more similar in morphology to vegetative cells than to akinetes of the wild type and thus, could have gone unnoticed (Wong and Meeks, 2002). If this were the case, *het*R would have a role in akinete differentiation, in contrast to heterocyst differentiation would not be required to trigger the process. These and other indications of a relationship between heterocysts and akinetes led to the conjecture that the heterocyst evolved from the akinetes (Wolk *et al*., 1994).

Wong and Meeks (2002) induced akinete development by culturing *N. punctiforme* in medium without phosphate. In phosphate starvation, cyanobacterial

strains *het*R mutant formed large cells that properties similar in overall structure of akinete. The large cells were tested on the chilling resistance and showed plating efficiency same as the wild type strain. Furthermore, mechanisms that protect cells from the effects of desiccation by akinete differentiation relate with oxidative damage. The synthesis of superoxide dismutase (SOD), mediates the disproportination of superoxide redicals to hydrogen peroxide and dioxygen. Cyanobacterium *A. cylindrica* Lemm. is known to use both Fe- and Mn- containing SODs to scavenge superoxide redicals (Canini *et al.*, 1992). Therefore, akinete differentiation is induced in Fe- and Mn- free medium. Shirkey and co-workers (2000) reported that active Fe-superoxide dismutase was abundant soluble protein and *sod*F mRNA was found in *N. commune* CHEN/1986 after prolonged storage in the desiccated state *sod* gene is the one way to investigate on akinete cell. Singh and co-workers (Singh and Dikshit, 1976; Singh and Sinha, 1965, Singh and Tiwari, 1969, 1970) identified mutants of four cyanobacterial genera, including *Anabaena*, in which akinete formation was lost (the Aki^- phenotype) while heterocyst formation was retained (the Het^+ phenotype). The results showed that at least one gene is specific to the process of akinete differentiation, but no such gene has yet been identified.

Genetic analysis of akinete differentiation would be greatly facilitated by the identification of a marker gene for developing or mature akinetes. Zhou and Wolk (2002) provided the first report of a gene that was expressed primary in akinetes. Akinetes and heterocysts of *A. variabilis* both form a conspicuous envelope, but they are distinguishable. Whereas, the pole of the heterocyst is perforated by a cytoplasmic channel that is surrounded by a thick layer of glycolipid, no such structure is found in the akinete. Often, the akinete is also more strongly pigmented and has a more granular

interior. Although very slight fluorescence at wavelengths characteristic of GFP emission was visualized with the wild-type strain, GFP-based fluorescence originated primarily from akinetes. Therefore, expression of *ava*K is primarily, perhaps completely, akinete specific.

3.3.6.3 Differentiation of heterocysts

Heterocysts are cells highly specialized in the fixation of atmospheric nitrogen under oxic conditions that some filamentous cyanobacteria differentiate when combined nitrogen becomes limiting. Changes in gene expression measured at level suggest that 15-25% of the genome is uniquely expressed in heterocysts (Lynn *et al.*, 1986). Heterocysts obtain photosynthate from nearby vegetative cells and provide fixed nitrogen as amino acids to the filament. Heterocysts are terminally differentiated cells that neither divide, consistent with the lack of the FtsZ protein (Fts: filamentous temperature sensitive) in these cell (Kuhn *et al.*, 2000), nor, after a certain point in the developmental process, revert to the vegetative cell state. Heterocyst death causes, in the case of intercalary heterocysts, breakage of the filament at the point occupied by the moribund heterocyst.

Heterocysts exhibit conspicuous differences, both in structure and function, with the vegetative cells from which they originate. These differences are aimed at the expression of the nitrogen fixation machinery, at increasing the efficiency of the nitrogen fixation reaction and at protection of the nitrogen fixation machinery against oxygen. The differential traits of the heterocyst include the presence of supplemental glycolipid and polysaccharide layers in the cell envelope, aimed at hampering the influx of gases; lack of activity of the photosystem II, avoiding photosynthetic oxygen

production; increased respiration, eliminating free oxygen and also contributing to the provision of energy for the nitrogen fixation reaction and lack of photosynthetic CO_2 fixation, thus avoiding distracting energy and reducing power to processes other than nitrogen fixation (Wolk *et al*., 1994).

The differentiation of "first generation" heterocyst, i.e. differentiation triggered by exhaustion of sources of combined nitrogen, the first event is perception of nitrogen stress. This lead to an increase in general proteolysis, in particular, to degradation of the phycobilliproteins, photosynthesis accessory pigments that may account for up to 50% of the cellular protein. Thus sign of differentiation shows the first microscope as a deficiency in fluorescence of the cells that start the route of development. Progression of differentiation produces the so-called proheterocysts, an intermediate stage that differs in shape and granulity from vegetative cells (Wolk *et al*., 1994). Proheterocysts undergo a series of traceable morphological changes (Nierzwicki-Bauer *et al.*, 1984; Sherman *et al.*, 2000) that leads to the formation of the heterocyst-specific envelope and reorganization of intracellular membranes, more or less concomitant with characteristic changes in cell metabolism such as an increase in respiration. Finally, expression of nitrogenase activity, that in some cyanobacteria is preceded by several genomic reorganization effected through site-specific recombinational events (Wolk *et al*., 1994). Based on the fact that certain mutants unable to form a proper heterocyst envelope are also unable to complete protoplast maturation. It has been suggested that the establishment of the barrier to oxygen might constitute a developmental checkpoint that could trigger the process of maturation (Fiedler *et al.*, 1998; Zhu *et al*., 2001).

Since heterocysts have lost the capacity of photosynthetic CO_2 fixation. The activity of nitrogen fixation is these cells depends upon the supply by the adjacent

vegetative cells of reduced carbon compounds to be used as sources of reductant and of the substrate necessary for the incorporation of ammonium derived from N_2 reduction. Sucrose is considered a likely candidate for reduced carbon vehicle (Wolk *et al*., 1994; Curatti *et al.*, 2002).

The distinctive morphological and physiological traits of the heterocysts are the consequence of a differential program of gene expression relative to that operating in the vegetative cells. Thus, a number of genes such as those encoding the enzyme nitrogenase are expressed only in the mature heterocyst or such as the *dev*BCA genes, preferentially during the intermediate states of heterocyst development. Whereas other sets of genes, e.g. those encoding ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), are actively expressed in the vegetative cells but not in the heterocyst. Some genes can still be expressed in both type of cells, as in the case of *gln*A, encoding glutamine synthetase. NtcA, the global nitrogen regulator, appears to play a crucial role at regulation of gene expression throughout the whole develpomental process. Some genes that have been identified as involved in heterocyst differentiation or function are summarized in Table 3.3.

Table 3.3. Some genes involved in heterocyst development or function (Golden and Yoon, 1998; Herrero *et al*., 2004)

Gene	Product homology or (putative) function	Reference (s)
Early events		
*ntc*A	Nitrogen regulation; autoregulatory gene	Frias *et al*., 1994; Wei *et al*., 1994
*pat*A	Pattern formation	Liang *et al.*, 1992
*pat*S	Intercellular inhibition of heterocyst formation	Yoon and Golden, 1998, 2001
*han*A	Similar to *E. coli* protein HU	Khudyakov and Wolk, 1996
*het*R	Autoregulatory gene; autoproteolysis and DNA-binding activities	Buikema and Haselkorn, 1991; Black *et al.*, 1993; Zhou *et al*., 1998; Huang *et al.*, 2004
Middle events		
*het*C	ABC-type exporter	Khudyakov and Wolk, 1997
*het*P	Unknown function	Fernandez-Pinas *et al.*, 1994
*het*L	Non essential positive-acting element	Liu and Golden, 2002

Table 3.3. (continued)

Gene	Product homology or (putative) function	Reference (s)
*het*F[a]	Required for localization of HetR in differentiation heterocysts	Wong and Meeks, 2001
*dev*H	Putative DNA binding protein	Hebbar and Curtis, 2000
*het*N	Pattern maintenance	Black and Wolk, 1994; Bauer *et al*., 1997; Callahan and Buikema, 2001
*het*M (*hgl*B), *hgl*C, *hgl*D, *hgl*E[a]	Structural genes for glycolipid biosynthesis	Black and Wolk, 1994; Bauer *et al*., 1997; Campbell *et al.*, 1997
*hgl*K, *dev*BCA	Transport and deposition of heterocyst envelope glycolipids	Fiedler *et al*., 1998; Black *et al.*,1995
*hep*K, *dev*R[a]	Two-component regulatory system; heterocyst polysaccharide biosynthesis	Campbell *et al.*, 1996; Zhu *et al.*, 1998; Zhou and Wolk, 2003
*hep*C, *hep*A	Heterocyst envelope polysaccharide biosynthesis	Zhu *et al*., 1998; Holland and Wolk, 1990
*abp*2, *abp*3	DNA-binding proteins required for expression of *hep*C and *hep*A	Koksharova and Wolk, 2002

Table 3.3. (continued)

Gene	Product homology or (putative) function	Reference (s)
*pkn*D	Protein kinase	Zhang and Libs, 1998
*prp*A, *pkn*E	Protein phosphatase and kinase, respectively	Zhang *et al.*, 1998
*pat*B	Putative helix-turn-helix and ferredoxin domains	Liang *et al.*, 1993; Jones *et al.*, 2003
Late events		
*hew*A	Autolysin required for heterocyst maturation	Zhu *et al.*, 2001
*pbp*B	Putative penicillin-binding protein	Lazaro *et al.*, 2001
*rfb*P	Undecaprenyl-phosphategalactose-phosphotransferase	Xu *et al.*, 1997
*xis*A, *xis*C, *xis*F	Excisases involved in DNA rearrangement	Golden and Yoon, 1998
*xis*H, *xis*I	Excision of the *fdx*N DNA-intervening element	Ramaswamy *et al.*, 1997
Function		
zwf[a], *opc*A[a]	Glucose-6-phosphate dehydrogenase and allosteric effector	Summers *et al.*, 1995; Hagen and Meeks, 2001
*cox*2,*cox*3 operons	Terminal respiratory oxidases	Jones and Haselkorn, 2002; Valladares *et al.*, 2003

Table 3.3. (continued)

Gene	Product homology or (putative) function	Reference (s)
*pet*H[b]	Ferredoxin-NADP$^+$ reductase	Razquin *et al.*, 1996; Valladares *et al.*, 1999
*fdx*H	Ferredoxin	Bohme and Haselkorn, 1988;
*fdx*N	Bacterial-type ferredoxin	Masepohl *et al.*, 1997b Mulligan and Haselkorn, 1989; Masepohl *et al.*, 1997a
nif genes	Nitrogenase strutural and maturation genes	Haselkorn *et al.*, 1983; Bohme, 1998
*hup*LS	Uptake hydrogenase	Carrasco *et al*., 1995; Oxelfelt *et al.*, 1998; Happe *et al.*, 2000
*glb*N[c]	Cyanoglobin	Potts *et al*., 1992; Hill *et al*., 1996
*gln*A	Glutamine synthetase	Frias *et al*., 1994; Tumer *et al.*, 1983
*arg*Ld	Arginine biosynthesis	Leganes *et al.*, 1998

Table 3.3. (continued)

Gene	Product homology or (putative) function	Reference (s)
cphAe, cphB	Cyanophycin synthetase and cyanophycinase	Ziegler et al., 2001; Picossi et al., 2004
idh	Isocitrate dehydrogenase	Martin-Figueroa et al., 2000; Papen et al., 1983

All genes first identified in *Anabaena* sp. strain PCC7120 except those identified in *N. punctiforme* (ATCC29133) ([a]), *Anabaena* sp. strain PCC7119 ([b]), *Nostoc commune* ([c]), *N. ellipsosporum* ([d]) or *A. variabilis* (ATCC29413) ([e]).

Heterocyst formation is arbitrarily divided into three phases: early events involved in initiation of development and pattern formation, middle events required for continued cellular differentiation and late events required for a functional nitrogen-fixing heterocyst (Golden and Yoon, 1998).

3.3.6.3.1 Early events and pattern formation

Heterocyst-forming cyanobacteria appear to respond to nitrogen deprivation in a series of stages, until differentiation is completed (in *Anabaena* sp. strain PCC7120, after about 20 h) (Wolk, 1996). Heterocyst differentiation and distribution has been studied at the molecular level almost exclusively in the *Nostocaceae*, in which heterocysts appear at semiregular intervals along the filament with a frequency of one heterocyst every 10-15 vegetative cells. Experiments designed to identify the earliest genes involved in heterocyst formation showed that nitrogen deprivation of *Anabaena*

sp. strain PCC7120 causes rapid activation of genes necessary for uptake and utilization of nitrate within 0.5 h (Cai and Wolk, 1997b). Three closely linked loci that showed rapid and strong induction were identified with a transposon bearing a *lux*AB reporter. The insertions were within the *nir*A (encoding nitrite reductase), *ntr*C (encoding nitrate/nitrite transporter) genes, between *nrt*D (encoding nitrate/nitrite transporter) and *nar*B (encoding nitrate reductase). The structure and expression of this nitrate assimilation gene cluster was also studied by another group (Frias *et al.*, 1997). These results showed that vegetative cell filaments starved for combined nitrogen first attempt to scavenge for nitrogen compounds before heterocysts are formed.

The DNA-binding protein NtcA is required for positive regulation of genes involved in nitrogen assimilation but also interacts with several other genes. The *Anabaena* sp. strain PCC7120 *ntc*A gene was identified by its homology to the *Synechococcus* gene (Frias *et al.*, 1993; Luque *et al.*, 1994), by NtcA (also known as BifA, VF1) interaction with DNA sequences between the site-specific recombinase gene *xis*A and a recombination site for the *nif*D element (Wei *et al.*, 1993). NtcA belongs to a family of prokaryotic regulatory proteins represented by Crp (cAMP receptor protein) (Frias *et al*., 1993; Wei *et al*., 1993). *Anabaena* sp. strain PCC7120 NtcA (BifA) also binds to other genes that are differentially regulated during heterocyst development including *gln*A (encoding glutamine synthetase), *rbc*L (encoding ribulose biphosphate carboxylase) and *nif*H (encoding dinitrogenase reductase) promoter region (Ramasubramanian *et al.*, 1994). NtcA is required for heterocyst development (Frias *et al*., 1994; Wei *et al*., 1994). *ntc*A mutants are unable to grow with nitrate or dinitrogen as the sole nitrogen source, but can be grown on media containing ammonia. *ntc*A mutants showed no sign of differentiation upon

combined nitrogen starvation, indicating that the NtcA protein is required at the initiation of the process. NtcA may respond not only to nitrogen status but also to the cellular redox status, which might be significant during heterocyst differentiation. NtcA interacts with the promoter sequence of the *Anabaena* sp. strain PCC7120 *gor* gene (encoding glutathione reductase), which is not known to be directly involved in heterocyst formation and appears to act as a repressor (Jiang *et al.*, 1997). It was previously suggested that NtcA might act as a repressor for the *rbc*LS operon (Ramasubramanian *et al*., 1994). In repression of *gor*, DNA-binding by NtcA is regulated *in vitro* by a redox-dependent mechanism involving cysteine residues of the NtcA protein (Jiang, *et al*., 1997).

As measured using transcriptional fusions to *lux*AB, a *het*R mutation does not block induction of *nir*A, *nrt*C, or *nrt*D, nor of another gene, *tln*6, that is activated at 4.5 h (Cai and Wolk, 1995). Mutants in *nir*A, *nrt*C, or *nrt*D remain competent to make heterocysts and fix nitrogen. Therefore, removal of ammonia elicits parallel responses that lead to nitrogen fixation and to an enhanced capacity to assimilate nitrate. However, an *ntc*A mutation blocks induction of *het*R (Frias *et al*., 1994), a *het*R mutation blocks induction (at 4.5-6 h) of *hep*A and *dev*A mutation in turn blocks activation (at 9 h) of *lux*AB (Black *et al*., 1993; Cai and Wolk, 1995). The *han*A gene encodes the histone-like HU protein that is absent from heterocysts and whose mutation results in a highly pleiotrophic phenotype that includes lack of heterocyst development (Khudyakov and Wolk, 1996; Nagaraja and Haselkorn, 1994). However, NtcA-binding site has a role at suppression of *han*A expression remains to be investigated.

HetR plays a central role in the control of heterocyst formation that exhibits DNA-binding (Huang *et al*., 2004) and autoproteolysis (Zhou *et al*., 1998) activities *in vitro*. Within 2 h of nitrogen deprivation, *het*R becomes more active in what are evidently developing heterocyst (Black *et al*., 1993; Haselkorn, 1995), proteolysis increases measurably (Thiel, 1990; Wood and Haselkorn, 1980) and liberated amino acids are excreted (Ownby *et al*., 1979; Thiel, 1990). HetR mutants fail to produce heterocysts and extra copies of *het*R on a plasmid cause formation of supernumerary heterocysts (Buikema and Haselkorn, 1991). The use of a *het*R-*lux*AB reporter fusion showed that *het*R is expressed in a spatial pattern and is positively autoregulated (Black *et al*., 1993). Normal expression of *het*R is blocked in *ntc*A (Frias *et al*., 1994) and *han*A (Khudyakov and Wolk, 1996) mutants, which may explain why these mutants fail to form heterocysts. A *het*R mutation blocks induction of genes required for later stages of heterocyst differentiation including *dev*A and *het*M (Cai and Wolk, 1997a). The *het*R mutation, however, does not block expression of several genes that are induced after nitrogen step-down, presumably in vegetative cells, but that are not required for heterocyst differentiation (Cai and Wolk, 1997a).

HetR has recently been shown to be an unusual serine-type protease after observing that the purified protein was unstable (Zhou *et al*., 1998). HetR protein from *het*R mutant strain 216, which cannot differentiate heterocysts (Buikema and Haselkorn, 1991), did not undergo proteolysis, suggesting that its proteolytic activity is required for HetR regulation of development. Antibodies against HetR were used to directly show that HetR protein levels increase in heterocysts three hours after induction and that the native protein may be post-translationally modified to become more acidic in heterocyst containing filaments (Zhou *et al*., 1998). For pattern

formation of heterocyst, *pat*A (Liang *et al*., 1992): PatA, requires for the increase in the expression of the *het*R gene in internal cells of the filament. *pat*A mutant suppressed the multiheterocyst phenotype normally produced by extra copies of the *het*R gene, thus, makes heterocysts mainly at the end of filaments.

Some genes have also been described that negatively affect heterocyst development. A conjugal cosmid clone was found to confer a heterocyst-suppression phenotype and subcloning experiments showed that overexpression of a small gene, *pat*S, blocks heterocyst differentiation. A *pat*S knockout mutant forms multiple contiguous heterocysts (Mch). Evidence suggests that PatS may function as a diffusible signal produced by pro-heterocysts to inhibit neighboring cells from differentiating. Cell-cell communication often plays a key role in signaling cell to adapt a specific identity and thereby to follow a particular developmental fate. A diffusible inhibitor has been suggested to regulate heterocyst pattern formation (Wolk *et al*., 1994; Yoon and Golden, 1998) and it is probable that PatS at least partially fulfills this role. Expression of *pat*S increases during several hours after nitrogen step-down in a patterned way in the cells that will become heterocysts, then decreases down to the initial level (Yoon and Golden, 1998, 2001). Although the way of regulation of *pat*S is currently unknown, the recent report (Huang *et al*., 2004) of binding of HetR to its promoter region must be taken into account. PatS seems to be involved in "*de novo*" heterocyst pattern formation upon combined nitrogen deprivation, by inhibition of the differentiation of neighboring cells (Yoon and Golden, 1998, 2001).

3.3.6.3.2 Middle events

A number of genes have been identified that are required for formation of a mature heterocyst (Table 3.3). Many of these genes are involved in the formation of the heterocyst envelope, which consists of glycolipid, lipopolysaccharide and polysaccharide components. The multilayered envelope is necessary to establish the microaerobic environment required for nitrogenase function. Other genes clearly encode elements of signal transduction pathways, but their inputs, outputs and interconnections are as yet unknown.

3.3.6.3.2.1 Heterocyst development

DevR and PatB are required for completion of heterocyst development. The *dev*R gene from *Nostoc* strain ATCC29133 is required for formation of mature heterocysts (Campbell *et al*., 1996). DevR is similar to receivers of two-component regulatory systems such as CheY and Spo0F and may be part of a phosphorelay that is required for the completion of heterocyst formation. A *pat*B mutant showed delays and incomplete heterocyst development causing filaments to accumulate more heterocysts than normal (Liang, *et al*., 1993). Its product is predicted to have a helix-turn-helix DNA-binding motif in its carboxy-terminal region and ferredoxin-like 4Fe-4S center in its amino-terminal region suggesting an involvement in redox regulation.

A gene that is involved in relatively early regulation of heterocyst differentiation, *het*C, encodes a 1,044 amino acid protein which shows very extensive similarity to a subclass of ATP-binding cassette (ABC) proteins that are involved in the export of certain proteins from bacterial cells (Khudyakov and Wolk, 1997; Muro-Pastor *et al.*, 1999; Xu and Wolk, 2001). *het*C mutants is arrested to form

proheterocysts, but do produce a cryptic pattern of nonfluorescent cells, which suggests that early stages of heterocyst differentiation have occurred. *het*C expression depend on HetR and on HetC itself, indicating autoregulation. Expression of *het*C takes place from a single NtcA-dependent promotor that is activated promptly upon combined nitrogen deprivation (Muro-Pastor *et al*., 1999). *het*C is approximately 1 kb upstream of *het*P, which is also required for normal heterocyst formation and causes multiple contiguous heterocysts to form when present in extra copies (Wolk, 1996; Leganes *et al*., 1994).

Expression of the *ntc*A gene itself is induced several fold during the early steps of heterocyst differentiation in a HetR-dependent and autoregulated manner based on activation of two regulated promoters; one generating transcription start point (tsp) -49 that is preferably used in the absence of combined nitrogen, early during heterocyst differentiation and that is active in mature heterocysts. Another one generating tsp -180 that appears to be transiently used during heterocyst development but not in mature heterocyst (Muro-Pastor *et al*., 2002; Ramasubramanian *et al*., 1996). Thus, a mutual dependence is observed in the expression of both regulatory genes *ntc*A and *het*R. Activation of the expression of *het*R at the initiation of heterocyst differentiation precedes that of *ntc*A (Muro-Pastor *et al.*, 2002). This implies that NtcA-mediated activation of *het*R expression does not require activation of the expression of the *ntc*A gene and makes it conceivable that the HetR-dependent activation of *ntc*A expression requires previous activation of *het*R expression. Conversely, the NtcA-dependent initiation of *het*C transcription is independent of HetR (Muro-Pastor *et al*., 2002). Because activation of *ntc*A expression is depend on HetR, these observations suggest that initiation of *het*C transcription does not require HetR-dependent increased

expression of the *ntc*A gene. Additionally, the observation that the NtcA-mediated activation of *het*R expression is not impaired in a *het*C mutant (Xu and Wolk, 2001; Muro-Pastor *et al*., 1999) indicates that HetC is not required for activation of *het*R expression.

A possible model for a sequence of events of activation of gene expression at the initiation of heterocyst differentiation implies independent activation of the *het*R and *het*C genes both operated by the initial low levels of NtcA protein already present in the filament exposed to combined nitrogen. Activation of *het*R by NtcA would be indirect and enhanced by autoregulation, whereas that of *het*C would be direct. Subsequently, the resulting increased cellular levels of the HetR protein would lead to activation of *ntc*A expression also enhanced by autoregulation (Fig. 3.14).

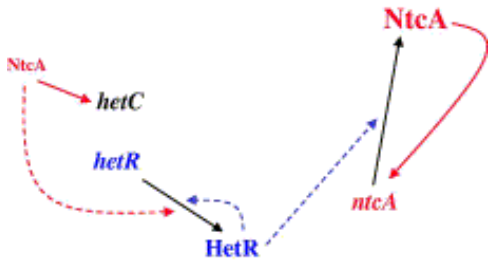

Figure 3.14 Some events of activation of gene expression at the initiation of heterocyst differentiation (Herrero *et al*., 2004). Black arrows: gene expression from transcription to the corresponding mature protein. Red solid arrows indicate NtcA promoted transcription activation. Dashed arrows indicate a positive action exerted by NtcA (red) or HetR (blue) on gene expression.

Other genes that participate in the heterocyst development and the establishment of the pattern of heterocyst distribution along the cyanobacterial filament have been described. In *N. punctiforme*, a gene named *het*F whose product appears to cooperate with HetR at positive regulation has been identified (Wong and Meeks, 2001). Similarly to the situation with *het*R, *het*F mutants are unable to develop heterocysts, whereas extra copies of *het*F induce formation of clusters of heterocysts. Activation of *het*R expression in *het*F mutant is delayed and not restricted to developing heterocysts, taking place even under nitrogen-replete conditions (Wong and Meeks, 2001). The *het*F gene is constitutively expressed and its relationship to NtcA is unknown. Another gene, named *het*L, whose overexpression produces Mch even in nitrate-containing medium, has been recently identified in *Anabaena* sp. strain PCC7120 (Liu and Golden, 2002). Although a *het*L-null mutant shows normal heterocyst development and diazotrophic growth, which might indicate a nonessential role of HetL in the process. *het*L overexpression can bypass the suppression of heterocyst differentiation provoked by extra copies of *pat*S, but cannot bypass the requirement for HetC or HetR (Liu and Golden, 2002). Interestingly, *het*L overexpression in *ntc*A mutant allows signs of initiation of heterocyst development, but differentiation could not proceed and the filaments became highly fragmented (Liu and Golden, 2002), consistent with a requirement for NtcA beyound the initial steps of differentiation. Thus, *het*L overexpression may have a positive effect on HetR activity or abundance (Liu and Golden, 2002), perhaps bypassing the requirement for NtcA or *het*R activation. However, it is also possible that HetL is not involved in the regulation of heterocyst differentiation in the wild-type strain (Liu and Golden, 2002).

The effect of manipulation of the expression of the N-control regulator NtcA and of genes *het*R, *het*F and *pat*S on heterocyst frequency and spacing pattern can be compared. While strains carrying multiple copies of *ntc*A (*N. punctiforme* (Wong and Meeks, 2002) or expressing the *ntc*A gene from a strong, constitutive promoter (*Anabaena* sp. strain PCC7120; E. Olmedo-Verd, A. Herrero, E. Flores and A. M. Muro-Pastor, unplusblished) develop heterocysts only in the absence of combined nitrogen and do so with wild-type spacing pattern and frequency. Overexpression of *het*R or *het*F or mutation of *pat*S leads to the formation of Mch in combined nitrogen-free medium. Moreover, overexpression of *het*R or inactivation of *pat*S but not overexpression of *het*F, leads to differentiation of heterocyst in the presence of nitrate (Yoon and Golden, 1998; Buikema and Haselkorn, 1991, 2001; Wong and Meeks, 2001). It is tempting to speculate that the action of NtcA could be primarily involved with the triggering and progression of differentiation of a given cell to a function heterocyst. While the action of the product of *het*R (and perhaps also *het*C, *het*F and *het*L) could be more directly connected to that of the negative factor PatS in determining the spacial distribution of heterocysts and prevention of PatS action inside the differentiating cell. When the cells sense nitrogen deficiency, the balance between the action of positive factors (NtcA, HetR, HetF, HetC and possibly HetL) and the suppression mediated by PatS (and later by HetN; see below) may lead to the decision of whether or not to differentiate and which particular cell will become a heterocyst.

3.3.6.3.2.2 Formation of heterocyst envelope

From 4 to 10 h, synthesis of heterocyst envelope layers is initiated and the shape of cell changes subtly (Wilcox *et al.*, 1973). The DevBCA exporter is essential

for heterocyst envelope formation (Fiedler *et al*., 1998). The *dev*BCA operon is expressed from a N-regulated, NtcA-dependent promoter early upon combined nitrogen deprivation (Fiedler *et al*., 2001). However, the increase of *dev*BCA transcript levels that is detected at intermediate stages of heterocyst development requires HetR in addition to NtcA (Fiedler *et al*., 2001). The *Anabaena* sp. strain PCC7120 *dev*A gene encodes a protein with high similarity to ATP-binding cassettes of ABC transporter. DevA may play a role in the transport of nutrients into heterocysts (Wolk, 1996). Whereas *dev*B and *dev*C encode proteins with similarities to membrane fusion proteins and to membrane-spanning proteins of ABC transports, respectively. Mutants affected in any of these three genes are blocked at an early stage of heterocyst development and the heterocyst glycolipid layer is missing. A *dev*A mutant fails to show induction of the *het*M gene fused to *lux*AB (Cai and Wolk, 1997a). The phenotype of these mutants suggests that completion of the envelope may be a developmental checkpoint (Fiedler *et al*., 1998). The *dev*H gene encoding a putative DNA-binding protein required for N_2 fixation in the heterocyst (Hebbar and Curtis, 2000) and the *cox*2 and *cox*3 operons encoding terminal respiratory oxidases also required for nitrogenase activity in the hetrocysts (Valladares *et al*., 2003).

A locus that includes the *het*M (also known as *hgl*B), *het*N and *het*I genes was originally thought to control heterocyst pattern (Black and Wolk, 1994). Further analysis of the *het*N locus suggests a role in glycolipid production (Bauer *et al*., 1997). *het*M (*hgl*B), *hgl*C and *hgl*D (only partially sequenced) encode domains silmilar to those found in polyketide or fatty acid synthases. RNA blot analysis showed that these genes are transcribed 6-12 hours after heterocyst induction. Inactivation of any these genes produced heterocysts incapable of nitrogen fixation in the presence of oxygen

and thin layer chromatography of these mutants showed that they are deficient in heterocyst-specific glycolipids.

The *N. punctiforme* (ATCC29133) *hgl*E gene also encodes a protein containing domain chraracteristic of polyketide and fatty acid synthases (Campbell *et al*., 1997). *hgl*E is required for production of heterocyst glycolipids and for aerobic nitrogen fixation. The synthesis of heterocyst glycolipids follows a pathway characteriatic of polyketide synthesis and involves similar large, multienzyme complexes.

Synthesis of heterocyst envelope polysaccharide is dependent on the gene *hep*A, which is activated starting between 5 and 7 h after nitrogen step-down (Holland and Wolk, 1990; Wolk *et al.*, 1993). The *hep*A promoter and *cis*-acting elements required for normal expression has been identified, as well as an upstream gene, *hep*C (Zhu *et al*., 1998). Inactivation of *hep*C caused contitutive expression of *hep*A and prevented synthesis of heterocyst envelope polysaccharide. A *hep*K mutation blocked both the synthesis of the heterocyst envelope polysacharide and induction of *hep*A (Zhu *et al*., 1998). HepK is predicted to be a sensory protein-histidine kinase (Zhu *et al*., 1998).

3.3.6.3.3 Late events

Upon completion of the envelope and augmentation of respiration, the protoplast of the differentiating cell becomes microaerobic. The programs of transcription and protein synthesis change extensively during the final hour of differentiation (Fleming and Haselkorn, 1974; Lynn *et al*., 1986). In particular, nitrogenase activity increases to its level in mature heterocysts. The defining late event during heterocyst formation is expression of nitrogen-fixation (*nif*) genes. For

many strains, programmed site-specific excision of DNA elements from *nif* operons occurs in heterocysts. These DNA rearrangements are required for functional expression of the *nif* genes. In addition, studies of the storage polymer cyanophycin (Leganes *et al*., 1998; Ziegler *et al*., 1998) and amino acid transport (Montesinos *et al.*, 1995, 1997) are expected to provide a better understanding of how fixed nitrogen is supplied to the filament.

In the past, additional nitrogen-fixation genes have been identified and characterized. The *Anabaena* sp. strain PCC7120 *nif*VZT genes have been cloned and form a second *nif* gene cluster that is probably an operon (Stricker *et al.*, 1997). A *nif*V knockout mutant was capable of diazotrophic growth that was only slightly impaired compared to that of the wild type. *A. variabilis* ATCC29413 fixes nitrogen under aerobic growth conditions in heterocysts and requires expression of either a Mo-dependent or V-dependent nitrogenase (Thiel, 1996). Under anaerobic conditions, a second Mo-dependent nitrogenase gene cluster, *nif*H, is expressed in vegetative cells (Thiel *et al*., 1995). The genes for this second Mo-dependent nitrogenase have been characterized (Thiel *et al*., 1997). A strain carrying a mutation affecting *nif*UHD in the *nif*H cluster did not fix nitrogen under anaerobic conditions until after heterocysts differentiated.

The role of two heterocyst-specific (2Fe-2S) ferredoxins was clarified by inactivation of their genes. It was concluded that the *fdx*H gene product of *Anabaena* sp. strain PCC7120 is important but not essential for nitrogen fixation (Masepohl *et al*., 1997b). *A. variabilis* ATCC29413 lacking the *fdx*N gene product was essentially wild type for nitrogen fixation (Masepohl *et al*., 1997a). Several additional genes that are required for normal heterocyst function and show developmental regulation are those

encoding uptake hydrogenase (Carrasco *et al*., 1995), *glb*N encoding cyanoglobin (Hill *et al*., 1996) and *pet*H encoding ferredoxin-$NADP^+$ reductase (Razquin *et al*., 1996).

Three heterocyst-specific DNA rearrangements occur in *Anabaena* sp. strain PCC7120 that involve the deletion of elements from the chromosome by site-specific recombination (Carrasco *et al*., 1995; Golden *et al*., 1985, 1987). Two rearrangements involve excision of 11 kb and 55 kb DNA elements from within *nif*D (encoding dinitrogenase alpha subunit) and *fdx*N (encoding a minor heterocyst-specific ferredoxin) genes, respectively (Golden *et al*., 1985, 1988). The *xis*A gene, which is on the *nif*D element and the *xis*F gene, which is on the *fdx*N element, encode site-specific recombinases required for the *nif*D and *fdx*N rearrangements, respectively (Golden and Wiest, 1988; Lammers *et al*., 1986; Carrasco *et al*., 1994). Correct excision of these elements is required for nitrogen fixation, but it is not clear if the elements serve other functions or if they are purely parasitic. The role of these binding sites in regulation of *xis*A expression is unknown. It has been hypothesized that binding of NtcA to them could exert a repressor role in vegetative cells (Chastain *et al*., 1990). The overlapping genes, *xis*H and *xis*I, downstream of *xis*F were shown to be required for excision of the *fdx*N element (Ramaswamy *et al*., 1997). *xis*H and *xis*I do not show similarity to any known genes. Surprisingly, extra copies of these genes force excision of the *fdx*N element in vegetative cells, indicating that they are involved in the cell-type specificity of this DNA rearrangement. The third rearrangement involves the deletion of a 10.5 kb element from within the *hup*L gene (Carrasco *et al*., 1995). *hup*L encodes the large subunit of a membrane-bound, NiFe uptake hydrogenase whose product recovers electrons expended in the formation of H_2 during nitrogen fixation (Carrasco *et al*., 1995; Matveyev *et al*., 1994) and is expressed late

during heterocyst development. The *xis*C gene, which is on the *hup*L element, encodes a polypeptide that is homologous to the site-specific recombinase XisA and distantly related to the integrase family of recombinase (Nunes-Duby *et al.*, 1998).

Anabaena sp. strain PCC7120 genes whose induction require NtcA and whose products act in the mature heterocyst include *pet*H (encoding ferredoxin: $NADP^+$ reductase), *gln*A (encoding glutamine synthetase), those in the *cph*BA1 (encoding proteins of cyanophycin metabolism) and *nif*HDK operons. Ferredoxin:$NADP^+$ reductase, which can contribute to the provision of the reduced ferredoxin required for the nitrogenase reaction and glutamine synthethase, responsible for the incorporation of the fixed nitrogen into carbon skeletons, are critical for the assimilation of nitrogen in heterocysts. The *pet*H gene is transcribed from two promoters, one constitutive with respect to the nitrogen source and another used in the absence of combined nitrogen and dependent on NtcA. The latter is the main promoter used in heterocysts, but it is also used in a *het*R mutant and in the wild type after a nitrogen step-down before mature heterocysts have developed (Valladares *et al*., 1999). The *gln*A gene is expressed from at least three promoters, one constitutive and two negatively regulated by ammonium and NtcA dependent (Frias *et al*., 1994). The *nif*HDK operon is expressed in *Anabaena* sp. strain PCC7120 under oxic conditions exclusively in the heterocyst (Elhai and Wolk, 1990) from a single N-regulated promoter that is not operative in the *ntc*A (Frias *et al*., 1994) or *het*R (A. Valladares, A. M. Muro-Pastor, A. Herrero and E. Flores, unplushished) mutants. An additional basis of the requirement for HetR and completion of heterocyst development for expression of the *nif* genes could originate in a negative effect of oxygen, consistent with the observed requirement of intact *cox*2 or *cox*3 genes for expression of nitrogenase activity (Valladares *et al*., 2003). PatB, a DNA-binding protein with a putative ferredoxin-like

domian expressed late during development and required for nitrogenase activity expression, may represent a sensor of redox state in the heterocyst (Jones *et al*., 2003). In heterocysts, promoters P_{cphB1}-1 that directs cotranscription of *cph*B1 (encoding cyanophycinase) and *cph*A1 (encoding cyanophycin synthetase) and P_{cphA1}-2 for monocistronic expression of *cph*A1 are N-regulated and used in an NtcA-dependent manner, although their requirement for HetR has not been investigated (Picossi *et al*., 2004).

In addition to its role as a transcriptional activator, NtcA appears to act as a repressor of some promoters during the course of heterocyst development (Herrero *et al*., 2001). Rubisco, encoded in the *rbc*LXS operon, is not expressed in heterocysts and in *Anabaena* sp. strain PCC7120 NtcA has been shown to bind to two sites in its promoter (Ramasubramanian *et al*., 1994). In summary, a hypothetical model for sequential activation of transcription of NtcA-dependent genes during heterocyst development is as presented in Fig. 3.15.

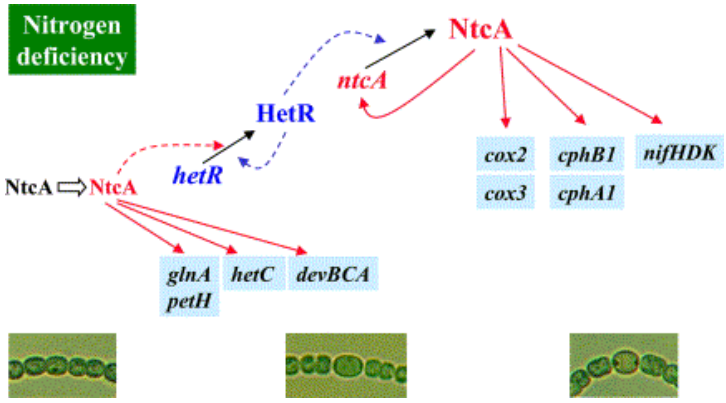

Figure 3.15 Hypothetical model for some steps of sequential activation of gene expression during heterocyst differentiation. Open arrow represents metabolic activation of the NtcA protein. Meaning of other types of arrows are as specified in the legend to Fig. 3.14. Temporal progression of morphological differentiation (shown on bottom) is only approximate (Herrero *et al*., 2004).

3.3.6.4 Additional factors

A few additional results bear mentioning because they are most likely to be heterocyst formation, although that relationship is not currently clear.

The *gln*B gene, encoding the PII protein, has been cloned from the heterocystous strain *N. punctiforme* (Hanson *et al.*, 1998). PII proteins are signaling molecules involved in response to the cellular nitrogen status. In *Synecchococcus* sp. strain PCC7942, the PII protein is differentially phosphorylated and is involved in the control of nitrate and nitrite uptake (Lee *et al.*, 1998). Six *Anabaena* sp. strain PCC 7120 sigma-factor genes have been identified: *sig*A encodes the principle sigma factor (Brahamsha and Haselkorn, 1991; Ramasubramanian *et al.*, 1995), *sig*B and *sig*C encode members of the σ^{70} group 2 (Brahamsha and Haselkorn, 1992). *sig*B and *sig*C are expressed under nitrogen-limiting conditions, but are not required for heterocyst differentiation or nitrogen fixation (Brahamsha and Haselkorn, 1992). Three genes, *sig*D, *sig*E and *sig*F, which encode group 2 sigma factors are individually dispensable for growth on dinitrogen. Although a *sig*D-*sig*E double mutant strain able to differentiate heterocysts, was unable to grow diazotrophically (Khudyakov and Golden, unpublished data).

Since Chunleuchanon and co-workers (2003) and Chairin (2002) demonstrated about population dynamic of N_2-fixing cyanobacteria in different ecosystems of Thailand and determined evaluation of uptake of nitrogen from cyanobacteria in rice plant. The results reported that cyanobacterial strain VICCR1-1 showed the morphology as *Nostoc* sp. (Fig. 3.16) and capable to fix nitrogen about 11 $\mu molC_2H_4$/mg chlorophyll a/h. ^{15}N labelled cyanobacteria at the rate of 4 g dried cells/pot were evaluated the availability of N from cyanobacteria for growth and yield

of Pathumthani-1 rice variety. The results indicated that N taken by rice plants which derived from cyanobacteria at different growth stages were as following: 29.7% at maximum tillering stage, 35.9% at flowering stage, 23.6% at harvest (Chairin, 2002).

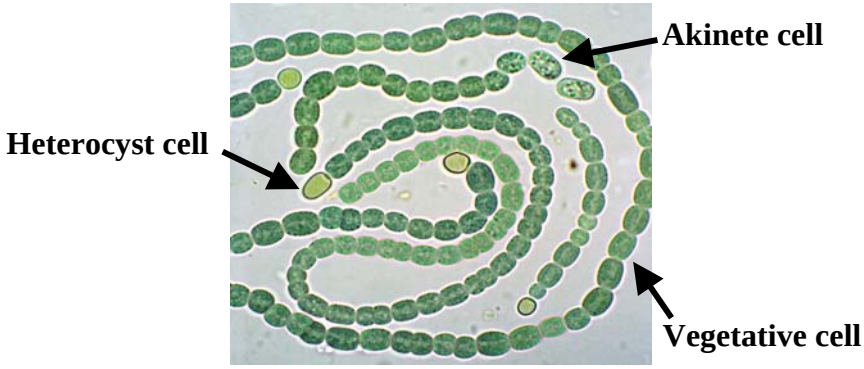

Figure 3.16 The cyanobacteial strain VICCR1-1.

Therefore, this research aimed to improve N_2-fixation efficiency for cyanobacteria as rice biofertilizer. The physiology and molecular genetic of N_2-fixing cyanobacteria were observed. Both of akinete and heterocyst development were induced to monitor the gene expression during differentiation. Furthermore, the akinete formation was induced to apply as inoculum in rice field. Since the montmorillonite clay had no nutrient for growth and easily dried when moisted, thus the akinete cell could not be germinated whereas capable to store for a long time. Therefore, the montmorillonite clay was used as carrier to prepare cyanobacterial inoculum.

3.4 Research Objectives

1. To induce akinete formation by chemical factors to apply as inoculum.
2. To determine expression of *het*R gene which involve in heterocyst differentiation.
3. To apply cyanobacteria as biofertilizer in rice field.

3.5 Materials and Methods

3.5.1 Cyanobacterial strain and cultivation

The cyanobacterial strain VICCR1-1 was grown on 50 ml of BG11 liquid medium (with N-source) (Richmond, 1986) with shaking at 150 rpm under continuous illumination 400 $\mu E/m^2/s$ at 28±2ºC for 30 days. Then, the cells were harvested by centrifugation at 4,000 rpm for 5 min and washed twice with steriled distilled water to eliminate remained N. The cells pellets were transferred to fresh media of BG11 and $BG11_0$ for determining the growth pattern. The number of total cells was enumerated by haemacytometer under 400X compound microscope (Nikon, Japan) every 3 days until 30 days. This experiment was compared with the amount of chlorophyll a which measurement followed by Wintermans and Demots (1965). The cell pellets were transferred to new BG11 medium which was modified to induce akinete and heterocyst cells such as: (i) BG11 (with N-source) (N^+); (ii) $BG11_0$ (without N-source) (N^-); (iii) $BG11_0$ without K_2HPO_4 and $FeNH_4$citrate (P^-&Fe^-); (iv) $BG11_0$ with various concentration of MoO_3 at 0, 0.25, 2 and 3 nM; (v) $BG11_0$ with various concentration of $MnSO_4.H_2O$ at 0, 2.5, 20 and 30 nM; (vi) $BG11_0$ with various concentration of $CaCl_2$ at 0, 0.12, 0.48 and 0.96 mM; (vii) $BG11_0$ with various concentration of K_2HPO_4 at 0, 5.5, 11 and 16.5 nM and (viii) $BG11_0$ with various concentration of $FeNH_4$ citrate at 0, 1.5, 12 and 24 mg/l. The strain in each condition was ultrasonicated at 60 amplitude for 15 sec (Ultrasonicator, Ultrasonic Processor GE100), then enumerated the number of vegetative, heterocyst and akinete cells by haemacytometer under 400X compound microscope (Nikon, Japan) every week until 4 weeks.

3.5.2 DNA isolation and PCR amplification for sequencing

Cyanobacterial strain VICCR1-1 was cultured in 125 ml Erlenmeyer flask containing 50 ml of $BG11_0$ liquid medium. The DNA extraction method was followed by Teaumroong *et al.* (2002). DNA template was amplified by PCR of the 16S rRNA gene with the universal bacterial primers fd1 (5'-AGAGTTTGATCCTGGCTCAG-3') and rd1 (5'-AAGGAGGTGATCCAGCC-3') (Weisburg *et al.*, 1991). The reaction mixture consisted of 40 µl of ddH_2O, 5 µl of 10X *Taq* buffer (20 mM Tris-HCl [pH 8.0], 100 mM KCl, 0.1 mM EDTA, 1 mM DTT, 50% glycerol, 0.5% Tween 20 and 0.5% Nonidet®-P40), 1.5 mM $MgCl_2$, 50 µM deoxynucleoside triphosphate, 0.5 µM of each primer, 1.25 U of *Taq* DNA polymerase (Promega, USA) and 5-10 ng of DNA sample. The reactions were run with the following holds cycles: 95ºC for 2 min; 35 cycles of 94ºC for 1 min, 55ºC for 1 min and 72ºC for 2 min; and 72ºC for 6 min. The PCR was performed in a Thermal cycler (GeneAmp®PCR System 9700, Perkin Elmer) and products were visualized using 1% agarose gel electrophoresis and stained with 0.5 µg/ml of ethidium bromide, then documented on Gel documentation and analysis (Ultra Violet Product, USA).

PCR products were purified by Wizard®SV Gel and PCR clean-up system (Promega, USA). Sequencing was performed using fd1 and rd1 primers, the Big Dye Terminator volume 3.0 and an ABI Prism™310 Genetic Analyzer (Applied Biosystems, UK). Sequencing reaction was conducted according to Performing DNA sequencing reactions of Applied Biosystems. Database comparisons were performed by using the software package of the NCBI.

3.5.3 Cyanobacterial colonization at rice root

Oryza sativa variety Pathumthani-1 was used as test plant. Seeds were surface sterilized and germinated as described by Nilsson and co-workers (2002). After germination, the seedlings were transferred to tubes containing 20 ml of $BG11_0$ liquid medium. The cyanobacterial strain VICCR1-1 was inoculated in to the plants after growth for 7 days. The plants were grown in 28±2ºC with a 12h/12h light/dark cycle with an average light irradiance of 400 μE/m^2/s for 30 days. The hormogonia were investigated including with determination cyanobacterial colonization with rice root and enumarated vegetative, heterocyst and akinete cells every 3 days until 30 days by haemacytometer under 400X compound microscope (Nikon, Japan) and determined amount of cell parallel to a three-tube MPN method (www, 2006).

To determine the cyanobacterial localization at root, the surfaces and freshly cut longitudinal and cross sections of rice roots were pre-fixed by 2.5%glutaraldehyde in 0.1 M phosphate buffer, pH 6.8. The material was post-fixed with OsO_4 in 0.1 M phosphate buffer, pH 6.8, then dehydrated in series concentration of ethanol (since 30% to 100%). The material was embedded in Spurr's resin. Two μM thick was sectioned with ultramicrotome (RMC, MTXultramicrotrome, USA). The sections were stained with toluidine blue and examined under light microscope (Nikon, Japan).

3.5.4 RNA isolation

Total RNA was isolated at specified time (every 4 h within 24 h, 48 h and 72 h) from 1.5 ml aliquots of induced cells in 50 ml of $BG11_0$ without K_2HPO_4 (P^-) for akinete induction and both of $BG11_0$ (N^-) and $BG11_0$ without $CaCl_2$ ($-Ca^{2+}$) for enhancing heterocyst cell. The cells were harvested by centrifugation at 5,000 rpm for

5 min. The cell pellets were washed with DEPC-treated water, vortexed and centrifuged at 5,000 rpm for 5 min. One milliliter of Trizol® reagent (Invitrogen, USA) was added and incubated at room temperature for 5 min. Then, phase was separated by adding 200 µl of chloroform, shaken by hand for 15 sec, incubated at room temperature for 3 min and centrifuged at 14,000 rpm for 15 min. The aqueous phase was transferred to a fresh tube, added 500 µl of isopropyl alcohol, then incubated at room temperature for 10 min and centrifuged at 14,000 rpm for 10 min. The supernatant was removed, washed the pellet with 1,000 µl of 70% absolute ethanol and centrifuged at 7,500 rpm for 5 min. The pellet was dried, dissolved in nuclease-free water and stored at -70ºC until used.

3.5.5 Reverse transcriptase PCR (RT-PCR)

The total RNA was treated with 1 U of RNase free DNase (Promega, USA) and incubated at 37ºC for 30 min. One microliter of stop solution (20 mM EGTA [pH8.0] at 25ºC) was added and incubated at 70ºC for 15 min. Two step RT-PCR was chosen for this study. The target RNA was combined with either 25 ng/µl of $Oligo(dT)_{15}$ primer or 2.5 ng/µl of random hexamers. The combined RNA was preheated at 70ºC for 5 min and kept on ice until the reverse transcription reaction was added. Reverse transcription reaction mix contained 4 µl of ImProm-II™ 5X reaction buffer, 3 mM $MgCl_2$, 0.67 mM dNTP mix, 20 U of ribonuclease inhibitor, 1 µl of ImProm-II™ reverse transcriptase (Promega, USA) and adjusted with nuclease-free water to 15 µl. Five microliters of RNA and primer mix were added into reverse transcription reaction mix, giving a final reaction volume of 20 µl. The solution was annealed at 25ºC for 5 min, extended at 42ºC for 60 min and inactivated reverse transcriptase at 70ºC for 15

min. Two microliters of the cDNA were amplified by PCR. The cDNA was checked for quality by PCR amplification of 16S rRNA gene with the universal bacterial primers PBA338F (5'-ACTCCTACGGGAGGCAGCAG-3') and PRUN518R (5'-ATTACCGCGGCTGCTGG-3') (Yang and Crowley, 2002). The following reaction mixtures and conditions were 2.5 µl of 10X *Taq* buffer (20 mM Tris-HCl [pH 8.0], 100 mM KCl, 0.1 mM EDTA, 1 mM DTT, 50% glycerol, 0.5% Tween 20 and 0.5% Nonidet®-P40), 200 µM deoxynucleoside triphosphate, 3 mM $MgCl_2$, 0.5 µM of each primer, 1.25 U of *Taq* DNA polymerase (Promega, USA), 0.1 mg/ml BSA, 14 µl of nuclease-free water and 2 µl of cDNA template. The reactions were run with the following holds cycles: 94ºC for 5 min; 35 cycles of 94ºC for 30 sec, 45ºC for 45 sec and 72ºC for 45 sec; and 72ºC for 10 min.

After the qualities of cDNA were confirmed, cDNA targeting based on *ava*K, *sod*F and *het*R genes were performed. Akinete differentiation depended on *ava*K and *sod*F genes, the primers for *ava*K gene were *ava*K (5'-GGAATTCCATATGATTAAGAGGCATTTTATATATTTGAGG-3') and *ana*K (5'-CGGGATCCTTAGCGTTCTTCAATGGGAAGACCAGGAGCATT-3') (Zhou and Wolk, 2002). The primer sequences for *sod*F gene were *sod*F-forward (5'-GAGTATCACTATGGCAAGCA-3') and *sod*F-reverse (5'-CTAAAGTCAATGTAGTAG-3') (Shirkey *et al*., 2000). Heterocyst differentiation depended on *het*R gene, the primers were forward (5'-AAGTGTGCAATATACATGAC-3') and reverse (5'-TCAATTTGTCTTTTTTCTTC-3') (Nilsson *et al.*, 2005). The following reaction mixtures and conditions for primers of *ava*K gene were 2.5 µl of 10X *Taq* buffer (20 mM Tris-HCl [pH 8.0], 100 mM KCl, 0.1 mM EDTA, 1 mM DTT, 50% glycerol, 0.5% Tween 20 and 0.5% Nonidet®-P40),

200 µM deoxynucleoside triphosphate, 3 mM $MgCl_2$, 1 µM of each primer, 1.25 U of *Taq* DNA polymerase (Promega, USA), 14.75 µl of nuclease-free water and 2 µl of cDNA template. The reactions were run with the following holds cycles: 94ºC for 5 min; 35 cycles of 94ºC for 30 sec, 45ºC for 1 min and 72ºC for 2 min; and 72ºC for 7 min. For primers of *sod*F gene, the reaction mixture consisted of 2.5 µl of 10X *Taq* buffer (20 mM Tris-HCl [pH 8.0], 100 mM KCl, 0.1 mM EDTA, 1 mM DTT, 50% glycerol, 0.5% Tween 20 and 0.5% Nonidet®-P40), 200 µM deoxynucleoside triphosphate, 1.5 mM $MgCl_2$, 0.4 µM of each primer, 1 U of *Taq* DNA polymerase (Promega, USA), 0.1 mg/ml BSA, 16.35 µl of nuclease-free water and 2 µl of cDNA template. The reactions were run with the following holds cycles: 95ºC for 2 min; 35 cycles of 95ºC for 1 min, 45ºC for 90 sec and 72ºC for 90 sec; and 72ºC for 10 min. For primers of *het*R gene, the reaction mixture contained 2.5 µl of 10X *Taq* buffer (20 mM Tris-HCl [pH 8.0], 100 mM KCl, 0.1 mM EDTA, 1 mM DTT, 50% glycerol, 0.5% Tween 20 and 0.5% Nonidet®-P40), 200 µM deoxynucleoside triphosphate, 1.5 mM $MgCl_2$, 1 µM of each primer, 1 U of *Taq* DNA polymerase (Promega, USA), 0.1 mg/ml BSA, 16.05 µl of nuclease-free water and 2 µl of cDNA template. The reactions were run with the following holds cycles: 92ºC for 5 min; 35 cycles of 95ºC for 30 sec, 55ºC for 30 sec and 72ºC for 30 sec; and 72ºC for 5 min. All RT-PCR were performed in Thermal cycler either GeneAmp®PCR System 9700 (Perkin Elmer, USA) and GeneAmp®PCR System 2400 (Perkin Elmer, USA) and products were visualized using 1% agarose gel electrophoresis and stained with 0.5 µg/ml of ethidium bromide, then documented on Gel documentation and analysis (Ultra Violet Product, USA).

3.5.6 Protein extraction and SDS-PAGE analysis

The cyanobacterial strain VICCR1-1 was grown in 125 ml Erlenmeyer flask containing 50 ml of BG11 liquid medium with shaking at 150 rpm under continuous illumination at 28±2ºC for 30 days. The cells were collected by centrifugation at 4,000 rpm for 5 min, then washed with steriled distilled water for 3 times. The cell pellets were transferred to fresh BG11, $BG11_0$ and $BG11_0$ without $CaCl_2$ liquid medium to induce heterocyst differentiation. After 30 days, 3 ml of cyanobacteria were centrifuged at 5,000 rpm for 5 min. The total protein of cyanobacterial cells was extracted according to the modified Mackenzie method (www, 2006). The cell pellets were resuspended with 450 µl of solubilizer buffer (160 mM sucrose, 100 mM Tris-HCl [pH8.3], 1 mM EDTA, 2% sodium dodecyl sulfate [SDS]) and added 105 µl of 150 mM dithiothreitol (DTT). The cell suspensions were placed on ice for 5 min and freezed at -70ºC for 10 min. Then, the tubes were sonicated for 15 min (TRU-SWEEP™ Ultrasonic cleaner, NJ), heated at 75ºC for 10 min and placed on ice for 5 min. The phases were separated by centrifugation at 14,000 rpm for 10 min, transferred to fresh microcentrifuge tube and stored at -20ºC until used. The amount of total proteins was measured followed by Bradford Protein Assay method (Bradford, 1976). The total proteins were performed on SDS-polyacrylamide gel electrophoresis (SDS-PAGE) of proteins (Bio-Rad, USA). Gels for SDS-PAGE were 10% acrylamide gel which the mixtures for separating and stacking gel followed by Sambrook and Russell (2001). The SDS-PAGE was run 120V for 90 min, then gels were stained with 0.25% Coomassie Brilliant Blue R-250 for 4 h and destained with 30% glacial acetic acid and 10% methyl-alcohol until the gel was cleared. The gel were vacuum dried on

filter paper at 55ºC for 2 h (Diaphramgm vacuum pump, Germany and Drygel Sr. Slab Gel Dryer Model SE1160, USA).

The interested protein from SDS-PAGE was cut and soak in nuclease-free water. The excised bands were sent to Bioservice unit, Bangkok. The tryptic digest of those bands were determined by liquid chromatography/mass spectrometry (LC/MS). The fragments similar in sequence were identified by BLAST search.

3.5.7 Stress tolerant of akinetes

The akinete cells were tested against various abiotic factors. The induced cells were incubated into in 50 ml of modified $BG11_0$ (without N-source) liquid medium with shaking at 150 rpm under continuous illumination 400 $\mu E/m^2/s$ at 28±2ºC for 7 days such as: (i) $BG11_0$ at pH 3, 4, 9 and 10; (ii) $BG11_0$ with 0.1 M, 0.3 M and 0.5 M NaCl and (iii) $BG11_0$ at 40ºC and 50ºC.

One milliliter of cell suspension was diluted with ten-fold dilution technique and one milliliter of each dilution was inoculated into a separate tube of $BG11_0$ liquid medium according to a five-tube MPN method (www, 2004). After incubation for 30 days, the broth tubes were observed for the presence or absence of growth and enumerated the number of cell according to the five-tube MPN table.

3.5.8 Cyanobacterial inoculum preparation

Cyanobacterial strain VICCR1-1 was grown in 5 liters of Erlenmeyer flask containing 4.5 liters of BG11 liquid medium under continuous aeration at 28±2ºC with a 12h/12h light/dark cycle with an average light irradiance of 400 $\mu E/m^2/s$ for 30 days. The cells were harvested by centrifugation at 4,000 rpm for 5 min and washed twice

with sterilized distilled water for 3 times to eliminate the remained N. Then, the cell pellets were cultured in 5 liters of Erlenmeyer flask containing 4.5 liters of $BG11_0$ with 24 mg/l $FeNH_4$ citrate (excess Fe) liquid medium under continuous aeration at 28±2ºC with a 12h/12h light/dark cycle with an average light irradiance of 400 μE/m^2/s for 30-45 days to induce akinete formation. Some of cell was harvested, ultasonicated (Ultrasonicator, Ultrasonic Processor GE100) at 60 amplitude for 15 sec and enumerated vegetative, heterocyst and akinete cell with haemacytometer under 400X compound microscope (Nikon, Japan). The whole induced cells were harvested by centrifugation at 4,000 rpm at 5 min and washed with sterilized distilled water for 3 times.

The cell pellets were homogeneously mixed with montmorillonite clay in ratio 1,000 ml of induced cells/kg montmorillonite clay to produce cyanobacterial inoculum. The inoculum was dried under shade zone for 3-5 days. The dried inoculum was blended with blender (Panasonic MX-J210GN, Japan) and stored at room temperature for 12 months. The population number in inoculum was determined every month by using a five tube MPN method (www, 2004).

3.5.9 Rice field trials

Rice field experiments were carried out in the total area of 20 m^2/treatment at farm of Suranaree University of Technology (SUT farm). Rice seedlings (*Oryza sativa*) Pathumthani-1 rice variety were planted with two crops during October 2004 to February 2005 (1^{st} crop: winter/dry season) and during May 2005 to September 2005 (2^{nd} crop: rainy season). Rice seedlings of 25 days old were transplanted in experimental field with 2×2 m^2 spacing. There were 4 and 6 treatments in this

experiments during 1st crop and 2nd crop, respectively, such as: (i) rice without nitrogen fertilizer, (ii) rice with chemical fertilizer recommend for rice (12-6-4 kgN-P_2O_5-K_2O/Rai), (iii) rice with cyanobacterial strain VICCR1-1, (iv) rice with montmorillonite clay, (v) rice with added akinete inoculum in 1st crop and (vi) rice with akinete inoculum (the treatments v and vi were obtained only in 2nd crop). Every rice field experiments were supplied 30 kg/Rai each of phosphorus and potassium. After cultivated for 7 days, the cyanobacterial strain VICCR1-1 and akinete inoculum were supplied in 3rd and 5th treatments, respectively. The information of rice was collected and analyzed by ANOVA.

3.5.10 Soil sampling and cyanobacterial population

The soil samples from rice field experiments were collected 5 samples in each treatment at initial planting and after harvesting. The population of cyanobacterial strain VICCR1-1 was determined by a five-tube MPN method (www, 2004), acetylene reduction assay (ARA) and detected with denaturing gradient gel electrophoresis (DGGE).

For ARA, each broth culture from MPN method was detected nitrogenase activity. Tubes were plugged with double septum before 10% of the head space air volumes was replaced with acetylene. Allow the incubation to proceed at 28±2°C with a 12h/12h light/dark cycle under average 400 μE/m^2/s light intensity for 24 h. One ml of gas mixture was withdrawn and analyzed by Gas chromatography (GC) equipped with capillary PE-Alumina column (AutoSystem XL, Perkin Elmer, USA). Ethylene production per tube per 1 h was determined by comparing the peak height in cm with that of known amount of chlorophyll a (Wintermans and Demots, 1965).

For DGGE, total genomic DNA from each broth culture, which were observed for the presence of cyanobacterial growth, were extracted according to Teaumroong *et al*. (2002). PCR products obtained with primer combination PBA338F-GC clamp (5'-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGACTCCTACGGGAGGCAGC-3') and PRUN518R (5'-ATTACCGCGGCTGCTGG-3') were used for analyzed with DGGE. The PCR was performed with a Thermal cycler (GeneAmp®PCR System 9700, Perkin Elmer). The PCR mixture contained 1 µl of diluted genomic DNA 1:100, 5 µl 10X *Taq* buffer (20 mM Tris-HCl [pH 8.0], 100 mM KCl, 0.1 mM EDTA, 1 mM DTT, 50% glycerol, 0.5% Tween 20 and 0.5% Nonidet®-P40), 200 µM deoxynucleoside triphosphate, 3 mM $MgCl_2$, 0.5 µM of each primer, 0.5 U of *Taq* DNA polymerase (Promega, USA), 0.1 mg/ml BSA and 32 µl of nuclease-free water. The sample was first incubated at 95ºC for 5 min to denature the DNA, then PCR conditions were 35 cycles at 94ºC for 30 sec, 45ºC for 45 sec and 72ºC for 45 sec, followed by elongation at 72ºC for 10 min. Before loading DNA samples on to the DGGE gel, the PCR products were incubated at 95ºC for 5 min and gradually cooled to 4ºC to avoid non-complementary annealing of DNA. Gels for DGGE were 10% polyacrylamide gel (10%-acrylamide and N, N-methylenebisacrylamide solution (37.5:1, v/v), 40% (v/v) formamide, 7M urea and 1X TAE) containing a linear gradient of the denaturant concentration ranging from 30% to 70%. The denaturing gradient gel was run for 720 min at 60ºC and 150V by the Dcode system (Bio-Rad, USA). After completion of electrophoresis, the gels were stained in an ethidium bromide solution (0.5 µg/ml) and documented on Gel documentation and analysis (Ultra Violet Product, USA).

3.6 Results and Discussion

3.6.1 Cyanobacterial strain

The genomic DNA of cyanobacterial strain VICCR1-1 was extracted and amplified target DNA based on 16S rRNA. Then, this strain was aligned in the database by using the software package of the NCBI. The result showed that the cyanobacterial strain VICCR1-1 was identity as *Nostoc entophytum* and *Nostoc* sp. PCC9231 92% (Fig. 3.17). Nucleotide sequence of *Noctoc* sp. strain VICCR1-1 (accession number DQ675573) was deposited in the GenBank.

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Query: 251 gacgancggtagctggttctgagtaggaagaccagccacactngggactgtagcacacg 310
           |||||| |||||||||||| ||||| |||| |||||||||||||| ||||||| || |||||
Sbjct: 217 cgacgatcggtagctggt-ctgag-aggatgaccagccacact-gggactg-ag-acacg 271


Query: 311 gcccagtactcctacgggaggtcagcagtcggggcaattttccgcaatggggcgaaagcc 370
           |||||| ||||||||||||||| ||||||| |||  |||||||||||||||| ||||||||
Sbjct: 272 gcccag-actcctacgggagg-cagcagtgggg--aattttccgcaatggg-cgaaagcc 326


Query: 371 tgacggatcaattaccgcgtgagggaggaaggctcttgggttgtaaacctcttttntcag 430
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Query: 431 ggaagaacacaatgacggtacctgtangaatcagcaacggctaactccgtgccagcagcc 490
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Query: 491 gcggtaatacggaggatgcaagcgttatnccggatggattgngcgtaaagcgtcc 545
           |||||||||||||||||||||||||||| |||||  ||||| ||||||||||||
Sbjct: 445 gcggtaatacggaggatgcaagcgttat-ccggaatgattgggcgtaaagcgtcc 498
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Figure 3.17 The alignment of 16S rRNA gene cyanobacterial strain VICCR1-1 database by using the software package of the NCBI.

3.6.2 Growth pattern of *Nostoc* sp. strain VICCR1-1

The *Nostoc* sp. strain VICCR1-1 was grown in BG11 (N-source supplement) medium with shaking at 150 rpm under illumination 400 μE/m^2/s 12h/12h (light/dark) at 28±2ºC for 30 days. The cells were transferred to fresh BG11 and $BG11_0$ (without N-source). The growth of *Nostoc* sp. strain VICCR1-1 under N supplement and N deprivation were monitored by chlorophyll a measurement and enumeration of the cells every 3 days for 30 days (Fig. 3.18 and 3.19). Based on chlorophyll a analysis, quantity of chlorophyll a exhibited the long lag phase (0-6 d; ranging between 0.21-0.25 mg of chlorophyll a) in N-supplement condition. It is exponentially increasing on day 12-15 (0.30-0.40 mg of chlorophyll a). Then the cells trended to increase continuously and still increased even at 30 d (0.89 mg of chlorophyll a). Likewise, Chairin (2002) demonstrated that the quantity of chlorophyll a of *Nostoc* sp. strain VICCR1-1 was exponentially increased at 6 d (0.025 mg of chlorophyll a) and still increased until 21 d (1.140 mg of chlorophyll a) (which was the end of this study). While the growth pattern of *Nostoc* sp. strains VICCR1-1 in to $BG11_0$ medium was somewhat different. The results were illustrated in Fig 3.19. After changing the medium, the amount of chlorophyll a immediately increased before 3 d and seemed to reach the logarithmic phase at 3 d (0.25 mg of chlorophyll a). Quantity of chlorophyll a was continuously increased until reached the peak at 18 d (0.89 mg of chlorophyll a), then this was dropped.

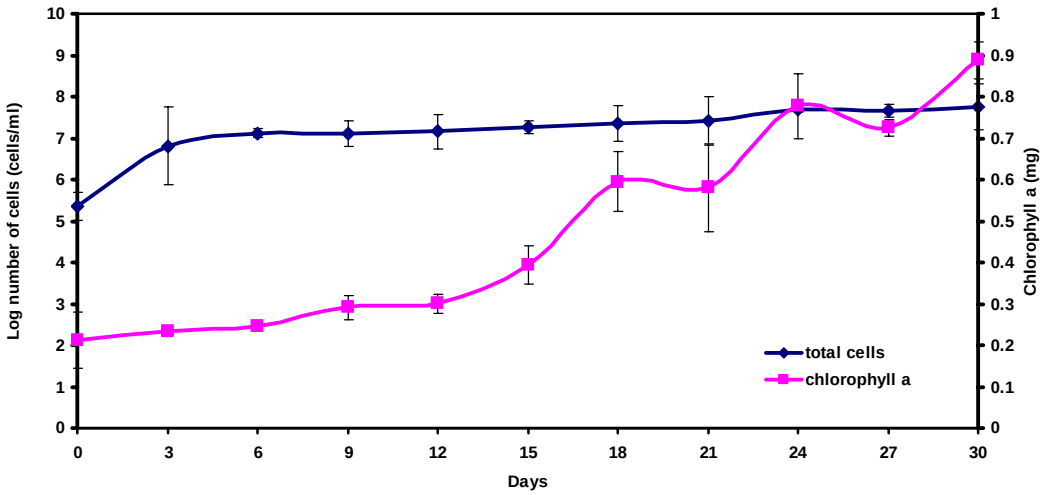


Figure 3.18 The growth pattern of *Nostoc* sp. strain VICCR1-1 in BG11 medium**.**

The inoculum size under N repletation was started at 2.27×10^5 cell/ml, amount of cells were increased up to 1 magnitude within 3 d (6.34×10^6 cell/ml). The total cell number as constantly displayed ranging between 7.10-7.25 log number of cells (1.28×10^7-1.79×10^7 cell/ml) during 6 d to 24 d (4.82×10^7 cell/ml) (Fig. 3.18). However, from previous study (Chairin, 2002) used inoculum size about 10^8 cell/ml, the cells were increased up to 1 magnitude at 21 d. The cells might perform the nutrient competition for growth and some cells might death or degraded during cultivation because of high inoculum size. Besides when nitrogen was step-down, the inoculum size was 2.27×10^5 cell/ml, the cells were increased up to about 2 magnitudes at 3 d (1.40×10^7 cell/ml). The total cells were rather steady until 18 d, then grown up to about 3 magnitudes at 21 d (1.28×10^8 cell/ml) (Fig. 3.19).

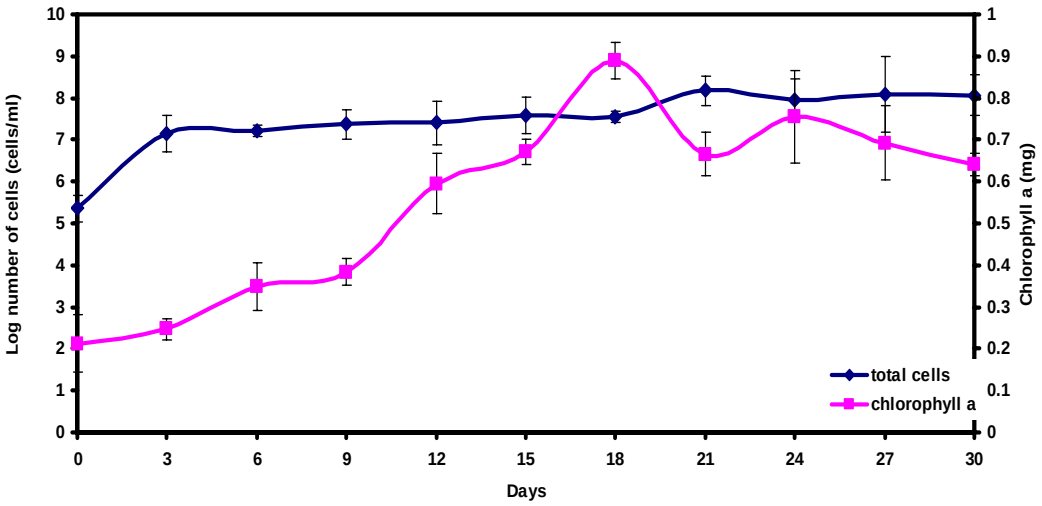


Figure 3.19 The growth pattern of *Nostoc* sp. strain VICCR1-1 in $BG11_0$ medium**.**

The cells in BG11 medium (the specific growth rate about 0.030) grew slower and used more time for growth than that of the cells in $BG11_0$ medium (the specific growth rate about 0.056) (Fig. 3.18 and 3.19). The results suggested that chlorophyll a measurement was corresponding with total cell enumeration. The chlorophyll a in $BG11_0$ medium as reached the peak of growth before the cells in BG11 medium because quantity of chlorophyll a was still increased at 30 d (BG11 medium), while the amount of chlorophyll a was peak at 18 d and dropped at 21 d ($BG11_0$ medium). And the cells in BG11 medium exhibited the lower magnitudes (2 magnitudes) than that of in $BG11_0$ medium (3 magnitudes). However, the pattern of chlorophyll a was not related to cell number. Actually, the number of cell will be increased until late of logarithmic phase and steady at stationary phase. The cyanobacterial death cells presented in colorless or pale color, thus the quantity of chlorophyll a was obviously decreased (Fig. 3.19). Besides before cell enumeration, the cyanobacterial cells were detached by ultrasonic. The single cell was difficult to distinguish between viable and death cell, hence the cells at stationary phase was not dropped.

3.6.3 Cyanobacterial rice root colonization

Rice seedlings were co-cultivated hydroponically with *Nostoc* sp. strain VICCR1-1 in $BG11_0$ liquid medium. The results of root attachment were investigated. The cell number was started at 4.9×10^5 cell/ml and increased up to 4.25×10^7 cell/ml (2 magnitudes) within 30 days when enumerated under haemacytometer. Whilst *Nostoc* sp. strain VICCR1-1 were grown as 4.3×10^6 cell/ml (1 magnitude) within 30 days when counted by three-tube MPN method (Fig. 3.20). However, the statistical variability of bacterial distribution is better estimated by using as many tubes as possible or practical (www, 2004). Since, the cyanobacterial cells were clumped formation, thus the number of cells might be biased by suction of the cell. Therefore, the amount of cells should be average value of the enumeration by haemacytometer and three-tube MPN method as 2.34×10^7 cell/ml. At 0 d, the number of cyanobacterial cells showed in proportion as 61.48%, 31.60% and 6.92% of vegetative, heterocyst and akinete cells, respectively. The number of heterocyst cells were ranging between 14.62% - 50.00% and the highest was 50.00% at 3 d (Fig. 3.21). Meanwhile, the akinete cell number showed ranging between 0.62% - 9.72% and the highest also was 9.72% at 3 d (Fig. 3.21). Due to the *Nostoc* sp. strain VICCR1-1 was changed from cultivation in BG11 medium under an average light intensity of 400 $\mu E/m^2/s$ with 12h/12h light/dark cycle in 28±2ºC for 30 days to $BG11_0$ medium, thus the heterocyst cells might be increased in order to adequately fix nitrogen. While the number of akinete cell was presented in early (3 d) and the late (30 d; the stationary phase) of co-cultivation.

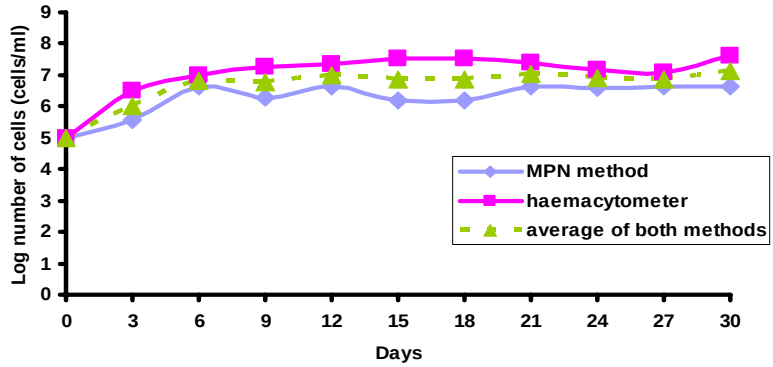


Figure 3.20 The number of cyanobacterial cells by using enumeration with haemacytometer and counted by three-tube MPN method.

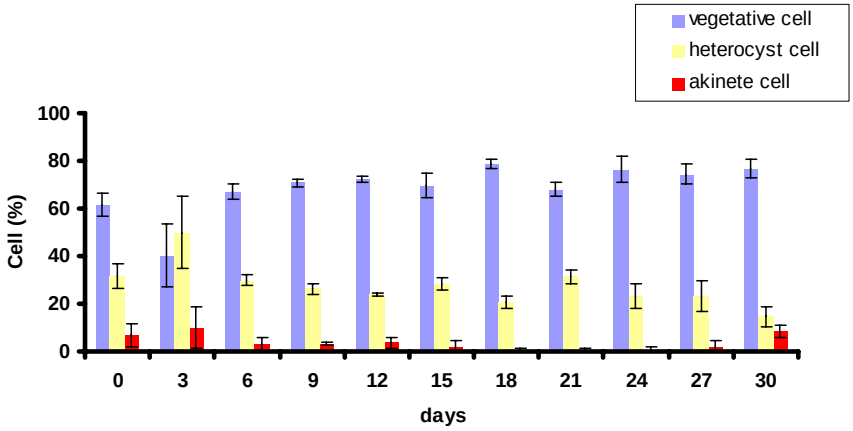


Figure 3.21 The cyanobacterial cell in term of percent vegetative, heterocyst and akinete cells.

The growth pattern of *Nostoc* sp. strain VICCR1-1 which co-cultured with rice seedling was enumerated under haematocytometer in compare with the free-living *Nostoc* sp. strain VICCR1-1 (Fig. 3.22). The inoculum sizes were about 2.28×10^5 cell/ml and 1.00×10^5 cell/ml of free-living and co-cultured cyanobacteria, respectively. The number of free-living cyanobacteria as increased up to 2 magnitudes within 3 days, whereas, only 1 magnitude increased was found in co-cultured cyanobacteria. The growth pattern presented constantly since 3 day until 18 day in both

cyanobacterial cells. The numbers of free-living cyanobacterial cells were getting higher at 21 d (1.5×10^8 cell/ml) and keep constant until 30 day. The co-cultured cyanobacterial cells were decreased at 21 d (2.62×10^7 cell/ml), this might due to the some cell was more colonized to the root surface (dropping number of hormogonia as shown in Fig. 3.24). The free-living cells were grown to about 3 magnitudes at 30 d, besides co-cultured cyanobacterial cells were increased to about 2 magnitudes at 30 d. The results were related with Steinberg and Meeks's information (1989), as the *Nostoc* sp. strain UCD7801, immediately after separation from pure cultures of a reconstituted symbiotic association with the bryophyte *Anthoceros punctatus*, exhibited a rate of light-dependent CO_2 fixation that was eightfold lower than that measured in the free-living growth state. Therefore, co-cultured cyanobacteria were low because the CO_2 fixation ability was dropped.

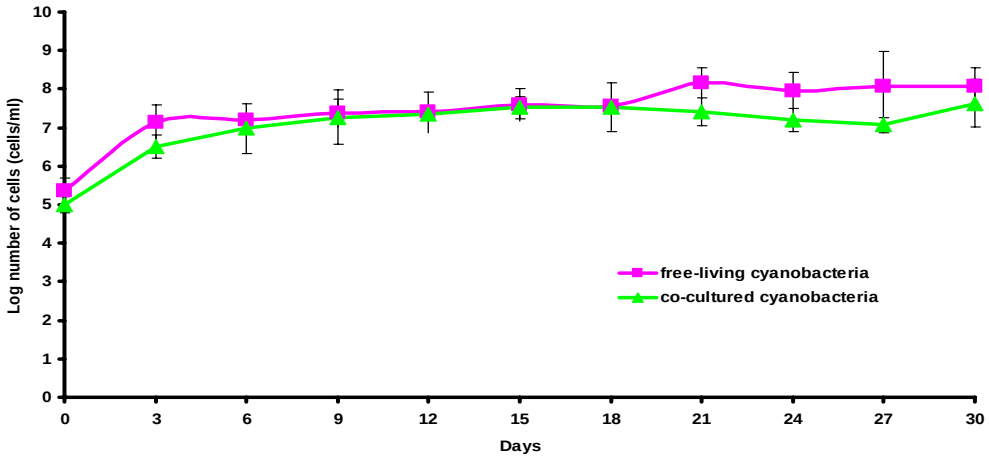


Figure 3.22 The growth pattern of *Nostoc* sp. strain VICCR1-1 which co-cultured with rice seedling was enumerated under haematocytometer comparison with the free-living *Nostoc* sp. strain VICCR1-1

The symbiotically competent *Nostoc* strains responded chemotactically to extracts of natural hosts, as well as to nonhost plants (Nilsson *et al.*, 2006). The various plant roots from nonhosts might attract bacteria; for instance, *Azospirillum* and the cyanobacterium *Nostoc* were extracted towards wheat (Zhulin *et al.*, 1988) and *Nostoc* towards alfalfa and *Solanum dulcamara* (Gorelova *et al.*, 1992, 1995). Moreover, Gantar and Elhai (1999) indicated that *Nostoc* sp. strain 2S9B which was originally isolated from solonetz (soil with a high concentration of NaCl) be able to associate with wheat.

The results demonstrated that the number of hormogonia was occurred in high number at early of inoculation and continuously decreased until disappearance on 27 d. Excreated mucilage from the nonhost *Oryza sativa* variety Pathumtani-1 might elicited chemotactic response, corresponding to ranging between 0-14 hormogonia per slide during 30 days of co-cultivation (Fig. 3.23 and 3.24). Nilsson and co-workers (2006) reported the chemotactic response among *O. sativa* showed approximately 40 and 20 hormogonia per microslide for *Nostoc* strain 8964:3 and PCC73102, respectively at 24 h to generate the highest number of attracted hormogonia. During the initial stages (1-3 d) of co-culture, mobile *Nostoc* hormogonia were common in the growth medium. As in the other symbiotic interaction (Rai *et al.*, 2000), these are probably important for reaching the site of colonization on the rice roots. However, this study rice roots were used, whereas Nilsson *et al.* (2006) used whole plant extracts. Therefore, the actual number of hormogonia per slide obtained in this experiment could not be compared directly to previous investigations. However, chemotaxis is known to vary at the strain level and with host species (Belsheim *et al.*, 1981; Gorelova *et al.*, 1995; Svircev *et al.*, 1997; Wei and Bauer, 1998). Chemotactic repressors might also be

involved (Chuiko *et al.*, 2002) as the same compound might function as an attractant for one microorganism and a repellent for another (Pandey and Jain, 2002). Furthermore, Nilsson *et al*. (2005) determined the competition among symbiotic cyanobacterial *Nostoc* strains forming artificial association with rice, and found that the attraction of chemotactic to rice varied depending on temperature and whether that plant had been grown with or without nitrogen. Using rice root for attraction, at lower temperature showed that number of hormogonia per microslide higher than higher temperature (i.e., approximately 250 and 80 hormogonia per microslide at 24ºC and 30ºC, respectively). While the amount of hormogonia per microslide were detected about 40 and 250 hormogonia per microslide when the co-culture had been grown with and without nitrogen, respectively.

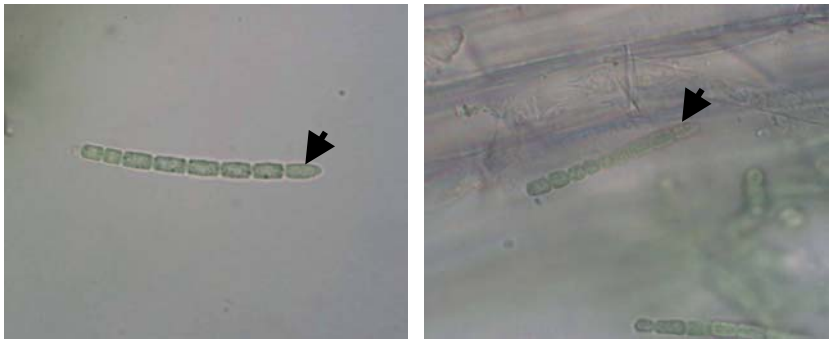


Figure 3.23 Hormogonia of *Nostoc* sp. strain VICCR1-1 at 9 d of co-cultivation.

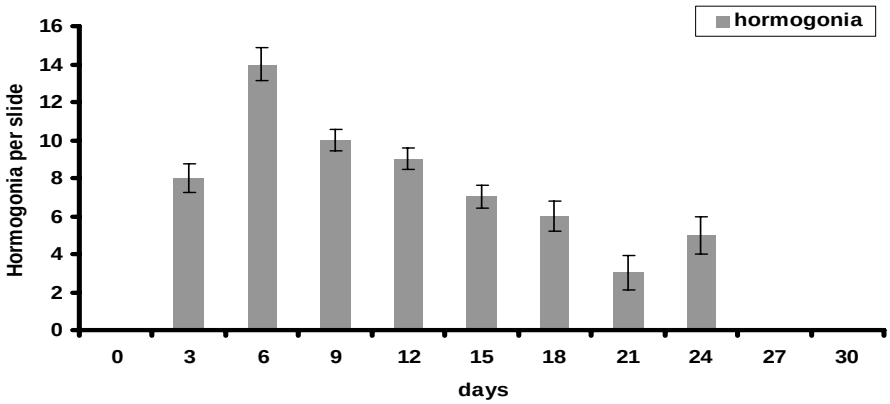


Figure 3.24 The hormogonial number per slide of *Nostoc* sp. strain VICCR1-1 toward *Oryza sativa* during 30 days cultivation.

On 24 d, the cyanobacterial cells seemed to penetrate into the intercellularly in the epidermal layer of the root (Fig. 3.25). Consequently, the transverse sections of the roots after 14 days of co-culture, using light microscope as examined (Fig. 3.26). The results showed that the *Nostoc* filaments were intimately associated with the root epidermis. The *Nostoc* filaments occurred in streaks or patches that followed the contours of the outer surface layer of the root epidermis. Association with root hairs was also observed. The *Nostoc* cells were not occurred intercelluarly in the epidermal layer of the root, which unlikely the phenomenon was found in the research of Nilsson *et al*. (2002, 2005). Thus, *Nostoc* sp. strain VICCR1-1 was most likely acted as epiphyte of rice root. This observation could represent early events in the process of colonization and, with time, the association may further (Nilsson *et al*., 2002). After association with the rice roots, the hormogonial state was followed by re-differentiation into mature vegetative filaments with heterocysts.

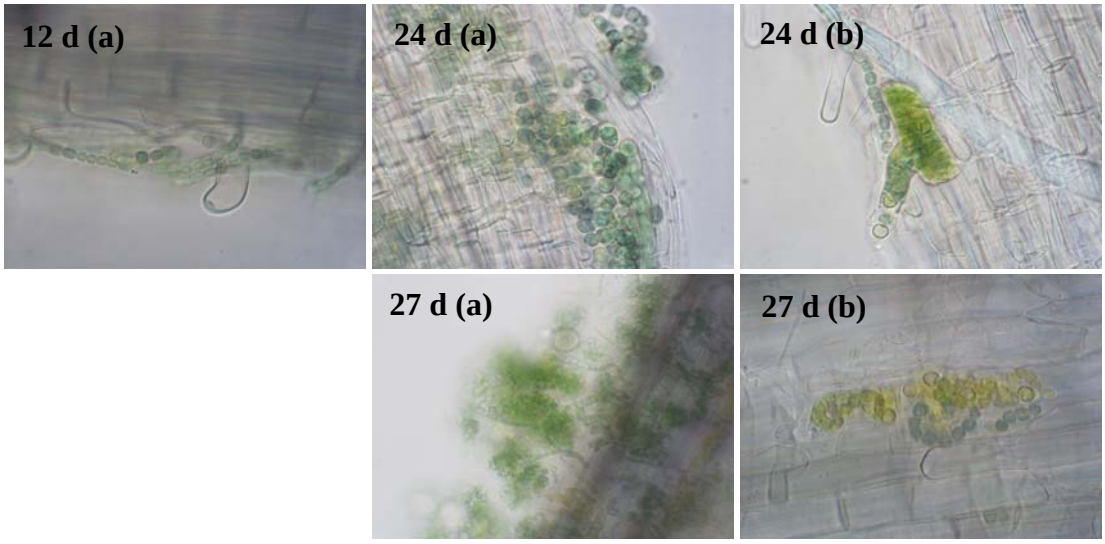


Figure 3.25 Colonization of rice roots at surface layer (a) and intercellularly in epidermal layer of root (b).

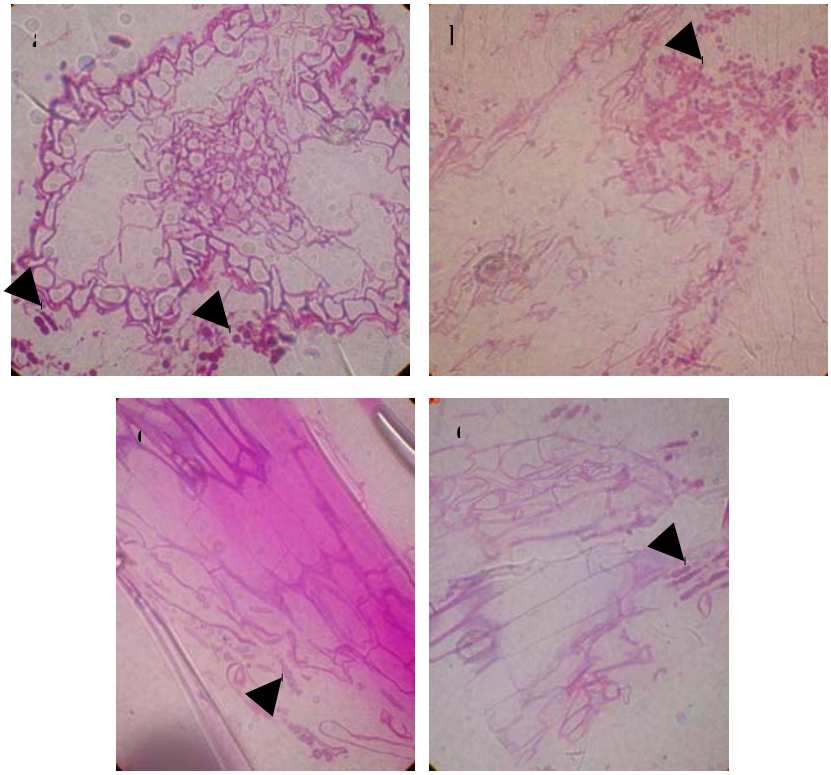


Figure 3.26 The transverse sections of the roots after 14 days of co-culture; (A and B) cross section of the infected rice root visualized through light microscope, cyanobacteria were seen as dark (arrow) and (C and D) longitudinal section of a resin-embedded root (arrow indicate the position of hormogonia).

3.6.4 Heterocyst and akinete induction

The *Nostoc* sp. strain VICCR1-1 was cultured in N-repetition medium with shaking at 150 rpm under continuous illumination 400 μE/m^2/s at 28±2ºC for 30 days. Then, the cells were transferred to induce heterocyst and akinete formation in various supplement or omitting of chemical factors such as; nitrogen, phosphorus, iron, molybdenum, manganese, calcium and copper. The types of cell were enumerated

under haemacytometer every week for 1 month. The inoculum size 5.9×10^7 cell/ml presented in form of heterocyst cell about 21.78% and akinete about 0.25% and the rest were vegetative cells.

The concentration of $CaCl_2$ was varied as 0, 0.12, 0.48 and 0.96 mM (normal was 0.24 mM in $BG11_0$ medium). The results were illustrated in Fig. 3.27. The heterocyst (%) was initially induced during 1st week, while akinete (%) was highly found at 3rd and 4th week (stationary phase), respectively. The heterocyst (%) was obviously high in absence $CaCl_2$, the heterocyst cell was continuously increased ranging between 29.85%-46.61% during cultivation and showed the highest number at 4th week. The resulted disagreed with the previous reports. Nitrogen step-down leads to increased Ca^{++} concentration which was recently found to be necessary for heterocyst development (Torrecilla *et al.*, 2004; Zhao *et al*., 2005). Torrecilla and co-workers (2004) found approximately three fold increase in the Ca^{++} level about 1 h after combined-nitrogen starvation. The Ca^{++} signal may act earlier than *het*R. The Ca^{++} level was found to be about 10-fold higher in heterocysts than in vegetative cells, which correlates with the levels of expression of *ccb*P (calcium-binding protein) (Zhao *et al*., 2005). Under nitrogen sufficiency conditions, CcbP may bind to Ca^{++} and maintain the free Ca^{++} at a low level and that the CcbP-Ca^{++} complex may serve as a Ca^{++} storage device (Zhao *et al*., 2005). Ca^{++} ions start to accumulate 45 min (Torrecilla *et al*., 2004) or 4 h (Zhao *et al*., 2005) after the withdrawal of combined nitrogen. Unknown mechanism in developing cells, which may increase the Ca^{++} levels, resulting in heterocyst differentiation (Zhao *et al*., 2005). Nevertheless, Torrecilla *et al*. (2004) demonstrated that when increased in external calcium concentration up to 5 mM, it had no significant effect on the magnitude and kinetics of

calcium transient. Therefore, the data suggested that the appearance of calcium transient is independent of an extracellular source of calcium and the main source of calcium seemed to be intracellular. As Zhao *et al*. (2005) recorded that the presence of external calcium inhibited heterocyst differentiation. The results from previous report might cause from the release of the amount of CcbP when provided external calcium in $BG11_0$ condition, was increased and CcbP would be bound with free Ca^{++}. Thus, CcbP-Ca^{++} might have a role in suppressing an early stage of heterocyst differentiation, as no external Ca^{++}amendment showed the highest heterocyst (%) in this study.

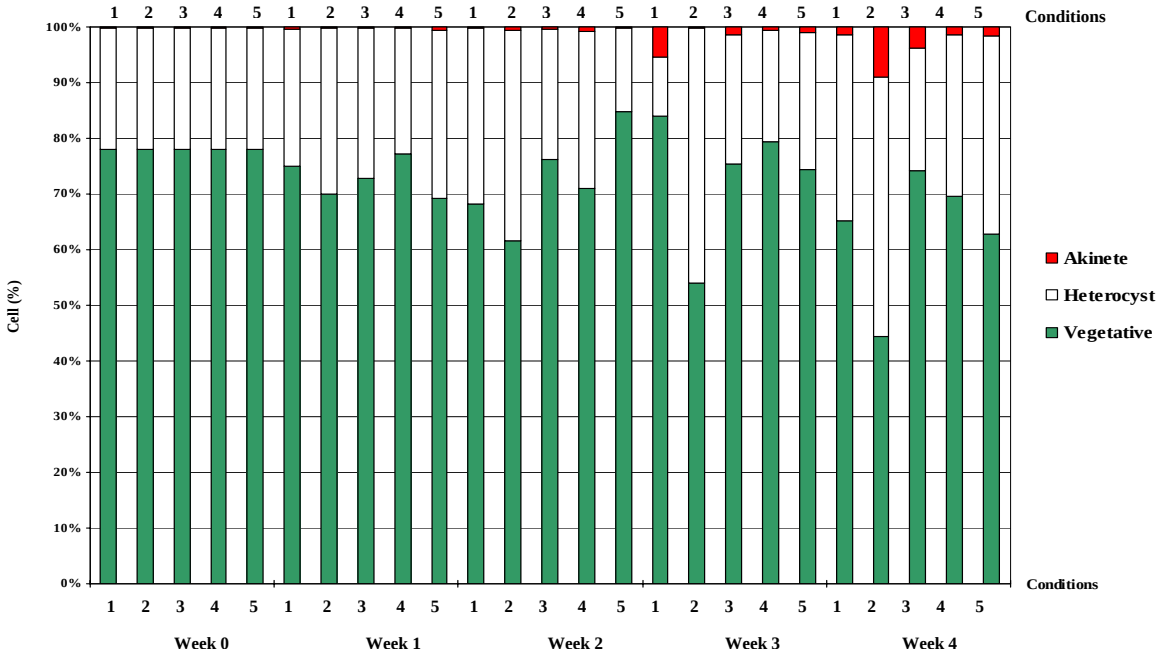


Figure 3.27 Heterocyst and akinete induction under $BG11_0$ (variations of $CaCl_2$ concentration) medium; No. 1-5 in X-axis described as (1) $BG11_0$ (normal condition), (2) 0 mM, (3) 0.12 mM, (4) 0.48 mM and (5) 0.96 mM $CaCl_2$.

The another experiment was to determine the % types of cell in the variation of $CuSO_4.5H_2O$ concentration, as 0, 1, 2 and 3 μM (normal was 0.32 μM in $BG11_0$ medium) under nitrogen starvation medium. The results presented that the % types of cell were rather not different from the % types of cell in normal medium (Fig. 3.28). Absence and 3 μM $CuSO_4.5H_2O$ supplement seemed to induce heterocyst formation. The number of heterocyst (%) was higher than that of in common medium. The cell information showed high amount of heterocyst (%) as 34.56% and 36.73% at 1st and 4th week, respectively, for absence $CuSO_4.5H_2O$ whilst 32.00% and 32.51% at 3rd and 4th week for 3 μM $CuSO_4.5H_2O$ concentration. Each concentration showed no different of heterocyst (%) ranging between 19.25%-36.73% and akinete (%) ranging between 0.07%-4.18%.

Eventhough a protease ought to act catalytically, whereas HetR which is serine-type protease required for heterocyst differentiation (Zhou *et al*., 1998; Dong *et al*., 2000), act stoichiometrically, based on the Cu^{++} induction experiment (Buikema and Haselkorn, 2001). Two μM copper was added to the *Anabaena* sp. PCC7120, the heterocyst frequency become 21, 23 and 29%, respectively, in ammonia, nitrate and nitrogen-free medium (Buikema and Haselkorn, 2001). So that, adding copper could obviously induce heterocyst differentiation in some case such as deprivation of nitrogen source (Zhao *et al*., 2005). However, the heterocyst cells were incapable to enhance with copper in some case, as the cell produced CcbP to bind Ca^{++} especially at early stage of heterocyst differentiation (Zhao *et al*., 2005). Thus, our studies showed the highest heterocyst (%) in absence $CuSO_4.5H_2O$ at 1st week. However, this reason could not be explained for heterocyst (%) at 4th week, it might because of the cells adaptation for surviving.

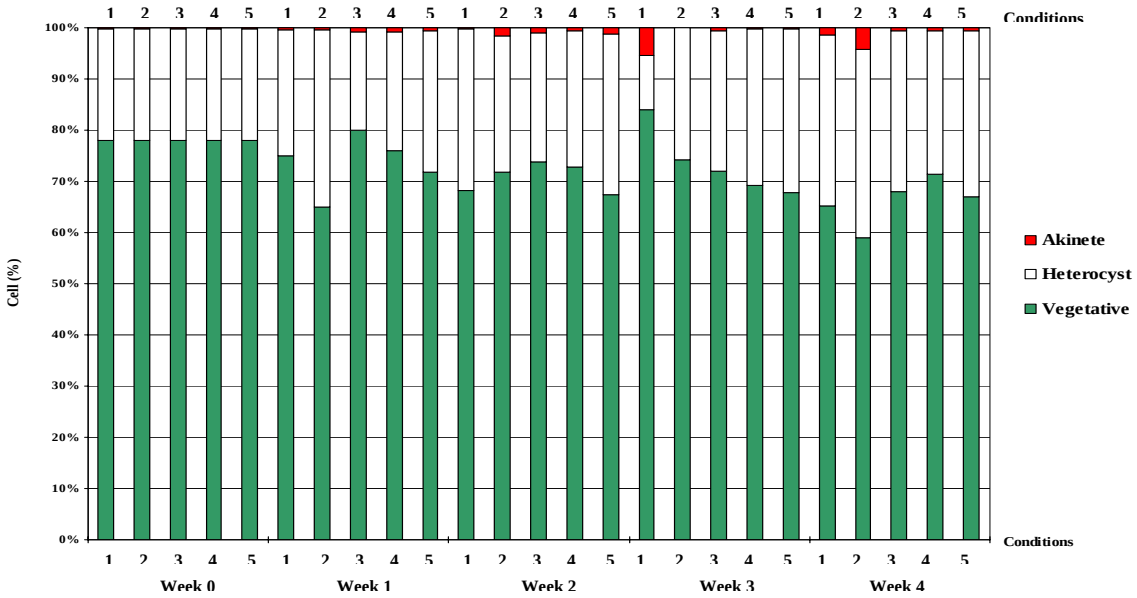


Figure 3.28 Heterocyst and akinete induction under $BG11_0$ (variations of $CuSO_4.5H_2O$ concentration) medium; No. 1-5 in X-axis described as (1) $BG11_0$ (normal condition), (2) 0 mM, (3) 1 mM, (4) 2 mM and (5) 3 mM $CuSO_4.5H_2O$.

In addition, the variations of MoO_3 concentration was carried out as 0, 0.25, 2 and 3 nM (normal was 1 nM in $BG11_0$ medium) (Fig. 3.29). The Mo also necessary for nitrogenase enzyme production which is storage in heterocyst cells (Dean and Jacobson, 1992). Thus, the MoO_3 should induce heterocyst differentiation as the heterocyst cells were started to produce during 1st week (ranging between 18.77%-31.35%). In addition, the akinete (%) was highly appeared in stationary phase (ranging between 0.74%-4.15%) at 3rd week. However, % types of cell in normal MoO_3 concentration showed the highest amount of heterocyst (33.35% at 4th week), when the experiment was compared with each MoO_3 concentration. The results suggested that the concentration of MoO_3 had no influence on the heterocyst

differentiation, even varied to ¼-fold (ranging between 16.19%-24.15% or added more 2-fold (ranging between 13.07%-21.13%) and 3-fold (ranging between 16.18%-22.67%) concentration.

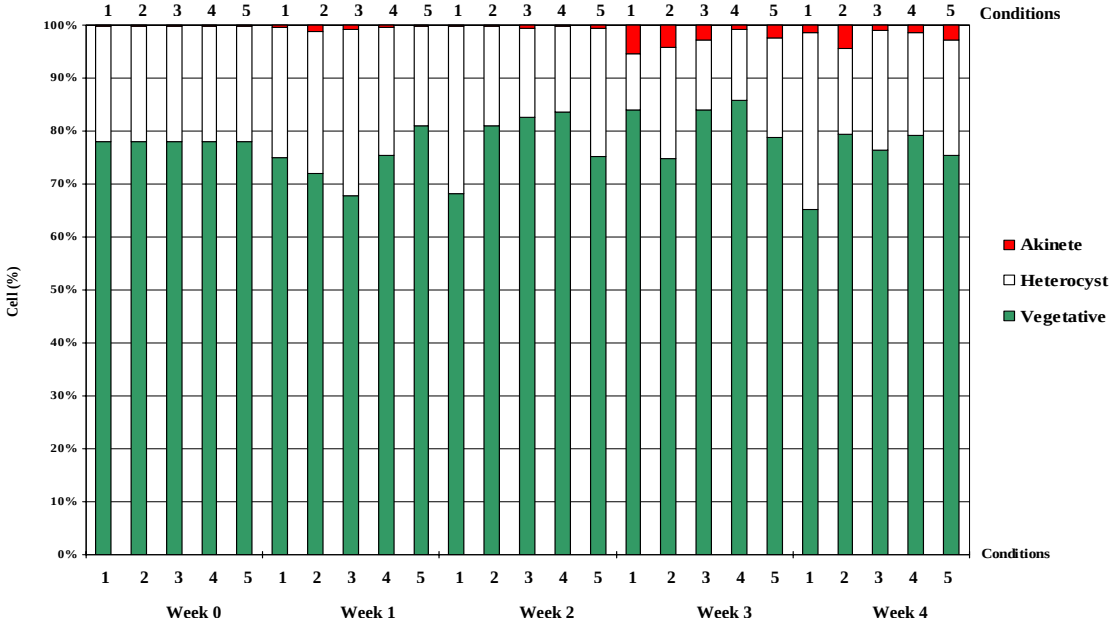


Figure 3.29 Heterocyst and akinete induction under $BG11_0$ (variations of MoO_3 concentration) medium; No. 1-5 in X-axis described as (1) $BG11_0$ (normal condition), (2) 0 nM, (3) 0.25 nM, (4) 2 nM and (5) 3 nM MoO_3.

The $FeNH_4$ citrate concentration was varied as 0, 1.5, 12 and 24 mg/l (normal was 6 mg/l in $BG11_0$ medium). The % types of cell was shown in Fig. 3.30, the heterocyst (%) in all conditions was increased at late of 1st week (ranging between 21.47%-29.13%). This might due to Fe is the one of the component of nitrogenase enzyme (Dean and Jacobson, 1992). Akinete (%) was highly formed at 3rd and 4th week due to the cells walked to stationary phase (ranging between 0.53%-3.13%) which was not different from normal concentration ($BG11_0$ medium). Declining of

$FeNH_4$ citrate concentration as ¼-fold (1.5 mg/l) induced amount of heterocyst (%) ranging between 13.50%-26.30%, which were lower than that of in normal condition (ranging between 10.58%-33.35%). Eventhough, the concentration of $FeNH_4$ citrate was varied as 2-fold (12 mg/l) and 4-fold (24 mg/l), however, the heterocyst (%) was likely lower than that of cultivation in normal condition ($BG11_0$ medium) ranging between 23.35%-29.13% and 16.11%-27.75% for 2-fold and 4-fold, respectively.

In fact, the variations of $FeNH_4$ citrate were also expected to play role in akinete induction. Since all aerobically growing organisms encounter toxic derivatives of molecular oxygen and thus are equipped with defense systems against oxidative stress (Farr and Kogoma, 1991; Halliwell and Gutteridge, 1989). Superoxide dismutase (SOD) is considered one of the key enzymes in the oxidative defense (Kim *et al.*, 1998). The reaction is catalyzed by cyclic oxidation and reduction of the transition metal in the active site of SODs (Halliwell and Gutteridge, 1989). Cyanobacteria are known to use both Fe- and Mn- containing SODs to scavenge superoxide radicals (Canini *et al*., 1992). Therefore, this also should enhance akinete development. However, the results indicated that increasing $FeNH_4$ citrate concentration was not directly effect to heterocyst and akinete induction.

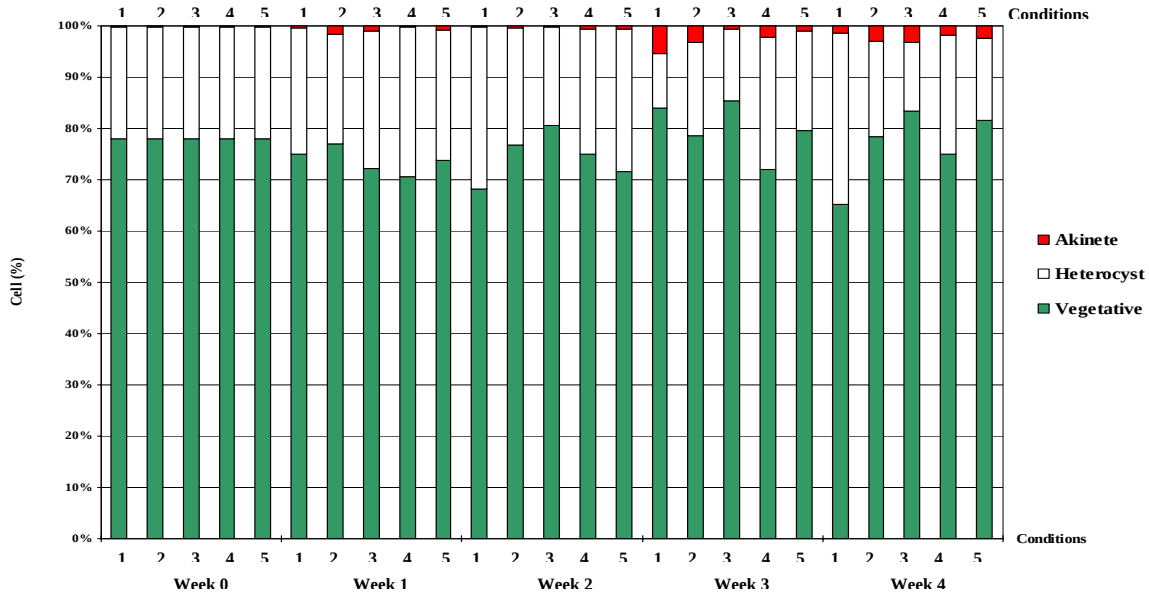


Figure 3.30 Heterocyst and akinete induction under $BG11_0$ (variations of $FeNH_4$ citrate concentration) medium; No. 1-5 in X-axis described as (1) $BG11_0$ (normal condition), (2) 0 mg/l, (3) 1.5 mg/l, (4) 12 mg/l and (5) 24 mg/l $FeNH_4$ citrate.

The % types of cell in the various concentration of $MnSO_4.H_2O$, as 0, 2.5, 20 and 30 nM (normal was 10 nM in $BG11_0$ medium), were investigated (Fig. 3.31). Heterocyst (%) was reached to the peak at 4th week (33.35%), while started to increase at 2nd week (31.41%) under normal condition ($BG11_0$ medium). On the other hand, akinete (%) was initially exhibited at 3rd week, this ought to grow up to stationary phase. The results indicated that $BG11_0$ medium able to induce heterocyst (%) in higher amount than varied $MnSO_4.H_2O$ concentration, even it was decreased or increased $MnSO_4.H_2O$ to ¼-fold, 2-fold and 3-fold, respectively. In fact, the variation of $MnSO_4.H_2O$ was expected to be the important role of akinete induction. Since, Mn- was the transition metal in the active site of SODs (Halliwell and Gutteridge, 1989).

However, this experiment seemed to imply that akinete development of this strain might not be directly induced by $MnSO_4.H_2O$.

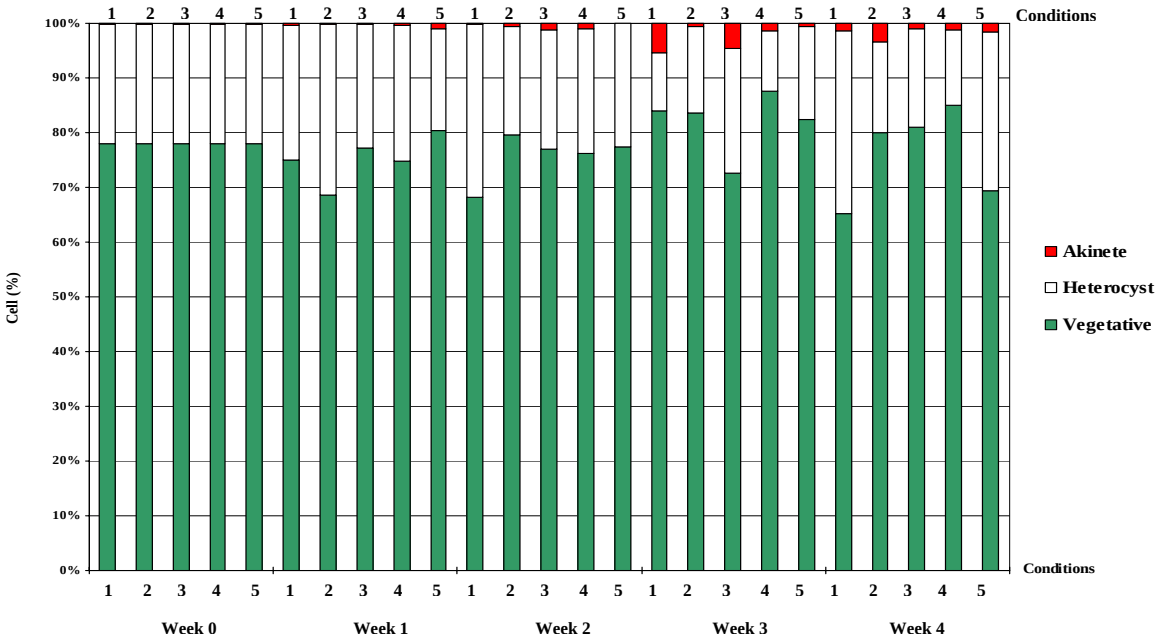


Figure 3.31 Heterocyst and akinete induction under $BG11_0$ (variations of $MnSO_4.H_2O$ concentration) medium; No. 1-5 in X-axis described as (1) $BG11_0$ (normal condition), (2) 0 nM, (3) 2.5 nM, (4) 20 nM and (5) 30 nM $MnSO_4.H_2O$.

In addition, K_2HPO_4 concentration was varied as 0, 5.5, 11 and 16.5 mM (normal was 22 mM in $BG11_0$ medium) and the results were shown in Fig. 3.32. Heterocyst (%) was ranging between 15.18%-23.93% under 5.5 mM K_2HPO_4, which showed the highest at 2nd week. The results implied that K_2HPO_4 concentration was not significantly effect to heterocyst formation. Besides K_2HPO_4 concentration seemed to play role on akinete development. The akinete formation was initially found at 1st week of cultivation, akinete (%) was abviously appeared under absence and lower K_2HPO_4 concentration. At 2nd and 3rd week showed similar results, the

highest akinete (%) was performed in 2nd week at 5.5 mM K_2HPO_4 (22.60%). For 3rd and 4th week, akinete (%) was dropped when growth reach to stationary phase. The results seemed to suggest that K_2HPO_4 was significant for akinete differentiation, as when cultured *N. punctiforme* in phosphate-limitation, akinetes were assayed after incubation for 10 d in the dark at 4ºC and found 10% akinete cells were detected (Wong and Meeks, 2002). However, the low K_2HPO_4 concentration, the cyanobacterial cells could grow better than that of absence K_2HPO_4. Thus, akinete (%) was high as in 3rd week and last week. The akinete (%) was no significant at the low concentration because of the late stationary phase. However, Olli and co-workers (2005) revealed a clear species-specific difference in the timing of peak akinete production. Generalizing over all the mecocosms, *A. cylindrica* akinetes were produced at the highest rate during the late phase of the bloom (approximately 1 week after the planktonic population peak). Besides the akinetes of *A. lemmermannii* were produced as the bloom progressed and there were no notable time shift the planktonic population and the akinete production rate.

Interestingly, in $BG11_0$ medium (without $FeNH_4$ citrate and K_2HPO_4) was displayed the highest akinete (%) about 21.17% at 3rd week. Although, the akinete (%) was highly appeared at 3rd and 4th week (only 3.13% and 2.97%, respectively) when lack of $FeNH_4$ due to the cells started to reach stationary phase which similarity to akinete (%) in normal condition (7th condition, Fig. 3.32). Meanwhile, without K_2HPO_4 supplement condition showed the highest akinete (%) about 11.27% at 1st week. These might due to $FeNH_4$ citrate had synergistic effect with K_2HPO_4, thus the akinete (%) performed obviously high in the lack of both chemical factors conditions.

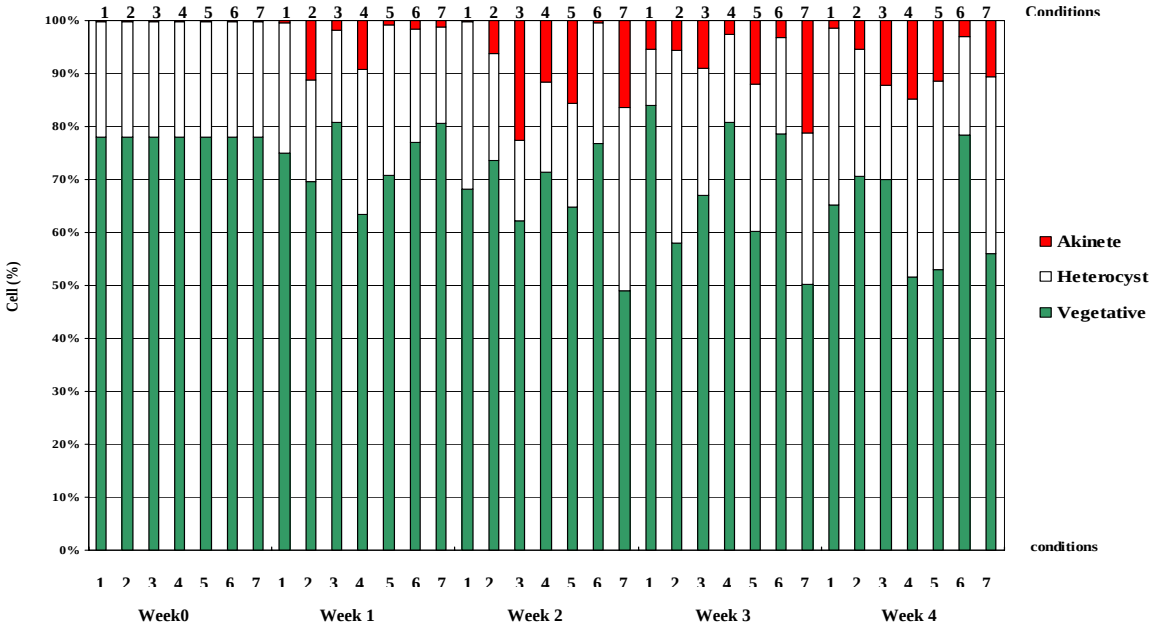


Figure 3.32 Heterocyst and akinete induction under $BG11_0$ (variations of K_2HPO_4 concentration) medium; No. 1-7 in X-axis described as (1) $BG11_0$ (normal condition), (2) 0 mM, (3) 5.5 mM, (4) 11 mM, (5) 16.5 mM K_2HPO_4, (6) $BG11_0$ (without $FeNH_4$ citrate) and $BG11_0$ (without both $FeNH_4$ citrate and K_2HPO_4)

3.6.5 Heterocyst differentiation and calcium ions

Since the previous experiment implied that $CaCl_2$ and K_2HPO_4 concentration had the effect to heterocyst and akinete induction, respectively. The importance of $CaCl_2$ to heterocyst formation was observed by changing the medium such as; i) $BG11_0$ to $BG11_0$ medium, ii) $BG11_0$ (without $CaCl_2$) to $BG11_0$ medium (normal), iii) $BG11_0$ to BG11 medium and iv) BG11 to $BG11_0$ (without $CaCl_2$) (Fig. 3.33). The cells in $BG11_0$ which were transferred to the same fresh medium was demonstrated similarly to % types of cell from $BG11_0$ (without $CaCl_2$) which changed to $BG11_0$ medium. When cell suspension was transferred to fresh same medium ($BG11_0$), the %

types of cell in each period was not different as heterocyst (%) ranging between 22.89%-33.32% (Fig. 3.33A). When the $BG11_0$ (without $CaCl_2$) was changed to be $BG11_0$ medium (normal), % types of cell was similar but higher than the previous condition as heterocyst (%) ranging between 19.06%-40.57% (Fig. 3.33B).

Torrecilla and others (2004) compared using 0 mM $CaCl_2$ and 0.2 mM $CaCl_2$ (normal condition; $BG11_0$) and reported that the cell number in the calcium transient in 0.25 mM $CaCl_2$ (almost 0.4 µM Ca^{++}), was higher than absence $CaCl_2$ (about 0.3 µM Ca^{++}). Since, the Ca^{++} may be involved in a very early step of the heterocyst differentiation process. Thus, when the medium was transferred to $BG11_0$ medium, heterocyst (%) should be high. In case of the $BG11_0$ changed to be BG11 medium, heterocyst (%) was not different at early cultivation (0-12 d) ranging between 20.60%-28.78%. This is due to the medium contained N-source. Heterocyst (%) then was initially increased at 18 d (ranging between 27.80%-41.54%) because of insufficient of N-source in medium (Fig. 3.33C). Interestingly, when BG11 transferred to $BG11_0$ (without $CaCl_2$) medium, heterocyst (%) was shown increasingly since 3 d until 30 d ranging between 46.68%-62.59% and the highest at 24 d (62.59%) (Fig. 3.33D). The results elucidated that heterocyst induction is independent to external Ca^{++} which is similar to the report of Zhao *et al*. (2005).

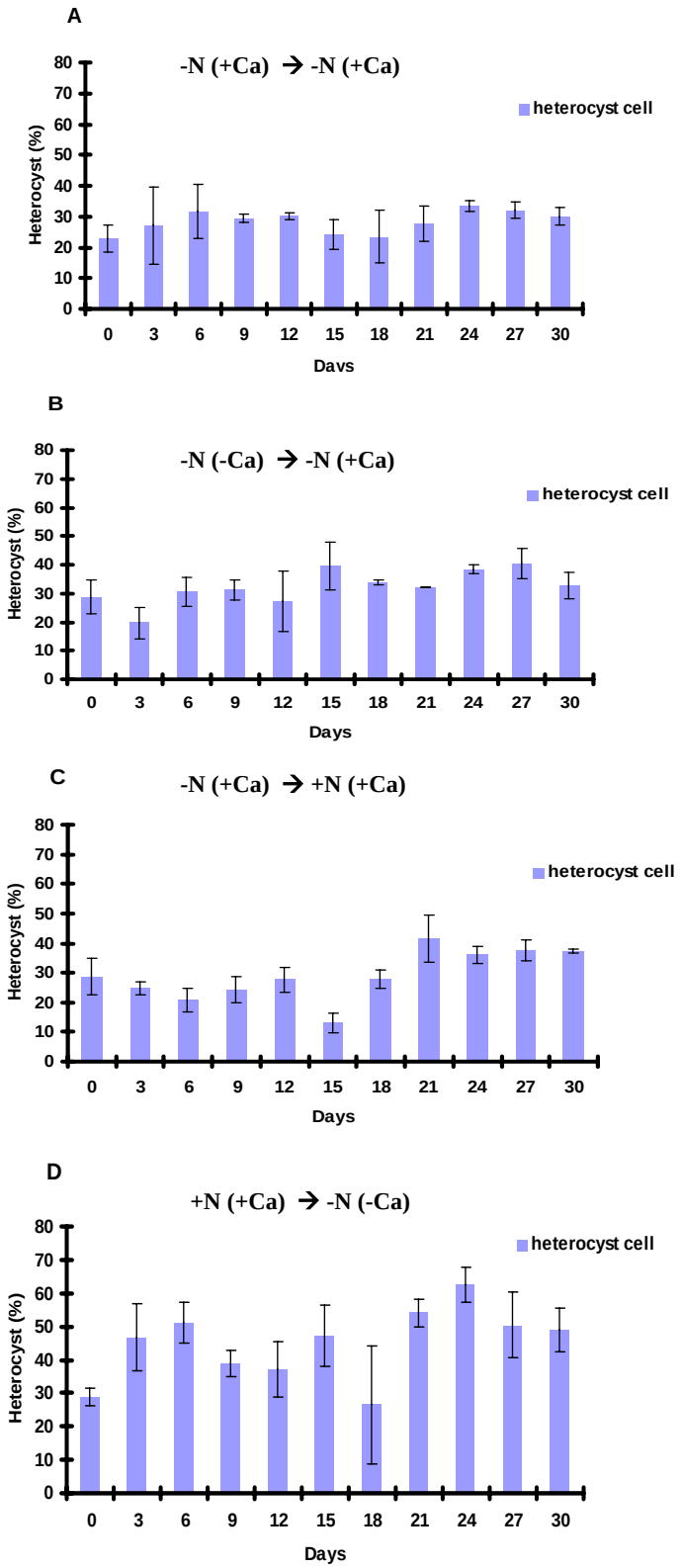


Figure 3.33 Heterocyst (%) based on changing media; (A) $BG11_0$ to $BG11_0$ medium, (B) $BG11_0$ (without $CaCl_2$) to $BG11_0$ medium, (C) $BG11_0$ to BG11 medium and (D) BG11 to $BG11_0$ (without $CaCl_2$).

3.6.6 Heterocyst differentiation and developmental patterns of gene expression

After the *Nostoc* sp. strain VICCR1-1 as grown in BG11 medium for 30 days and transferred to fresh medium which able to enhance heterocyst development ($BG11_0$ and $BG11_0$ (without $CaCl_2$) media) as well as compared with N-supplement medium. The heterocyst cells forming under every condition were enumerated and found that at 0 d, there was 27.47% of heterocyst cell. The heterocyst (%) was fluctuated during early 24 h in every experiment, especially in N-repetition medium. The percentage of heterocyst cells were high and steady about 35.89%-54.33% at 12 d to 30 d (Fig. 3.34A). In nitrogen step-down condition, the heterocyst (%) presented low fluctuate which was continuously increased at 3 d to 15 d (32.85%-48.55%). Then the heterocyst (%) slightly dropped at 18 d and keep constant since day 21 until day 30 (about 27.50%) (Fig. 3.34B). Otherwise, the heterocyst (%) performed in high and steady pattern initially at 2 d and trended to exponentially increasing at 18 d to 30 d (48.63%-57.44%) in N-deprivation which lack of $CaCl_2$ medium (Fig. 3.34C). The results indicated that the heterocyst formation in N-starvation was induced faster than that of cell in combined-nitrogen medium (Fig. 3.34). Thus, the heterocyst differentiation initially increased at 2 d and 3 d (early 1^{st} week) in $BG11_0$ and $BG11_0$ (without $CaCl_2$), respectively. Besides the heterocyst induction in fresh BG11 medium contained N-source, thus induction of heterocyst formation might not need for nitrogen fixation in 1^{st} week. On the other hand, the heterocyst differentiation was necessary in N-deprivation medium. The medium which lack both nitrogen and $CaCl_2$, trended to induce heterocyst (Fig. 3.34C). Because the intracellular free Ca^{++} ion was the role of heterocyst differentiation (Torrecilla *et al*., 2004). Whereas, there was extracellular

free Ca^{++} ion in $BG11_0$ medium, thus it might enhance CcbP production to bind Ca^{++} and inhibited heterocyst formation in late of growth (Fig. 3.34B).

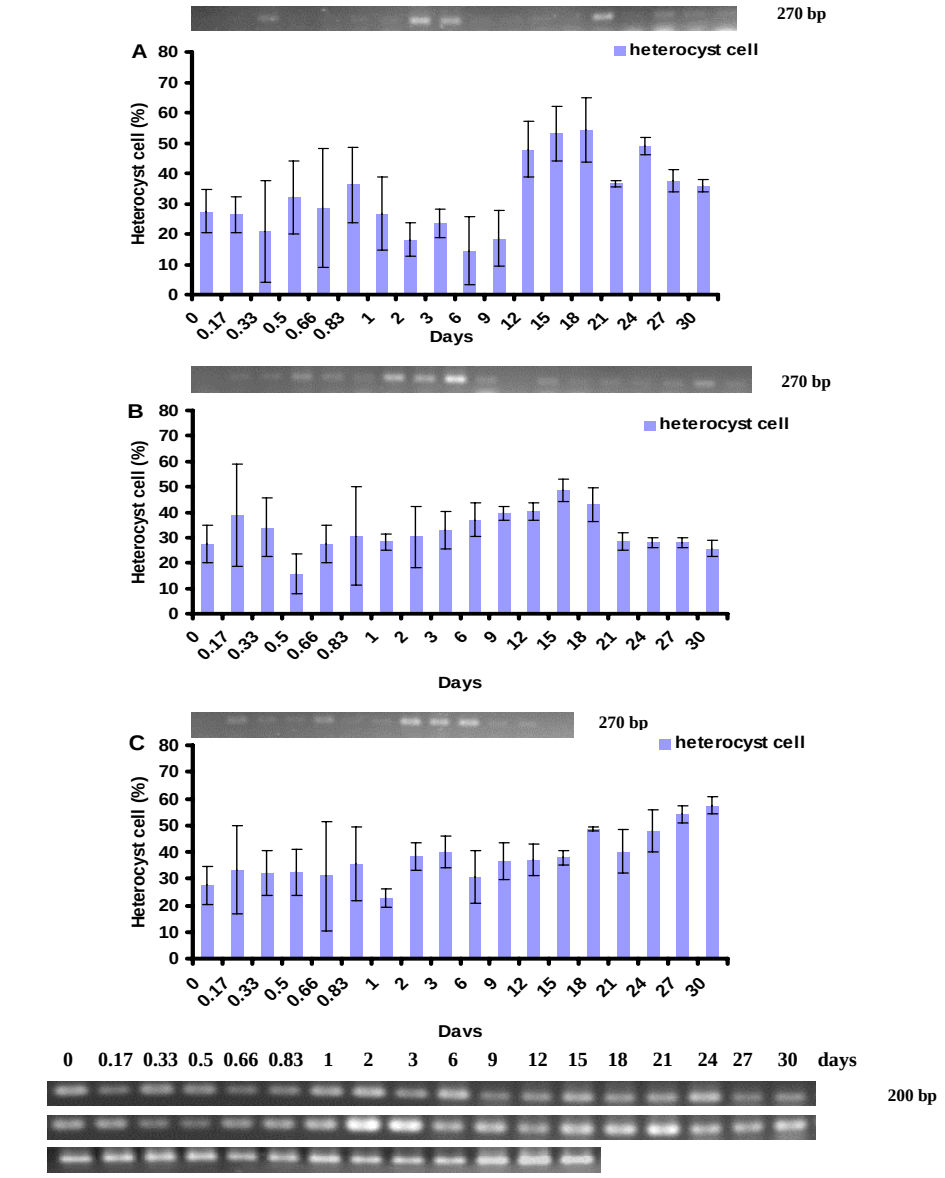


Figure 3.34 Heterocyst cell (%) and developmental patterns in *het*R gene expression in different conditions at period experiments; (A) BG11 medium, (B) $BG11_0$ medium, (C) $BG11_0$ (without $CaCl_2$) and (D) 16S rRNA gene expression in BG11 medium, $BG11_0$ medium and $BG11_0$ (without $CaCl_2$), respectively.

In the meantime, *het*R expression was determined by reverse-transcription PCR because HetR plays a role in regulation of heterocyst differentiation (Adams and Duggan, 1999; Buikema and Haselkorn, 2001; Meeks and Elhai, 2002). The 16S rRNA was included as a control for equal amount of mRNA template (Fig. 3.34D). The results indicated that *het*R was expressed in size 270 bp in every period of the experiments except at 0 d. The level of expression of *het*R in BG11 medium was illustrated in Fig. 3.34A. The *het*R transcription activity was low during early cultivation, however, the *het*R transcription activity was high at 8 h, higher at 3 d and 18 d (the highest at 2 d). However, the *het*R expression was peaked at 8 h when compared with the intensity of 16S rRNA gene (Fig. 3.34A and D). In $BG11_0$ medium, the low *het*R expression level was observed in initial growth of cyanobacterial cells (0-20 h) and started to decrease again at 6 d to 30 d, while the high *het*R expression level was observed at 1, 2 and 3 d (Fig. 3.34B). *het*R intensity was compared with 16S rRNA intensity and showed *het*R expression since 4 h which peaked at 3 d (Fig. 3.34B and D). For $BG11_0$ (without $CaCl_2$), the *het*R transcription activity was also low at early of growth, on the other hand, the high of *het*R transcription activities were exhibited at 2, 3 and 6 d, then declined continuously from 9 d to 15 d (Fig. 3.34C). However, the hetR expression was peaked at 6 d when compared with 16S rRNA intensity (Fig. 3.34C and D).

The level of *het*R expression was shown within 8 h in fresh combined-nitrogen medium. The amount of *het*R transcription activity reached its maximum level within 3 d in early cultivation. However, there was reached the highest level at 18 d again. *het*R transcription might be maximum level in early stage (2-3 d) when transferred to new medium and highly transcription level once when decreased N-source in the

medium (Fig. 3.34A). Otherwise, the *het*R expression's results after nitrogen step-down indicated that the *het*R transcription activities were initially decreased within 4 h (Fig. 3.34B and C) as reported of *Anabaena* sp. PCC7120 *het*R expression by real-time PCR analysis (Huang *et al.*, 2004). Expression of *het*R was apparent a few hours after removing combined nitrogen from the growth medium (Buikema and Haselkorn, 2001). The transcription activity increased at 1 d, 2 d and maximum at 3 d, these results were related with Huang *et al*. (2004) which suggested that the *het*R mRNA showed maximum value at 1 d after nitrogen step-down. However, the transcription profiles of *het*R in free-living *Nostoc* strain 0102 isolated from *Gunnera* symbiosis performed high intensity of band from RT-PCR at 6 d, whereas *het*R in the symbiosis was overexpressed (Wang *et al.*, 2004).

In this study, the nitrogen step-down along with lack of $CaCl_2$, the *het*R level was reached maximum at 2, 3 and 6 d. Zhao and teams (2005) reported that the total cellular-free Ca^{++} concentration increased rapidly 4 h after nitrogen step-down, whereas the phase of fast Ca^{++} concentration was about 1-2 h behind the phase of HetR increased. The *het*R transcription activities of both of nitrogen step-down conditions ($BG11_0$ and $BG11_0$ without $CaCl_2$) showed the similarly results (Fig. 3.34B and C). Torrecilla and others (2004) suggested that the appearance of the calcium transient was independent of an extracellular source of calcium and thus, the main source of calcium seemed to be in the intracellular.

When heterocyst cell (%) and *het*R expression level were compared (Fig. 3.34), the data indicated that no relationship between both of experiments. In contrast previous report (Wang *et al*., 2004), cyanobacterial genes expression involved in heterocyst differentiation along a plant symbiosis development profile was determined

(*Nostoc* sp. and *Gunnera*). The experiment found that the levels of expression of *het*R in *Nostoc* isolated along the *Gunnera* stem were varied significantly. So that, in cyanobionts at apex (stage 1) with a low heterocyst frequency, the *het*R transcription activity was low. Before reaching the middle part of the developmental profile, the *het*R transcription activity increased to a maximum (stage 3 which heterocyst frequency recorded about 30-40%). *het*R expression then declined continuously and was negligible in the oldest tissue (stage 8). Wang and co-workers (2004) indicated that the genes showed distinct expression profile related to the developmental profile of the host, suggesting that the plant has an important regulatory role. However, our studies determined in the form of free-living, therefore the results might be different. Moreover, there were several genes that involved *het*R expression. In the timetable of heterocyst development, genes required for initiation and morphogenesis are basically divided into two groups: (i) transcription of *het*R, a gene essential to initiation of heterocyst development and of *het*C, a gene required for very early heterocyst development, is up-regulated within 3.5 h after deprivation of fixed nitrogen (Black *et al*., 1993; Xu and Wolk, 2001); (ii) from 5 to 10 h, *hep*A, *het*M and *dev*A, the genes involved in formation of the heterocyst envelope, are induced (Holland and Wolk, 1990; Cai and Wolk, 1997a; Maldener *et al.*, 1994). *het*N is induced between 6 and 12 h after transfer into nitrogen-free medium (Bauer *et al*., 1997). *het*R, *het*C, *hep*A, *dev*A and *het*N are expressed specifically or primarily in heterocysts or proheterocysts. Furthermore, PatA is required for the increase in expression of *het*R gene in internal cells of the filament (Buikema and Haselkorn, 2001) and PatS inhibited its DNA binding activity provided the first mechanistic view of HetR function and regulation. The previous studies seemed to imply that the genes involved heterocyst differentiation

should express in early of cultivation, thus middle and late of cultivation might not obviously perform transcription of *het*R gene, as indicated in this study. Besides two-component signal transduction systems are also important machineries for bacteria to regulate cell differentiation and other physiological processes in response to environmental or intracellular changes (Albight *et al.*, 1989). The simplest two-component regulatory systems consist of a sensor histidine kinase, often located in the cytoplasmic membrane and a cytoplasmic response regulator. Upon sensing a certain signal, an input domain of the sensor histidine kinase modulates the activity of its transmitter domains, which then auto-phosphorylates an internal histidine residue and transfers the phosphoryl group to a response regulator. Consequently, transcription of particular genes or various other functions are regulated (Ning and Xu, 2004). However, many prokaryotic signaling systems have multiple components, interconnections with other regulatory circuits or feedback loops (Stock *et al.*, 2000). In the genome of *Anabaena* sp. PCC7120, there are 203 two-component signal transduction genes, 73 encoding sensory kinases, 77 encoding response regulators and 53 encoding hybrid sensory kinases and response regulators (Kaneko *et al*., 2001).

3.6.7 Akinete differentiation and developmental patterns of gene expression

P-limitation medium was used for inducing akinete development as well as with BG11 medium. At 0 d, the akinete cells appeared about 2.30% (Fig. 3.35). Since the cyanobacterial cells were grown in BG11 for 30 days, then transferred to fresh media. Thus the akinete cells were high at early of new cultivation (1-2 d) in both of media. For BG11 media, the akinete cells showed high percentage (2.89%) again at 24 d (stationary phase) (Fig. 3.35A), while the akinete cell were initially increased since

day 6 (ranging between 4.66%-16.64%) and fluctuate highly level of akinete cell (%) in P-limitation medium (Fig. 3.35B).

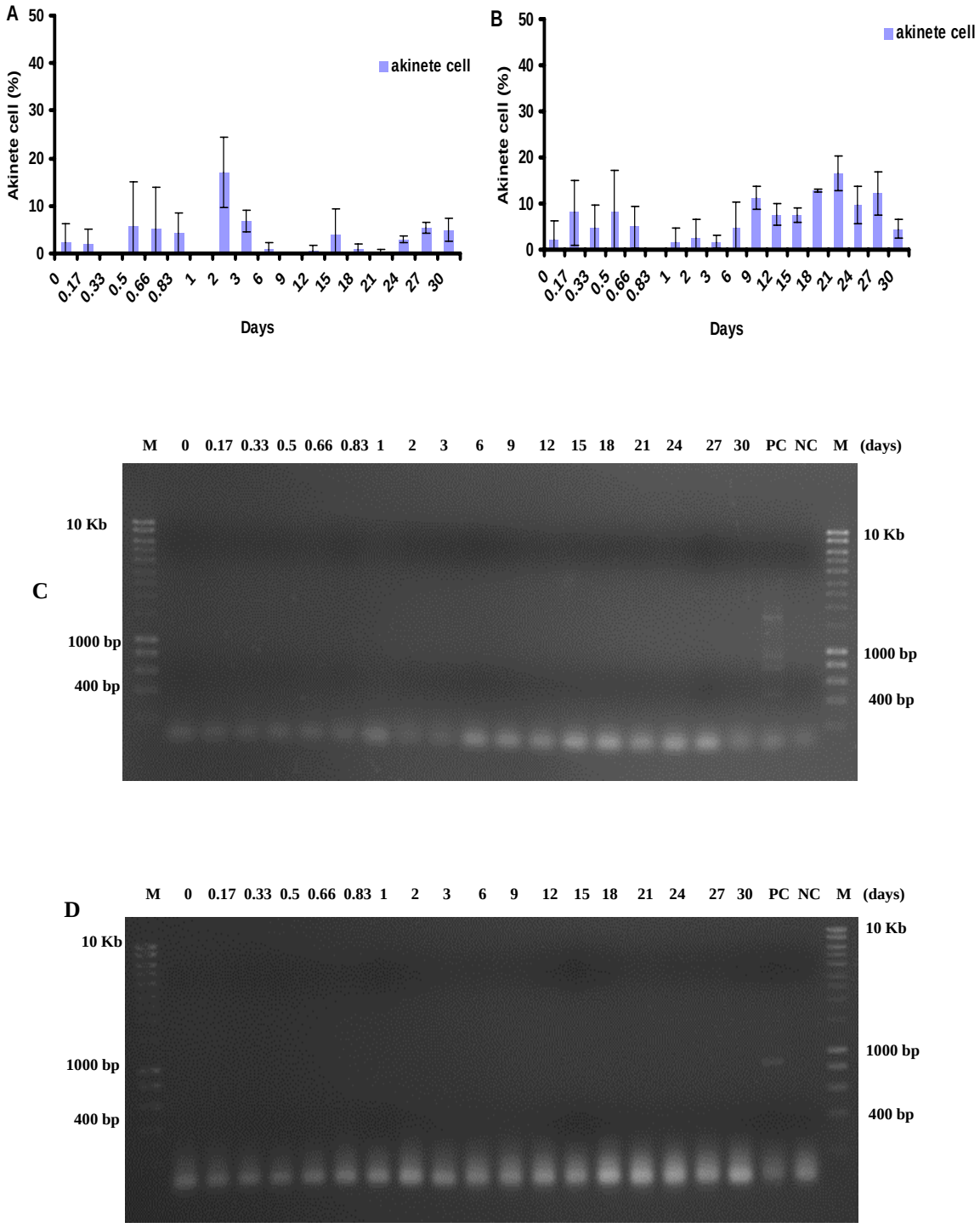


Figure 3.35 Akinete cell (%) in (A) BG11 medium, (B) $BG11_0$ (without K_2HPO_4), (C) represented *sod*F gene expression and (D) represented *ava*K gene expression at period experiments.

*sod*F gene was used for investigating on akinete cell differentiation due to *sod*F mRNA was found in *N. commune* CHEN/1986 after prolonged storage in the desiccated state (Shirkey *et al*., 2000). Furthermore, *ava*K gene also was detected in this experiment, since this gene was identified as an akinete marker gene in *A. variabilis* (Zhou and Wolk, 2002). mRNA was isolated and expression patterns were analyzed (using a two step RT-PCR). The 16S rRNA was induced as a control for equal mRNA template (Fig. 3.35D). The PCR product in every period of experiments based on *sod*F and *ava*K genes was not detected (Fig. 3.35C and D). Eventhough the genomic DNA showed band product size 427 bp and 800 bp, respectively. This might indicated that the amount of mRNA containing *ava*k gene was very low, hence *ava*K transcription activity was insufficient to detect (P.C. Wolk, personal communication). In addition, the quantity of *sod*F released from the cells approach the magnitude of that released by rehydrating *N. commune* (Shirkey *et al*., 2000). Upon rehydration of desiccated cells, there was a turnover of *sod*F mRNA within 15 min, the fact that *sod*F mRNA is abundant after 24 h of rehydration emphasizes the possible importance of SodF during the recover phase (Shirkey *et al*., 2000). Since our studies were not rehydrated cyanobacterial cells, therefore, *sod*F expression might insufficient to detect as *ava*K gene.

*het*R also is required for akinete differentiation and *het*R is actively expressed in akinete (Leganes *et al*., 1994). The *het*R expression levels in P-limitation condition ($BG11_0$ without K_2HPO_4) were demonstrated in Fig 3.36A. The 16S rRNA was included as a control for equal amount of mRNA template (Fig. 3.36C). The *het*R transcription activity was firstly appeared at 3 d then decreased at 6 d, continuously increased again until reached maximum level at 30 d. The *het*R intensity was compared with 16S rRNA and showed the highest expression at 27 d (Fig. 3.36A and

C). These results seemed to relate to akinete cell (%) in the period experiments. Furthermore, *het*R transcription activity was corresponding to heterocyst cell (%) which contrast with *het*R expression levels in previous studies (Fig. 3.36B). This might be due to different conditions, there are several factors that influence heterocyst formation when induced heterocyst differentiation.

Thus, only *het*R transcription might not appropriate for detecting cell differentiation during heterocyst development. Nevertheless, the akinete differentiation was mainly induced in P-limitation medium, therefore, *het*R transcription was related with common heterocyst formation. Consequently, *het*R is required for both akinete and heterocyst differentiation, supporting the view that heterocyst may have evolved from akinetes, or that the two cell types may share a common ancestor (Wolk *et al*., 1994).

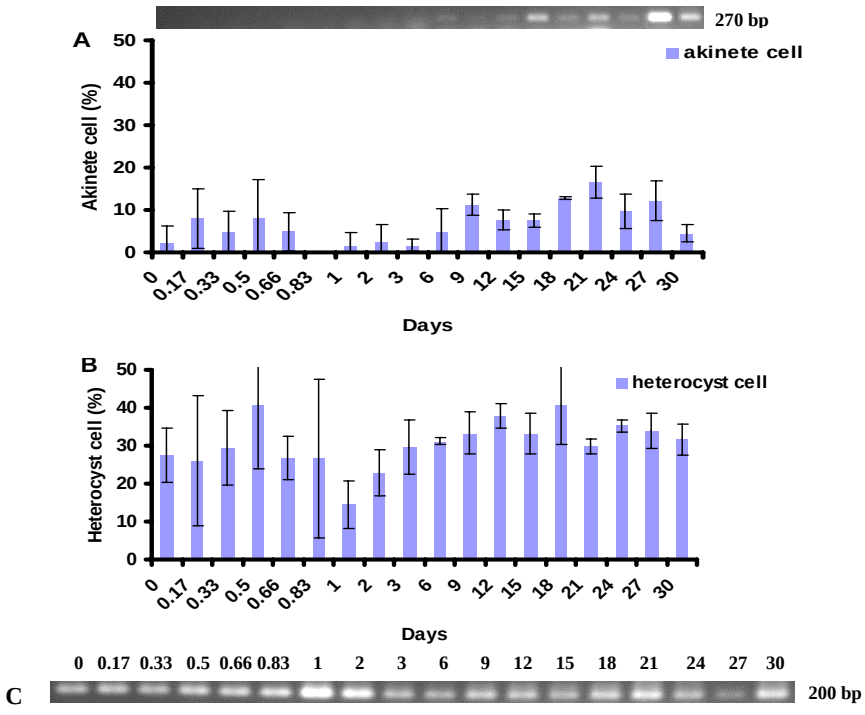


Figure 3.36 *het*R transcription activity, akinete cell (%) in $BG11_0$ (without K_2HPO_4) (A), heterocyst cell (%) in $BG11_0$ (without K_2HPO_4) (B) and 16S rRNA gene expression in $BG11_0$ (without K_2HPO_4) (C)**.**

3.6.8 Tryptic peptides and homology searching

Since nitrogen starvation and lacking $CaCl_2$ including nitrogen conditions could induce heterocyst formation. Thus, the total proteins from cyanobacterial cell in both conditions were extracted and analyzed on SDS-PAGE (Fig. 3.37). A 140 KDa and 72 KDa bands were appeared in both BG11 and $BG11_0$ media, meanwhile only 45 KDa was found in $BG11_0$ (without $CaCl_2$) medium. The protein with a molecular mass of 45, 72 and 140 KDa were excised from SDS-PAGE gels and collected for protein microsequencing. The sequences were determined by liquid chromatography mass spectrometry (LC/MS). Fragments similar in sequence were identified, by BLAST search. A 72 KDa from BG11 medium was presented ten proteins similar to chaperonin GroEL (HSP60 family) *Nostoc punctiforme* PCC73102 (40.99% amino acid identity; Fig. 3.38). Six proteins showed 23.74% amino acid identity similar to ribulose 1,5-biphosphate carboxylase, large subunit (*N. punctiforme* PCC73102) (Fig. 3.39). In addition, a 72 KDa from $BG11_0$ medium was exhibited eight proteins similar to chaperonin GroEL (HSP60 family) *N. punctiforme* PCC73102 (35.71% amino acid identity; Fig. 3.40). Therefore, a 72 KDa protein was expected to be chaperonin GroEL (HSP60 family), while *Oscillatoria* sp. presented GroEL with a molecular mass 60 KDa (Yamazawa *et al.*, 1999). cpn60 is widely distributed from bacteria to eukaryotic organelles and is involved in the folding (Goloubinoff *et al.*, 1989), assembly (Goloubinoff *et al*., 1989), translocation of other polypeptides (Bochkareva *et al.*, 1988; Lecker *et al*., 1989). The alternative promoters of *gro*E operon will be involved depending on the environmental conditions (Ohtaka *et al.*, 1992). More recently, these proteins were found to assist the assembly process of recombinant ribulose 1,5-biphosphate carboxylase/oxygenase (Rubisco) cloned from prokaryotic

source (Ellis, 1990; Zeilstra-Ryalls *et al.*, 1991). Wang and Tabita (1992) noted that Rubisco which serves as the cone stone enzyme of Calvin-Benson Bassham reducing pentose phosphate pathway of carbon dioxide assimilation (Li and Tabita, 1997), appeared to associate with a protein that migrated in the approximate position of cpn60 (GroEL) of *R. sphaeroides*; in the presense of ATP. The interaction of Rubisco with ATP led to a further interaction of the enzyme with a major heat shock and chaperonin protein of *R. sphaeroides*, the cpn60 or GroEL protein. The Rubisco polypeptides in *Anabaena* are obviously folded by simultaneous synthesis of chaperonin proteins cpn60 (GroEL) and subsequently assembled to yield active enzyme (Badger, 1980; Tabita and Colletti, 1979). Two proteins were co-related functional each other, Li and Tabita (1997) suggested that a chaperonin-like function of Rbx, which is juxtaposed and cotranscribed with the *rbc*L and *rbc*S genes encoding from I ribulose 1,5-biphosphate carboxylase/oxygenase.

Thus, a 72 KDa protein in this study, was similar to chaperonin GroEL (HSP60 family) *N. punctiforme* PCC73102 which function including with ribulose 1,5-biphosphate carboxylase/oxygenase (Rubisco). So that, this protein was importance for growth and CO_2 fixation able to occur both supplied and without N-supplement conditions. Therefore, this protein was routinely expressed in both conditions (Fig 3.37).

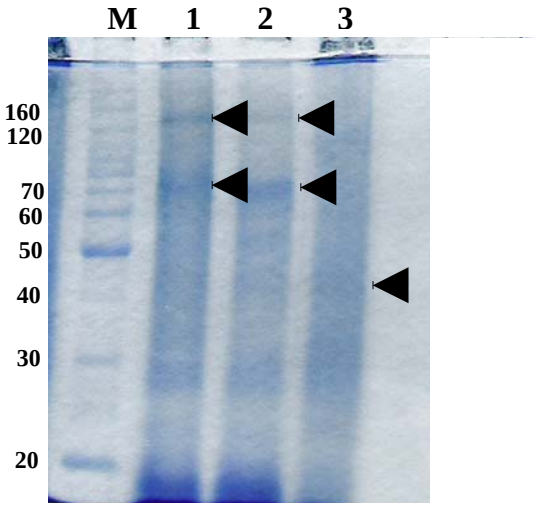


Figure 3.37 SDS-PAGE-10% gel protein of *Nostoc* sp. strain VICCR1-1 stained by coomassie brilliant blue R-250. Vertically arrayed numbers are sizes of standards in kilodaltons. The arrows indicate potential interested-band protein (lane M; BenchMark™ Protein ladder marker, lane 1; *Nostoc* sp. strain VICCR1-1 in BG11 medium, lane 2; *Nostoc* sp. strain VICCR1-1 in $BG11_0$ medium and lane 3; *Nostoc* sp. strain VICCR1-1 in $BG11_0$ (without $CaCl_2$).

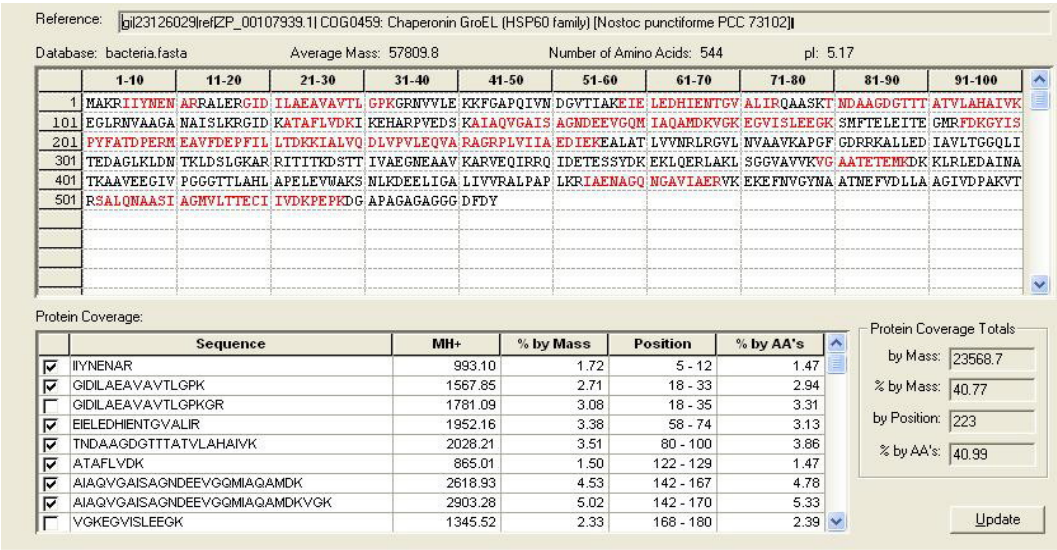


Figure 3.38 A 72 KDa from BG11 medium, showed 40.99% amino acid identity with chaperonin GroEL (HSP60 family) *Nostoc punctiforme* PCC73102**.**

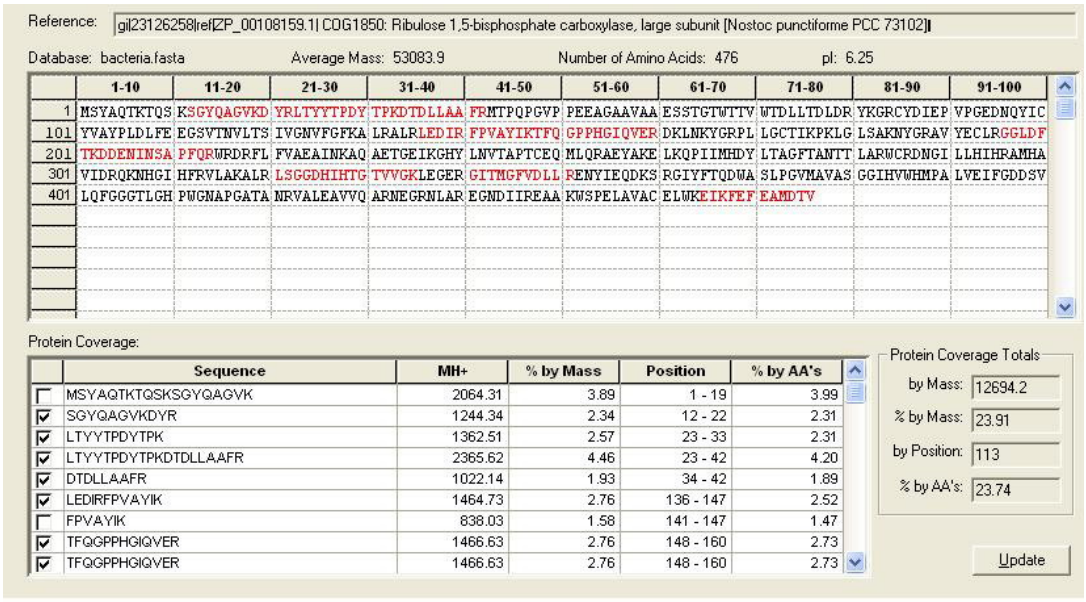


Figure 3.39 A 72 KDa from BG11 medium, showed 23.74% amino acid identity with ribulose 1,5-biphosphate carboxylase, large subunit (*N. punctiforme* PCC73102).

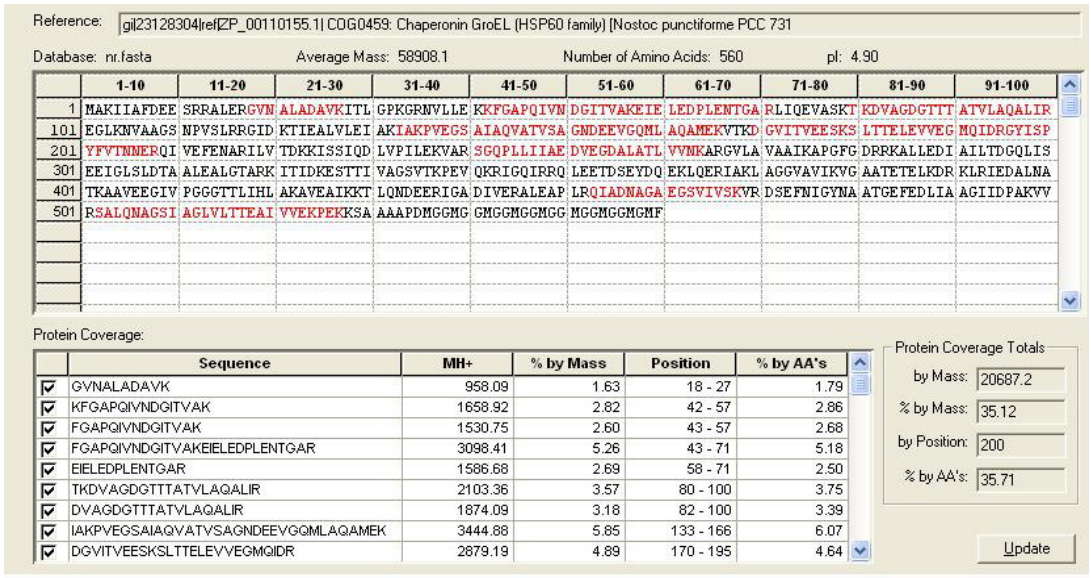


Figure 3.40 A 72 KDa from $BG11_0$ medium, showed 35.71% amino acid identity with chaperonin GroEL (HSP60 family) *N. punctiforme* PCC73102.

A 140 KDa from BG11 medium was similar to DNA-binding ferritin-like protein (oxidase damage protect) (49.46% amino acid identity; Fig. 3.41). The ferritin-like proteins shown functionally to bind inorganic iron can be classified in two different categories: (i) the heme-b containing "bacterioferritins" (Bfr) found in microorganisms such as *E. coli* (Yariv *et al*., 1981; Frolow *et al.*, 1994) and *Azotobacter vinelandii* (Steifel and Watt, 1979) and (ii) non-heme containing ferritins such as those expressed by *Helicobacter pylori* (Doig *et al.*, 1993; Frazier *et al*., 1993) and by the *E. coli gen-165* gene (Izuhara *et al.*, 1991; Hudson *et al*., 1993). Ferritins are proteins involved in the safe storage a timely delivery of iron for biosynthesis in eukaryotic organisms (Frankel and Blakemore, 1991; Theil, 1987; Crichton, 1990; Eisenstein *et al.*, 1990). *A. variabilis* display sequence similarity with mammalian and proven baterial ferritins and conserve all or most of the seven ferroxidase center amino acids (Bozzi *et al*., 1997).

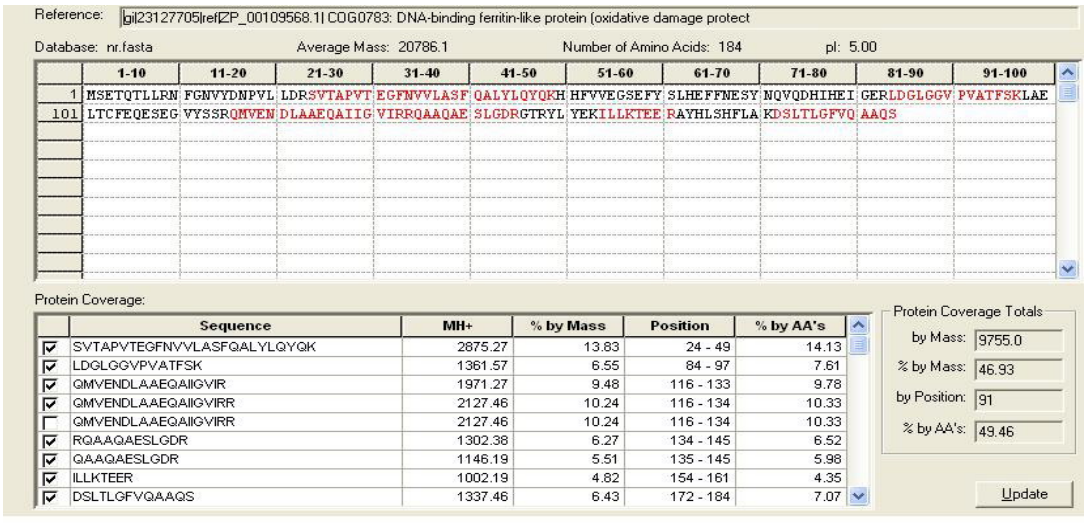
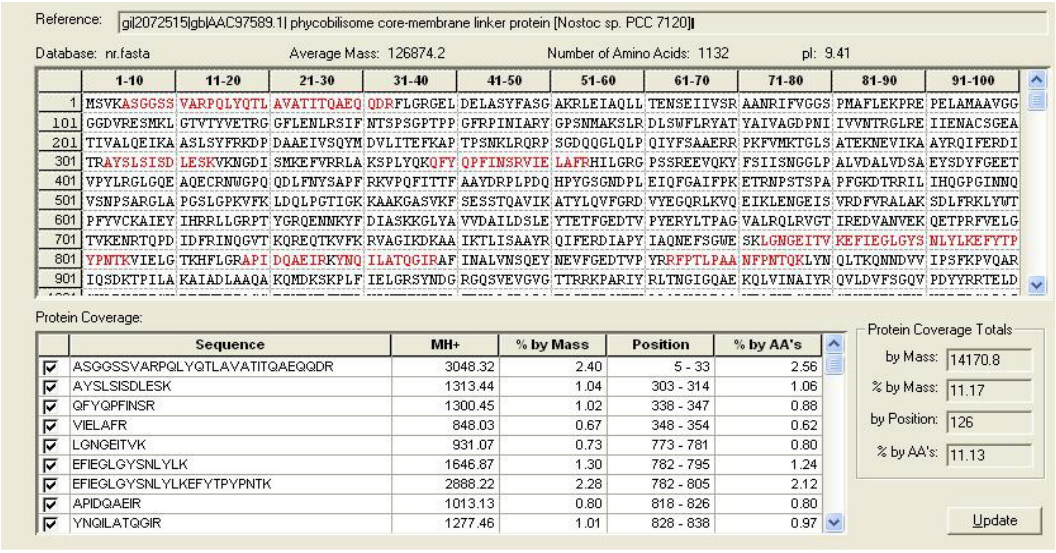


Figure 3.41 A 140 KDa from BG11 medium, showed 49.46% amino acid identity with DNA-binding ferritin-like protein (oxidase damage protect).

Moreover, a 140 KDa band from BG11 showed seven proteins which were similar to phycobilisome core-membrane linker protein (*Nostoc* sp. PCC7120) (11.13% amino acid identity; Fig. 3.42). Likewise, a 140 KDa band from $BG11_0$ was also similar to phycobilisome core-membrane linker protein (*Nostoc* ap. PCC7120) (7.24% amino acid identity; Fig. 3.43). Phycobilisomes are multiprotein complexes composed of allophycocyanin, phycocyanin and frequently phycoerythrin (Gray *et al.*, 1973) which serve as primary light-harvesting antennae for photosystem II reaction centers in cyanobacteria (Glauser *et al*., 1992). Phycobilisomes are primary assembly also requires non-chromophore-bearing phycobilliprotein; as 'linkers' (Glauser *et al*., 1992). However, size comparisons of the core-membrane linker phycobilliproteins of *Mastigocladus laminosus* and *Anabaena* sp. PCC7120 reveal an apparent molecular mass of 120 KDa, 95 KDa for *Synechococcus* sp. PCC7002 and 99 KDa for *Calothrix* sp. PCC7601 (Glauser *et al*., 1992). These indicated that the molecular mass of protein was species-specific of microorganisms. This protein was necessary for photosynthesis, thus it was commonly expressed in both with and without N-supplement in the medium (Fig. 3.37).



	Sequence	MH+	% by Mass	Position	% by AA's
☑	ASGGSSVARPQLYQTLAVATITQAEQQDR	3048.32	2.40	5 - 33	2.56
☑	AYSLSISDLESK	1313.44	1.04	303 - 314	1.06
☑	QFYQPFINSR	1300.45	1.02	338 - 347	0.88
☑	VIELAFR	848.03	0.67	348 - 354	0.62
☑	LGNGEITVK	931.07	0.73	773 - 781	0.80
☑	EFIEGLGYSNLYLK	1646.87	1.30	782 - 795	1.24
☑	EFIEGLGYSNLYLKEFYTPYPNTK	2888.22	2.28	782 - 805	2.12
☑	APIDQAEIR	1013.13	0.80	818 - 826	0.80
☑	YNQILATQGIR	1277.46	1.01	828 - 838	0.97

Figure 3.42 A 140 KDa band from BG11, showed, 11.13% amino acid identity with phycobilisome core-membrane linker protein (*Nostoc* sp. PCC7120).

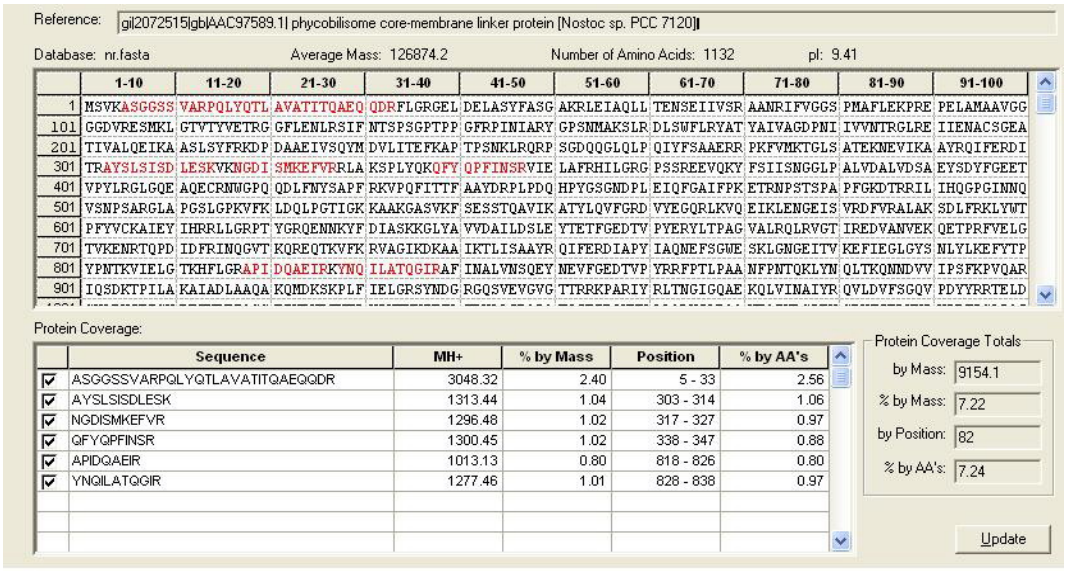


Figure 3.43 A 140 KDa band from $BG11_0$, showed 7.24% amino acid identity with phycobilisome core-membrane linker protein (*Nostoc* ap. PCC7120).

In fact, a 72 KDa; chaperonin GroEL (HSP60 family) and ribulose 1,5-biphosphate carboxylase, large subunit and 140 KDa; phycobilisome core-membrane linker protein and DNA-binding ferritin-like protein which are importance for growth, were found in BG11 and $BG11_0$ media. Cyanobacterial cells in $BG11_0$ (without $CaCl_2$) should also have those proteins but could not be detected in this experiment. This might be due to this condition could induced high number of heterocyst cells which lacked the photosystem II. The amount of protein might very low and no able to detect by coomassie brilliant blue R-250 staining.

Only the protein of 45 KDa was expressed in $BG11_0$ (without $CaCl_2$) medium. The protein band was similar to outer membrane protein (*N. punctiforme* PCC73102) with low amino acid identity of 11.32% (Fig. 3.44). One of the major functions of every bacterial cell envelope is to allow sufficient transport of nutrients and metabolites into and out of the cell (Hoiczyk and Hansel, 2000). Cyanobacteria have

developed different transport system and as in other bacteria these transport systems can be discriminated according to the energy source used for transport, the complexity of the transport machinery, or the chemical nature of the translocated substrates (Paulsen *et al.*, 1998). The transport system generally use the following example; (i) cyanobacterial porins as examples for diffusion-based import; (ii) ATP-binding cassette (ABC) transporters as examples of oligomeric transporters; (iii) the question of "Bayer bridges" acting as transport routes for the translocation of components within the cell wall (transmigration) and (iv) the junctional pore complex (JPC) as an example for a multicomponent export machinery (Hoiczyk and Hansel, 2000). Porins, which are nonspecific diffusion channels for small solutes (Benz and Bauer, 1988) and transport systems for substrates which are unable to diffuse passively through hydrophilic pores (Paulsen *et al*., 1998). In most gram-negative bacteria, porins are usually stable trimmers formed by polypeptides of 30 to 40 KDa (Schulz, 1993; Weiss *et al*., 1991). The porins characterized so far in cyaobacterial cell walls are bigger and composed of monomers of about 50 to 70 KDa (Hoiczyk and Hansel, 2000). Heterocyst differentiation is dependent with intracellular free Ca^{++} ion as shown in $BG11_0$ (without $CaCl_2$) medium. Thus, outer membrane, expected porins, might be up-regulated because there were not calcium in medium and formed heterocyst were induced. The outer membrane was expressed to prevent and maintain intracellular Ca^{++} in the cell for early heterocyst development.

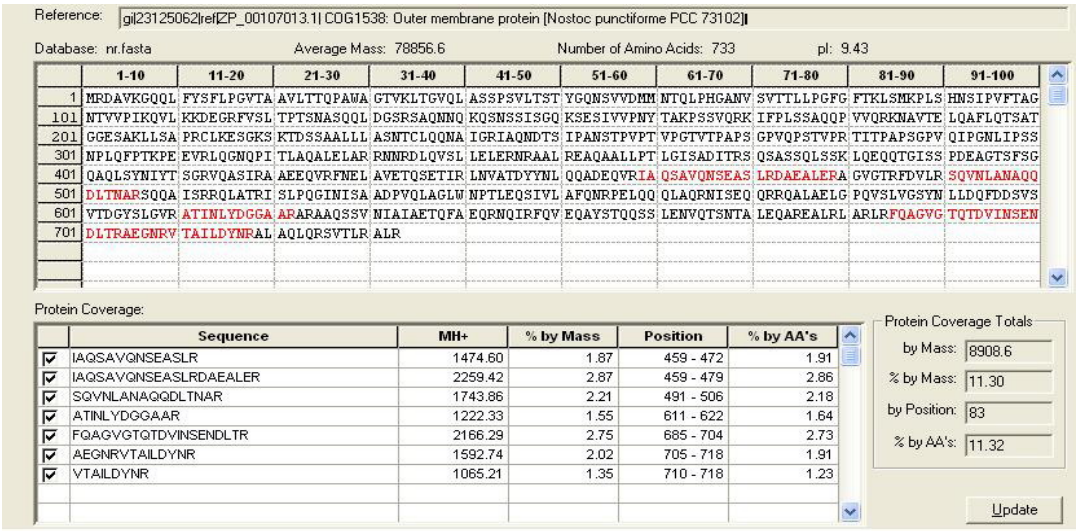


Figure 3.44 A 45 KDa was showed in $BG11_0$ (without $CaCl_2$) medium, showed 11.32% amino acid identity with outer membrane protein (*N. punctiforme* PCC73102).

3.6.9 Akinete cell and stress tolerant

Since akinete cells able to be induced under deprivation of $FeNH_4$ citrate and K_2HPO_4 medium and/or only K_2HPO_4 limitation (Fig. 3.45). Figure 3.45 showed akinete cells since 6 d, however, the number of akinete cells was too low when compared with the other conditions because of low growth rate when lack of K_2HPO_4 (Fig. 3.46). The results demonstrated that the number of akinete cell was high in condition with excess $FeNH_4$ citrate at 18 d and without FeNH4 citrate condition at 27-30 d. The number of cells in $BG11_0$ and $BG11_0$ (with excess FeNH4 citrate; 24 mg/l) were exponential increasing during 9-18 day. The akinete cell number of in $BG11_0$ (with excess $FeNH_4$ citrate; 24 mg/l) was higher than cells in $BG11_0$. Therefore, condition with excess $FeNH_4$ citrate was chosen for akinete production. Then, the *Nostoc* sp. strain VICCR1-1 was grown with shaking at 150 rpm under continuous

illumination 400 µE/m^2/s (24 mg/l) at 28±2ºC for 3-4 weeks in $BG11_0$ including excess $FeNH_4$ citrate.

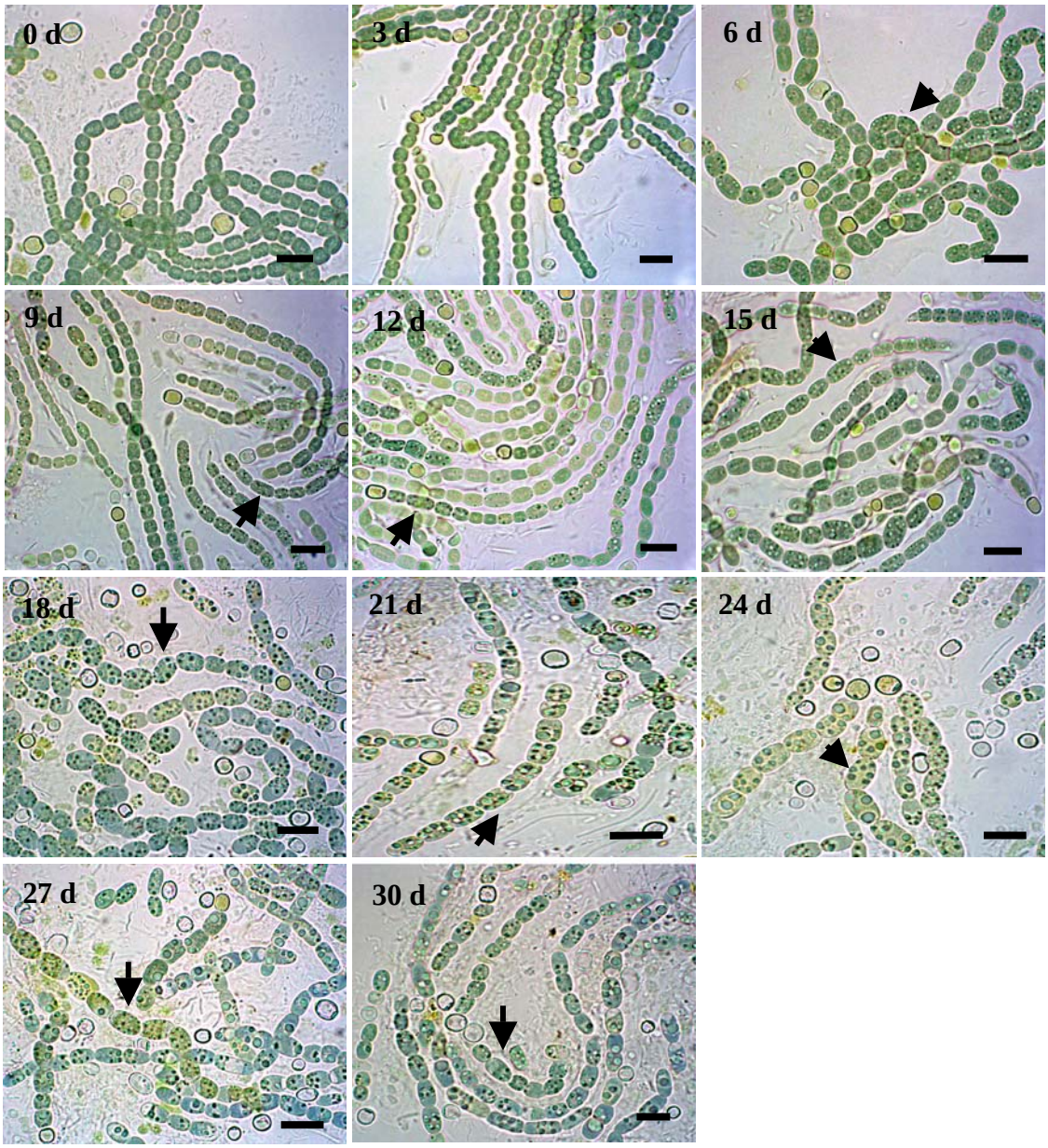


Figure 3.45 *Nostoc* sp. strain VICCR1-1 in P limitation every 3 days until 30 days (bar = 20 µm). The arrows showed represented akinete cell.

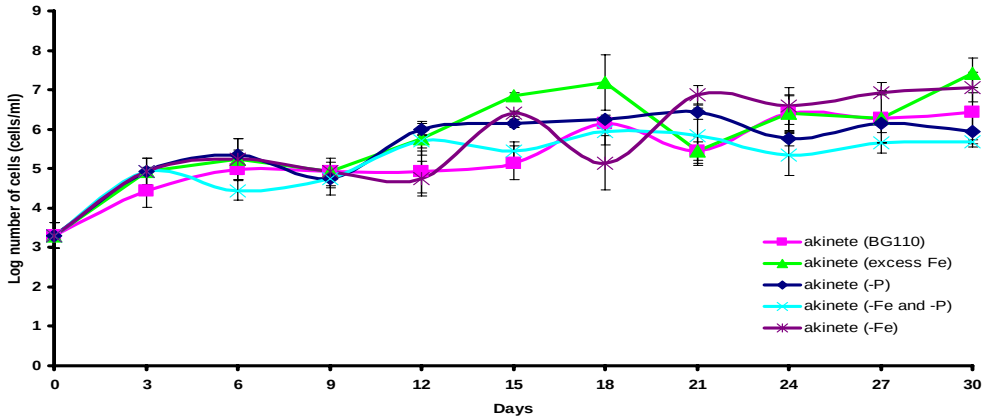


Figure 3.46 Comparison the number of akinete cells in various conditions.

The akinete cell inoculum size was adjusted to 5.1×10^5 cell/ml, then were transferred to test against various abiotic factors for 7 days. Then, the cells were enumerated in 5 ml of $BG11_0$ medium under continuous illumination 400 µE/m^2/s (24 mg/l) at 28±2ºC for 50 days based on 5- tube MPN method (Table 3.4)

Table 3.4. The akinete cells under stress tolerant conditions.

Conditions (7 days)	The amount of cells based on 5-tube MPN method (cells/ml)
Salt tolerant	
0.1 M NaCl	3.3×10^7
0.3 M NaCl	7.9×10^7
0.5 M NaCl	1.3×10^7
Acid and alkaline tolerant	
pH 3	2.0×10^5
pH 4	1.1×10^6
pH 9	2.7×10^6
pH 10	9.2×10^7
Thermotolerant	
40ºC	2.3×10^6
50ºC	No growth
Normal condition	4.9×10^6

The akinete cells were capable to tolerate against and growing well the high salt concentration as the cells were increased 2 magnitudes under $BG11_0$ consisted of

0.1 M, 0.3 M, and 0.5 M NaCl, comparison with normal condition (increased 1 magnitude; 4.9×10^6 cell/ml) (Table 3.4). However, the cell grew well under 0.3 M NaCl, this condition might suit for germination of this strain. Imashimizu and co-workers (2005) added 0.2 M NaCl to the culture medium and reported that cellular cAMP was rapidly increased in N_2-fixing cyanobacteria, *Anbaena* sp. PCC7120. It is postulated that the change in NaCl concentration was sensed by a specific signal-receptor histidine kinase, such as Hik33, which is located in the cytoplasmic membrane of *Synrochocystis* PCC6803 (Marin *et al*., 2003). The report concluded that NaCl induced expression of genes related to hetorocyst envelope formation in *Anbaena* sp. PCC7210, possibly via a CyaC-cAMP signal transduction system (Imashimizu *et al*., 2005).

For acid and alkaline tolerant, the number of cell at pH 4 and pH 9 were increased for 1 magnitude but not different from the cell in normal condition (Table 3.4). However, the cells were germinated well at pH 10, as increase up to 2 magnitudes. Besides, the cyanobacterial cells were dropped to about 2.0×10^5 cell/ml at pH3 when compared with the growth in normal condition ($BG11_0$). The optimal pH for cyanobacterial growth seems to range from 7.5 to 10.0 and the lower limit is about 6.5 to 7.0 (Holm-Hansen, 1968), however, the acid tolerant depended on species-specific cyanobacterial cell. Anand and co-workers (1990) reported that cyanobacteria are capable of shifting the pH of the external medium to suit its growth within 6 days in unbuffered medium. *Nostoc calcola* though unable to shift the pH in a buffered medium survived at pH 5 to pH 10 and is considered as alkalotolerant strain (Anand and Revathy, 1992).

The *Nostoc* sp. strain VICCR1-1 was not grow at 50ºC because the optimal temperature of cyanobacterial growth is about 30-35 ºC (Roger and Reynaund, 1979). However, this strain could grow at 40ºC and the cells able to increased up to 1 magnitude (Table 3.4).

The results suggested that akinete cell of the *Nostoc* sp. strain VICCR1-1 able to tolerate to salt (about 0.5 M NaCl), pH 4, 9 and 10 and high temperature as 40ºC. When environmental condition changes gradually, organisms can acclimate to the new conditions by sensing the changes and expressing a large number of previously unexpressed genes, with the resultant synthesis of large numbers of proteins and metabolites that are involved in protection against environmental stress (Murata and Suzuki, 2006). The general features of the plasma and thylakoid membranes of cyanobacterial cells are similar to those of the chloroplasts of higher plants in terms of lipid composition and the assembly of membranes. Therefore, cyanobacteria might be expected to sense as powerful model systems for studies fo the molecular mechanisms of the responses and acclimation to stress (Murata and Wada, 1995; Glatz *et al.*, 1999).

3.6.10 Cyanobacterial inoculum

The *Nostoc* sp. strain VICCR1-1 was induced to form akinete by cultivation in $BG11_0$ (excess $FeNH_4$ citrate) under continuous aeration at 28±2ºC with 12h/12h light/dark cycle with an average light irradiance of 400 $\mu E/m^2/s$ for 30 days. The akinete cells were harvested and stored in montmorillonite clay at room temperature (4.4×10^7 akinete cell/g montmorillonite clay). The amount of induced *Nostoc* sp. strain VICCR1-1 in akinete inoculum was enumerated compared with vegetative cells in montmorillonite clay, on the basis of five-tube MPN method every 4 week for 1

year (Fig. 3.47). The results demonstrated that *Nostoc* sp. strain VICCR1-1 could be maintained in the form of akinete cell in montmorillonite clay. The population number was found 4.0×10^6 cell/g montmorillonite clay at 0 d. The cells were dropped 1 magnitude within 4 weeks. Whereas the cells showed constant amount of cell until 24th week and trended to constant. Meanwhile, the number of cells was started with 4.0×10^6 cell/g montmorillonite clay, the cells then dropped at 16th week and still constant up to 56th week (3.3×10^5 cell/g montmorillonite clay).

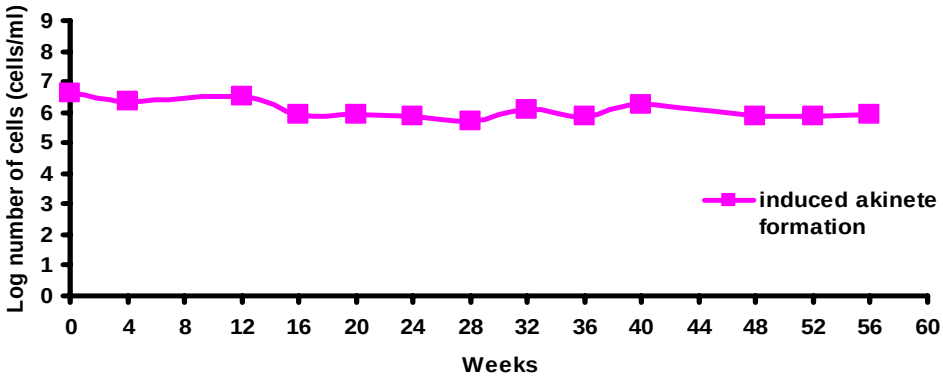


Figure 3.47 The number of akinete inoculum after storage at room temperature for 1 year.

Akinetes are considered as propagating or perennating, bodies exhibiting resistance to adverse conditions, mainly cold and desiccation (Herrero *et al*., 2004). Some metabolic activities such as CO_2 fixation are very low in akinete cells, the rate of respiration is often high (Adams and Duggan, 1999). Also, akinetes have been shown to make at least a few proteins, so that they seem to maintain, although low metabolic activity (Thiel and Wolk, 1983). Thus, *Nostoc* sp. strain VICCR1-1 which were induced to be akinete formation could maintain in montmorillorite clay for long time (Fig. 3.47). Since, montmorillonite clay was not suitable for cyanobacterial growth but

easy to dry, thus the akinete cells were not germinated (contains SiO_2 = 56.60%, Al_2O_3 = 16.18%, FeO_3 = 5-7%, Na_2O = 2.4-3.0%, MgO = 1.5-2.0%, K_2O = 0.3-0.5% and TiO_2 = 1.2-1.5%) (Umpush and Kiattikomol, 2004), whereas prompted to germinate under favorable conditions. In addition, the cyanobacterial cells could not survive in montmorillonite clay, thus the cells might be degraded during maintain in that clay. The results suggested that induced akinete cells maintain in montmorillorite clay were suitable for apply as cyanobacterial inoculum.

3.6.11 Enhancement of rice production by cyanobacterium inoculum

O. sativa Pathumthani-1 rice variety was planted in rice field trials (SUT farm). The cyanobacterial inoculum was inoculated in rice fields to enhance in rice production. There were two crops during October 2004 to February 2005 (1^{st} crop) and during May 2005 to September 2005 (2^{nd} crop). The treatments consisted of; i) rice without nitrogen fertilizer (control), ii) rice with chemical fertilizer recommend rate for rice (12-6-4 kgN-P_2O_5-K_2O/Rai), iii) rice with vegetative cell of *Nostoc* sp. strain VICCR1-1 (without montmorillonite clay), iv) rice with akinete inoculum, (v) rice with montmorillonite clay (without *Nostoc* sp. strain VICCR1-1 and (vi) rice with akinete inoculum amendment (treatment v and vi were provided only 2^{nd} crop). Montmorillonite clays inoculated with cyanobacteria and incorporated into rice paddy under field conditions significantly increased rice production in terms of grain yield (Table 3.5).

The 1st crop, the numbers of rice and panicle per a clump of rice showed (columns 2 and 4) no significant in plots 2 and 4, while the control no supplied N was significantly lower than the other treatments. The seeds (column 6) from adding vegetative cell (row 3) and akinete inoculum (row 4) were significantly higher than the treatment with and without N fertilizer, respectively. The grain yield was observed and found that using cyanobacteria (both vegetative cell and akinete cyanobacterial inoculum) showed not significantly different with N-fertilizer application. However, they were highly significant different to control (column 8). Also, Hall and co-workers (2002) used immobilized cyanobacteria in rice field and found that the grain yield of immobilized *Anabaena* showed similar to N-fertilizer plot. The 2nd crop, There were not significant difference between the plots supplied with N fertilizer, akinete inoculum, added akinete cyanoacterial inoculum in 1st crop and only momtmorillonite clay in terms of the numbers of rice and panicle per a clump of rice. Interestingly, the field that obtained vegetative cells was not significant difference with control not supplied N fertilizer. The seeds were the highest at rows 4 and 5 which related to adding akinete inoculum and showed no significant with the other treatment. Likely the seeds, the grain yield was also high when the plots supplied akinete inoculum and higher than that of N fertilizer supplement. While the control treatment (row 1) was similar to using vegetative cells (row 3). The plot supplied only montmorillonite clay (row 6) showed that the montmorillonite clay alone has no effect to the rice and grain yield.

The increase of grain yield in each treatment (%) was compared with grain yield of no N-fertilizer treatment (columns 10 and 11). The results showed that 1st crop had the highest of % increase grain yield in rice with vegetative cells (row 3) and

the higher in rice with N-fertilizer (row 2) than rice with akinete inoculum (row 4). This might because akinete inoculum needed the time for germination, thus using vegetative cells showed % increase grain yield higher tha using akinete inoculum. In contrast, % increase grain yield in 2nd crop was lower than 1st crop, especially in rice with vegetative cells showed the lowest % increase of grain yield (1.51%). The grain yield was increased about 46.24% and 49.46% in akinete inoculum and added akinete inoculum in 1st crop treatments, which were higher than using N-fertilizer (37.63%). However, % increase grain yield in rice with only montmorillonite clay was 15.05% which was not relate to grain yield when use akinete inoculum. The grain yield of each treatment was compared with N-fertilizer treatment (columns 12 and 13). The results indicated that the grain yield in most treatments was highly decreased, there were increasing of grain yield about 7.29% when rice grew with vegetative cells in 1st crop, 6.25% and 8.59% in 2nd crop when rice grew with akinete inoculum and added akinete inoculum in 1st crop, respectively. On the other hand, Mishra and Pabbi (2004) reported about the effect of cyanobacterial biofertilizer inoculation on rice yield at a farmer's field. The grain yield was increased about 12.26%-19.48% which was different from thise research. Mishra and Pabbi (2004) studied at a farmer's field area about 4-9 ha, whereas, this research carried out area about 0.002 ha (20 m^2/treatment). Thus % increase grain yield in this research seemed to over than Mishra and Pabbi's information (2004).

Since the rice grew well in 2nd crop because it was raining period. Thus, the leaves might cover the rice field, then the light was insufficient for photosynthesis of cyanobacteria. So that, the data from vegetative cells treatment was not different from control. Surprisingly, the treatment plot which has ever added akinete inoculum in 1st

crop but the 2nd plot was not added both N-fertilizer and akinete inoculum, still showed high grain yield. Therefore, the results suggested that the cyanobacterial cells were remained in 2nd crop and led to produce high grain yield. The 2nd crop showed higher rice growth and grain yield than 1st crop, this might because the seasons were different (1st crop: winter/dried season and 2nd crop: rainy season. The arid soils (mostly found in winter/dried season) are probably very inhospitable to many microorganisms including plants because the temperature is high and water is severely limited (Roger and Reynaud, 1982). The paddy soils provided a favourable environment for the growth of cyanobacteria with respect to their requirement for light, water, high temperature and nutrient availability. This could be the reason cyanobacteria highly grow abundance in paddy soils than in arid soils (Watanabe and Yamamoto, 1971). Thus, the rice growth and grain yield in 2nd crop showed higher than 1st crop. The akinete inoculum (row 4) was demonstrated high rice growth and grain yield in both crops. Therefore, the akinete inoculum could be used in both seasons.

3.6.12 Cyanobacterial population in rice field

The cynobacterial populations in soils were monitored during rice cultivation and post-harvesting in 1st crop by combination of five-tube MPN method, Acetylene reduction activities (ARA) and denaturing gradient gel electrophoresis (DGGE). The results were showed in Table 3.6.

Table 3.6. The estimation of population of *Nostoc* strainVICCR1-1 in rice field.

Treatments	During rice cultivation (cfu/m^2)			Post-harvesting (cfu/m^2)		
	MPN*	ARA*	DGGE*	MPN*	ARA*	DGGE*
1	1.00×10^8	2.85×10^7	1.02×10^5	9.20×10^6	8.10×10^6	N.D.
2	8.82×10^7	1.94×10^7	1.00×10^7	5.90×10^7	2.33×10^7	1.02×10^7
3	1.67×10^8	1.45×10^8	2.04×10^7	2.42×10^7	1.89×10^7	2.50×10^5
4	1.16×10^8	5.52×10^7	1.76×10^5	1.15×10^8	4.15×10^7	1.62×10^6

1: no N fertilizer (control), 2: N fertilizer, 3: vegetative cells and 4: akinete inoculum

Bacterial population (initiation) 1.0×10^8 cell/g of soil

*MPN was detected by observation of cyanobacteria in the tubes. For ARA and DGGE, the both cultures were derived from 5- tube MPN method. The tubes which showed nitrogenase activity (ARA) and/or band similar to the *Nostoc* sp. strain VICCR1-1.

The soil properties were 1.86% organic matter, 15.62 ppm P, 56.3 ppm K and pH 6.59. The indigeneous bacterial populations in soils were about 8.47×10^6 cfu/g soil (aerobic condition) and 1.67×10^6 cfu/g soil (anaerobic condition). The akinete cells were inoculated in montmorillonite clay in amount of 4.4×10^7 cfu/g montmorillonite clay and supplied in the field about 2.8×10^6 cfu/m^2. While the cyanabacterial cells were supplied directly in the field in amount of 1.05×10^7 cfu/m^2. The cyanobacterial cells were found about 10^5 cfu/g soil during rice cultivation and post-harvesting by enumeration with five-tube MPN method (columns 1 and 4, Table 3.6). Each tube was detected nitrogen fixation ability by ARA and most conditions showed not different with five-tube MPN method. However, genomic DNA from appeared cyanobacteria in MPN method, were extracted and run on DGGE based on the analyses of the 16S rRNA (Fig. 3.48). PCR products that have a same length can be separated by DGGE on the basis of malting domain structure and nucleotide composition (Fischer and

Lermann, 1983; Kogure *et al.*, 1980). DGGE is a widely used tool for screening microbial community dynamics in large numbers of samples and environments yielding diversity patterns as well as phylogenetic information (Muyzer, 1999). This technique can be used to assess the genotypic diversity in environmental samples and to judge the purity and uniqueness of isolated strains. Isolated cultures can be assigned to field populations based on the comparison of their DGGE profile and sequence information from profile bands can be used to characterize the organisms that are present (Janse *et al.*, 2003). The DGGE profiles from soil samples were compared with DGGE profiles of *Nostoc* sp. strain VICCR1-1. The cells were still steady in plots supplied N fertilizer and added akinete inoculum (rows 2 and 3). Besides the control not supplied N fertilizer and provided directly vegetative cells, were decreased to about 10^2 cfu/g soil during rice cultivation. Post-harvesting, the number of cyanobacterial cells absented in control, constant the number of cell in N fertilizer and stepped down about 10^3 cfu/g soil in both plots contained vegetative cells and akinete inoculum. Teske and co-workers (1996) also investigated the sulfate-reducing bacterial populations of a stratified marine water column, Mariager Fjord, Denmark by DGGE based on 16S rRNA parallel to MPN and reported that both strategies are in fact highly valuable indicators of real phenomena: the highly differentiated pattern of bacterial activity, the abundance of uncultered bacteria.

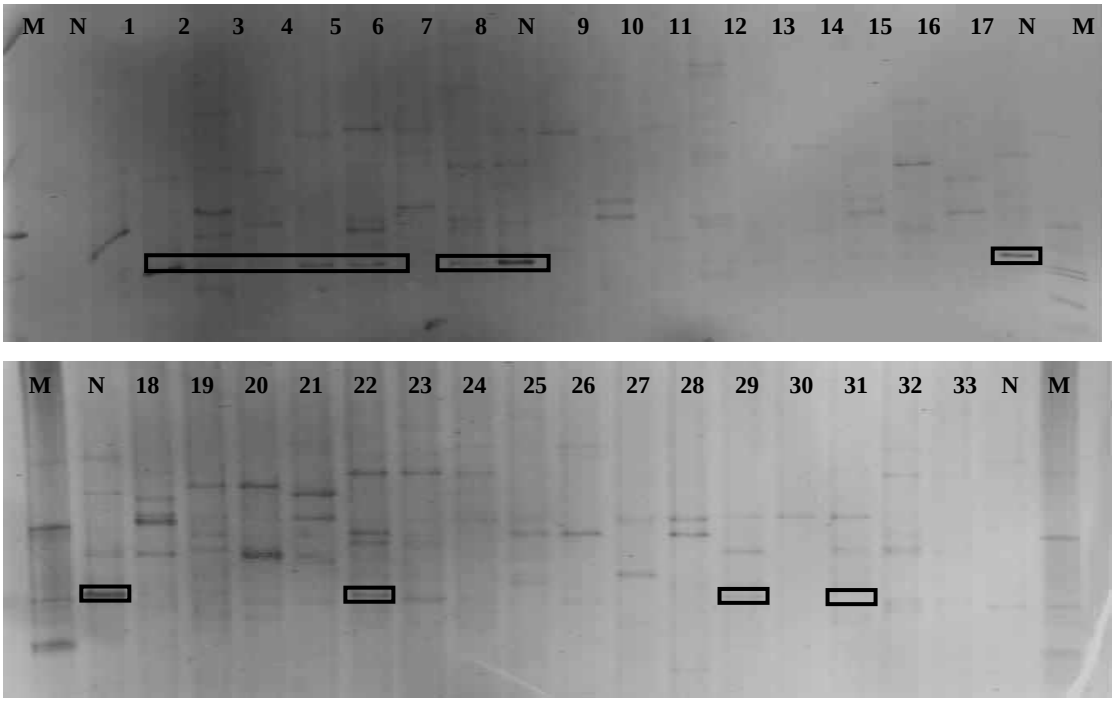


Figure 3.48 The representation of MPN-DGGE band profiles based on 16S rRNA. Lane M; Marker; lane N; *Nostoc* sp. strain VICCR1-1, lane 1; B3-3 (10^7), lane 2; B3-2(10^7), lane 3; B3-1 (10^7), lane 4; B3-2 (10^5); lane 5; B3-1 (10^5), lane 6; B3-3 (10^4), lane 7; B3-2 (10^4), lane 8; B3-1 (10^4), lane 9; B2-2 (10^5), lane 10; B2-1 (10^5), lane 11; B2-3 (10^4), lane 12; B2-2 (10^4), lane 13; B2-1 (10^4), lane 14; B1-2 (10^6), lane 15; B1-3 (10^4), lane 16; B1-2 (10^4), lane 17; B1-1 (10^4), lane 18; M5-2 (10^4), lane 19; M5-1 (10^4), lane 20; M4-2 (10^5), lane 21; M4-1 (10^5), lane 22; M4-3 (10^4), lane 23; M4-2 (10^4), lane 24; M4-1 (10^4), lane 25; M3-1 (10^5), lane 26; M3-3 (10^4), lane 27; M3-2 (10^4), lane 28; M3-1 (10^4), lane 29; M2-3 (10^5), lane 30; M2-2 (10^5), lane 31; M2-1 (10^5), lane 32; M2-3 (10^4), lane 33; M2-2 (10^4). B: plot supplied cyanobacterial cell and M: plot supplied cyanobacterial inoculum. The boxes showed band product were similar to *Nostoc* sp. strain VICCR1-1.

The results suggested that *Nostoc* sp. VICCR1-1 was found in all treatments because this strain was isolated from rice in rotation with other crops. This strain was found in amount 2.5×10^5 cfu/m^2 and 1.62×10^6 cfu/m^2 in treatments 3 and 4, respectively. The cells still remained in the field after harvesting the yield about 1.0×10^7 cfu/m^2 for plot supplied N fertilizer. This might be due to the plot supplied N fertilizer had more nutrient than the other plots. Furthermore, the cyanobacterial inoculum (akinete form) could survive in the environment better than cyanobacterial cells (vegetative form). In treatment 1 (control: no N fertilizer) there were not found *Nostoc* sp. strain VICCR1-1 after harvesting, this might because fluctuating during cultivation and harvesting. The results implied that cyanobacterial cells able to apply as biofertilizer in the rice field, especially in form of akinete inoculum.

3.7 Conclusions

The 853 isolates of N_2-fixing cyanobacteria obtained from soil samples collected from different ecosystems including mountainous, forest and cultivated areas in the central, northern and northeastern region of Thailand were examined (Chunleuchanon *et al*., 2003). The *Nostoc* sp. strain VICCR1-1 was isolated from rice in rotation with other crops cultivation showed the highest nitrogen fixation efficiency about 11 μmol C_2H_4/h/mg total chlorophyll a. The database of 16S rRNA from this strain was aligned with BLAST search and showed 92% identity with *Nostoc entophytum* and *Nostoc* sp. PCC9231. Then, the vegetative cells of *Nostoc* sp. strain VICCR1-1 were induced in order to form heterocysts and akinetes on basis of nutrient modification. Absence $CaCl_2$ was played the role of heterocyst differentiation and showed able to induced about 46.61%. The lack of $CaCl_2$ condition was confirmed the

influence to heterocyst development by changing the medium. The heterocysts were induced up to 62.59% when transferred the cyanobacterial cells from BG11 to $BG11_0$ (without $CaCl_2$) medium. Phosphorus and iron were found to be the critical composition in akinete differentiation, especially lack of both elements. The number of akinete cells could be increased up to 21.17% when compared with culturing in normal condition ($BG11_0$ medium). The gene expression which involved heterocyst and akinete differentiation was observed. Although *het*R is the master of heterocyst induction, however, the results suggested that only *het*R expression could not be the indicator for heterocyst development particularly for *Nostoc* sp. strain VICCR1-1. Whereas *het*R also related to akinete cell, the two cell types may share a common ancestor. *sod*F and *ava*K expression were also provided for detect during akinete differentiation. However, the number of akinetes might not sufficient for detection. Furthermore, the total proteins were extracted and compared from three conditions such as: i) BG11 medium, ii) $BG11_0$ medium and iii) $BG11_0$ (without $CaCl_2$). There were 72 KDa and 140 KDa which expected to be chaperonin GroEL (HSP60 family) and phycobilisome core-membrane linker protein. Besides 45 KDa (expected to be outer membrane protein, porins) was up-regulated only when growth in $BG11_0$ (without $CaCl_2$) medium.

Akinete cell germination was tested under various stress conditions. They can well germinate under the broad range of pH from 4 to 10, at high temperature as 40ºC and high salinity as 0.5 M NaCl. In order to prepare akinete inoculum, it was homogeneously mixed with montmorillonite clay at 4.4×10^7 cfu/g montmorillonite clay. The akinetes could survive in the montmorillonite clay in constant number until 1 year. To test the effect of *Nostoc* sp. as biofertilizer with rice plantation, inoculum

was applied in amount 2.8×10^6 cfu/m^2. After harvesting, the grain yields from chemical-N fertilizer, cyanobacterial cells and cyanobacterial inoculum treatments were not significantly different. To monitor the persistence of *Nostoc* sp. strain VICCR1-1 after harvesting, the MPN-DGGE technique using 16S rRNA gene was employed. The results indicated that the remaining population is at 1.0×10^7 cfu/m^2, 2.5×10^5 cell/m^2 and 1.62×10^6 cell/m^2 in treatments supplied N-fertilizer, vegetative cells and akinete inoculum, respectively. Thus, this approach might be useful to produce N_2-fixing cyanobacteria inoculum in the form of akinete and apply as biofertilizer in rice cultivation system.

3.8 References

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