EFFECTS OF LIGHT AND SALINITY ON PIGMENT CONTENT IN, AND SPECTROSCOPIC DISCRIMINATION OF, FILAMENTOUS CYANOBACTERIA ISOLATED FROM SALINE SOIL IN NAKHON RATCHASIMA, THAILAND

Ms. Somchanh Bounphanmy

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Environmental Biology

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อิทธิพลของแสงและความเค็มที่มีต่อปริมาณของสารสี และการวินิจฉัยเชิงสเปกโทรสโกปีแบคทีเรียสีเขียวแกมน้ำเงินแบบเส้นสายแยกจากพื้นที่ดินเค็มในจังหวัดนครราชสีมา ประเทศไทย

นางสมจัน บุญพันมี

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
สาขาวิชาชีววิทยาสิ่งแวดล้อม
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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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การศึกษาครั้งนี้ประกอบด้วยสองส่วนที่สำคัญ คือ การทดสอบอิทธิพลของแสงและความเค็มต่อปริมาณสารสีของแบคทีเรียสีเขียวแกมน้ำเงินแบบเส้นสาย จากพื้นที่ดินเค็มอำเภอคง พิมาย และโนนสูง จังหวัดนครราชสีมา ประเทศไทย ในส่วนที่สองเป็นการใช้ FTIR สเปกโทรสโกปีเพื่อวินิจฉัยแบคทีเรียสีเขียวแกมน้ำเงินบนพื้นฐานคลื่นแสงอินฟราเรด ATR-FTIR สเปกโทรสโกปีถูกนำมาใช้เป็นครั้งแรก เพื่อวินิจฉัยลักษณะของแบคทีเรียสีเขียวแกมน้ำเงินบนดินเค็ม การศึกษาเปรียบเทียบความชุกชุมของแบคทีเรีย ลักษณะและความเค็มของดิน ดำเนินการในดิน 30 ชุด ความชุกชุมของสาหร่ายสีเขียวแกมน้ำเงินที่ค่าต่ำในดินทรายและมีความเค็มสูง สาหร่ายสีเขียวแกมน้ำเงิน 7 ชนิด ถูกแยกให้ปลอดเชื้อและเลี้ยงในห้องปฏิบัติการ โดย 5 ชนิด ถูกนำมาทดสอบอิทธิพลของแสงและความเค็มต่อปริมาณสารสี พบว่าทุกชนิดมีความคงทนต่อสี่ระดับต่ำแรกที่ทดสอบ (0 0.5 1.0 1.5 และ 2.5 กรัม NaCl/ลิตร) และความคงทนต่อสามระดับต่ำแรกของความเข้มข้นแสง (20 40 60 และ 80 µmol $m^{-2}s^{-1}$) ไม่มีผลต่อการเติบโตของสาหร่ายสีเขียวแกมน้ำเงินในระยะแรก แต่ใน *Phormedium laminosa*, *Phormedium* sp และ *Oscillatoria limosa* การเติบโตเพิ่มขึ้นในสองระดับแรกของความเข้มข้นของแสง ซึ่งเกิดในระยะสุดท้ายของการเติบโต เมื่อเลี้ยงในระดับความเข้มแสง 80 µmol $m^{-2}s^{-1}$ เป็นเวลานานจะมีผลทำให้ปริมาณของคลอโรฟิลล์ลดลงในทุกชนิด ความเค็มสูงสุดมีผลยับยั้งการเจริญเติบโตทุกชนิด ยกเว้น *Oscillatoria limosa* ส่วน *Scytomena javanicum* ระดับคลอโรฟิลล์เอ เพิ่มขึ้นใน 20 µmol $m^{-2}s^{-1}$ และปริมาณบีตาแคโรทินมีค่าสูงสุด ในระดับความเค็ม 1 กรัม NaCl/ลิตร สาหร่ายสีเขียวแกมน้ำเงินทั้ง 7 สายพันธุ์ได้นำมาศึกษาอินฟราเรดสเปกโทรสโกปี มากกว่า 700 คลื่นแสง โดยวัดคลื่นแสงในช่วงระยะแรกระยะกลาง และระยะสุดท้ายของการเติบโต เพื่อศึกษาการเปลี่ยนแปลงองค์ประกอบของแมโครโมเลกุลของเซลล์ในช่วงการเติบโต และการเปลี่ยนแปลงนี้อาจมีผลต่อการจัดจำแนกพื้นฐานข้อมูลจากคลื่นแสง เทคนิคทางสถิติและ Principal Component Analysis (PCA) ถูกนำมาใช้เพื่อแปลข้อมูลของระบบ Systematic variance ในระบบข้อมูลของคลื่นแสง ข้อมูลถูกจัดแบ่งเป็นสองกลุ่ม คือ Training และ Testing โดยกลุ่ม

แรกถูกนำมา สร้าง PCA ของแต่สายพันธุ์ แล้วถูกนำไปใช้เพื่อจัดจำแนกคลื่นแสงในชุดหลัง โดยวิธี Chemometric คือ Soft Independent Modeling by Class Analogy (SIMCA) ผลที่ได้แสดงให้เห็นว่าวิธีทางสเปกโทรสโกปี เป็นวิธีที่มีความแม่นยำสูงและรวดเร็ว ในการนำมาใช้เพื่อจัดจำแนกแบคทีเรียสีเขียวแกมน้ำเงินบนดินเค็มโดยไม่มีการทำลายเซลล์

สาขาวิชาชีววิทยา
ปีการศึกษา 2547

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SOMCHANH BOUNPHANMY : EFFECTS OF LIGHT AND SALINITY ON PIGMENT CONTENT IN, AND SPECTROSCOPIC DISCRIMINATION OF, FILAMENTOUS CYANOBACTERIA ISOLATED FROM SALINE SOI IN NAKHON RATCHASIMA, THAILAND. THESIS ADVISOR : ASSOC. PROF. SOMPONG THAMMATHAWORN, Ph.D. 168 PP. ISBN 974-533-428-6

CYANOBACTERIA/PIGMENT/SPECTROSCOPY/FTIR/ATR/PCA/SIMCA

There are two main aspects to the dissertation. The first involves studying the distribution of filamentous cyanobacteria found in the saline soil at the Ampur Khong, Pimai and Ampur Nonsung, Nakhon Ratchasima province, Thailand and examining the effects of varying photon fluxes and salt concentration on some isolated strains. In the second part FT-IR spectroscopy was explored as means of rapidly discriminating the isolated filamentous cyanobacteria based up on their IR spectra. Specifically, Attenuated Total Reflectance (ATR), Fourier Transform Infrared (FTIR) spectroscopy was used for the first time to characterize terrestrial cyanobacteria. There were thirty study sites covering a wide range of soil types and salinity levels. Patterns of soil salinity, soil types and abundance of cyanobacteria were compared. Cyanobacteria appeared to be less abundant when soil had a high sand content and were more saline. Seven filamentous strains were isolated and cultured in the laboratory. Five were used in experiment investigating the effects of photon fluxes and salinity on growth and pigment content. All strains appeared to be tolerant of the four lowest salinity level in the range applied (0, 0,5, 1,0, 1,5, 2,5 g NaCl L^{-1}) and to the three lower photonfluxes (20,40, 60 and 80 μmol $m^{-2}s^{-1}$). There was no effect on initial growth rates in these

treatments, however in *Phormidium laminosum*, *Phormidium* sp. and *Oscillatoria limosa* growth rate increased under the lower two photon fluxes in the late exponential phase of growth. Prolonged exposure to photon fluxes of 80 μmol $m^{-2}s^{-1}$ appeared to result in chlorosis in all species. The highest salinity inhibited growth in all species except *Oscillatoria limosa*. In *Scytonema javanicum* chlorophyll *a* levels were elevated at the lowest photon fluxes and β-carotene levels were highest at salinity of 1gNaCl. L^{-1}. All seven strains were used for the IR spectroscopy study. More than 700 absorbance spectra were acquired. Spectra were acquired at early, mid and late exponential phases to study the change in the macromolecular composition of the cells during growth and how this might affect classification based on spectra information. The multivariate statistical techniques Principal Component Analysis (PCA) was used for the extraction and interpretation of the systematic variance in the spectral data set. The data set was divided into training and testing sets. The training sets were used to develop PCA models representing the cyanobacterial strains and these were used to classify spectra from the testing sets using a chemometric method known as Soft Independent Modeling by Class Analogy (SIMCA).The results showed that spectroscopic method has the potential to be used as an accurate, rapid and non-destructive means of classifying terrestrial cyanobacteria.

School of Biology

Academic Year 2004

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Co-advisor's Signature _____

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Somchanh BOUNPHANMY

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LIST OF ABBREVIATIONS

ATR	Attenuated Total Reflectance
CB	Cyanobacteria
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
FTIR	Fourier transform Infrared spectroscopy
PCA	Principal Component Analysis
PC	Principal component
SIMCA	Soft Independent Modeling by Class Analogy
VS	Versus
^{o}C	Degree Celsius
EC	Electro conductivity
EDTA	Ethylene diamine tetraacetic acid
et al	et alia (and others)
g	gram
β	Beta
(m,μ)L	(mili, micro)Liter
μmol	micro mol
UV	Ultraviolet
ppm	part per million
pp	page
%	percent

LIST OF ABBREVIATIONS (Continued)

mm	millimeter
mL	milliliter
cm	centimeter
nm	nanometer
HPLC	High Performance Liquid Chromatography
h	hour
g/L	gram per liter
conc.	concentrated

CHAPTER I

INTRODUCTION

1.1 Significant of the study

Cyanobacteria are ancient and diverse group of photosynthetic, nitrogen fixing microorganisms, which inhabit many different and extreme environments. The diverse distribution of cyanobacteria indicates a high degree of biological adaptation, which has enabled them to thrive and complete effectively in nature (Burja *et al*., 2002). As they fix nitrogen cyanobacteria have been used for the reclamation of barren and alkaline soil acting as a biofertilizer and organic manure (Babu, Joshi,and Viswanathan, 1998; Malam Issa *et al*., 2001). Algae, including cyanobacteria provide organic food to the marine food web and perform over 90% of the total photosynthetic activity, and account for about 50% of total planetary primary productivity (Babu, Joshi,and Viswanathan, 1998). Besides their conversion of atmospheric nitrogen into a form that can be utilized by plants and other microorganisms, cyanobacteria play an important role in reducing soil erosion by helping bind the particles of soil together, increasing the soil organic matter content and in producing certain substances which enhance the growth of higher plants (Gopalaswamy and Kannaiyan, 2002). Nitrogen-fixing cyanobacteria are being used sporadically as a supplement to chemical nitrogen fertilizers for rice cultivation in rice-growing countries, including India and Bangladesh. Cyanobacterial biofertilizers may reclaim the problem soils such as acid soils and saline soils, improve the fertility status and may supplement 25-35%

nitrogen for rice cultivation in these soils and this kind of biofertilizer may be used in improving the soil environment (Hashem, 2001). In the last decade, the utilization of microbial sources, particularly microalgae for the production of feed, food, food additives, pharmaceuticals, and fine chemicals is growing in importance (Wood *et al*., 1999). Carotenoids, a photosynthetic pigment of plant and algae, have gained outstanding importance in the food and pharmaceutical areas. Apart from being essential for plant and algae growth and photosynthesis, in recent years carotenoids have become used extensively as safe, natural colorant for food, feed, and cosmetics, and are also the main source of vitamin *a* for human consumption (Britton, 1991). In the developing countries vitamin *a* deficiency is still a problem. According to an estimate of the World Health Organization (FAO, 1999), 250000 – 500000 children go blind every year due to deficiency of vitamin *a*. Moreover, interest in carotenoids has recently focused on their importance in physiology and their potential use in cancer prevention, as many studies confirm their anti-oxidative properties and particularly the positive effects on the health status of elder people. Many observations have been published with regard to the antioxidant activity of carotenoids such as: inhibition of mutagenesis (Alasalvar *et al.,* 2005), Chidambara *et al*., 2005); enhancement of the immune response (Oliver and Palou, 2000); and inhibition of the tumor development (Palozza *et al*., 2001; Del Campo *et al*., 2000; Oliver and Palou, 2000). Because microalgae can synthesize a large amount of carotenoids a considerable amount of research has been carried out, mostly during the last 15 years, on the possibility of growing cyanobacteria, species on an industrial scale for health food products. This work has largely focused on the genus *Spirulina,* which are cultivated in large open-air ponds in several countries (Jones, 1993*)*

There are many factors limiting cyanobacterial growth including physical, chemical and biological ones (Pilar, Susana and Jorge, 2001). Light and salinity are the major factors depressing growth of cyanobacterial cells. Cyanobacteria require relatively low photon flux densities to utilize sunlight for photosynthesis, while in the nature these species are vulnerable to the exposure of intense sunlight including UVB, long wave ultraviolet radiation (UV-A radiation), visible radiation and heat (Babu, Joshi and Viswanathan, 1998). High salinity in soil and in aqueous environment is an important physical factor that affects growth and survival of living organisms (Aran and Aphichart, 2003). Inhibition of cyanobacteria by salinity may be mediated by inhibition of nitrogenase activity (Mahmoud *et al*., 2003). However, the response of cyanobacteria to salinity differs from one strain to another. Presumably there is a large range of optimal salinity conditions from freshwater strains to saltwater strains to hyper-saline strains. Adaptation to different salinities may occur developmentally via osmoregulation or evolutionarily via modification of the enzymes of the photosynthetic apparatus (Telor, 1979).

Nakhon Ratchasima province is situated in the northeastern part of Thailand, and has the largest area of salinity-affected agricultural land in the country, which is the cause of low productivity or non-productivity particularly in highly terraced areas. A number of research programs on ecology of cyanobacteria in Thailand and elsewhere indicates that the paddy field ecosystem provide a suitable environment for growth of cyanobacteria. Moreover, some strains of cyanobacteria in Thailand have found to be salt tolerant with a high ability for nitrogen fixation (Lavan, 1990), which makes them potentially suitable candidates for use as a source of biofertilizers in agriculture in the salt-affected areas.

Utilisation of microalgae is often limited because specific strains are difficult to identify. Up until now, cyanobacterial identification and classification has mostly relied upon morphological characterization and genetic methods. In the case of many algae, and in particular the cyanobacteria, morphological features are very limited, requiring the researcher to resort to time consuming and expensive genetic methods. The development of an inexpensive, rapid and non-destructive method of molecular composition measurement in algae would be useful and have significant commercial value, and other purposes (Kansiz *et al*., 1999). In the past five years, a number of research groups have conducted a series of projects and tested the applicability of the spectroscopic technique to characterize prokaryotic organisms (Lasch, Boese, Patrico, and Diem, 2002). Kirschner and Naumann (2003) observed that in the last decade, several papers were published on the use of Fourier-transform infrared (FT-IR) spectroscopy as a means of rapidly differentiating and identifying microorganisms. Most of these studies concerned themselves with identifying of discriminating human pathogens, but the study of Kansiz *et al*. in 1999 demonstrated that FT-IR spectroscopy, in conjunction with chemometrics methods, can consistently discriminate between cyanobacterial at the strain level. This study extends the approach to soil-born cyanobacteria but uses a different methodology (Attenuated Total Reflectance FTIR spectroscopy; ATR FT-IR) to that employed by Kansiz *et al*. (1999), which is easier and more rapid and less expensive because an FTIR microscope is not required.

1.2 The objectives of the study

This research aims to study the distribution of cyanobacteria in different soil types ; to investigate effects of light and salinity on pigment, and on growth rate of filamentous cyanobacteria. Another important objective of the study is to use the spectroscopic methods combined with chemometrics methods as a new tools to discriminate between cyanobacterial strains. The detail objectives are delineated below:

1. To investigate the distribution of cyanobacterial strains in different soil types and salinity levels;

2. To investigate the effects of light and salinity on growth rate, pigments, particularly chlorophyll a and β-carotene in the cell;

3. To apply a new spectroscopic methods, Attenuated Total Reflectance FT-IR spectroscopy, in combination with the chemometrics methods, PCA and SIMCA in discrimination cyanobacterial strains.

1.3 The scope of study

1.3.1 The study areas

The field work of research had been performed in the Nakhon Ratchasima province which has the most diverse range of saline soils on the northeast part of Thailand. The sampling points were randomized in three Ampurs/ Districts (Ampur Nonsung, Ampur Pimai and Ampur Khong) within 10 villages as shows in the Table 1.

1.3.2 The experimental approach

The research has been carried out with attention to the following observations, experiments, and analyses:

1. collection and analysis of soil samples to investigate the effect of different soil types and salinities on the distribution and abundance of cyanobacteria;

2. identification of collected cyanobacteria from sampling points by conventional (morphological) methods;

3. experimental manipulation of light and salinity in cultures of five strains of filamentous cyanobacteria representing two filamentous non heterocystous genera, *Oscillatoria* and *Phormidium*, the heterocystous genus *Nosto*c, and a species of the branching genus *Scytonema*, and measurement of growth rate and chlorophyll a and β-carotene concentrations;

4. identification of cyanobacteria using spectroscopic, Attenuated Total Reflectance FT-IR, and chemometrics, PCA and SIMCA methods.

1.3.3 Sampling times

1. The field work was taken three times: during dry season on March 28, 2003, beginning of the rainy season on May 25, 2003 and end of the rainy season on October 15, 2003.

2. Samples for measurement of growth rate and chlorophyll were taken every two days continuously from the first day of culture until stationary growth phase.

3. Samples for measurement of β-carotene were taken at the middle of exponential growth phase of experimental species.

4. Samples for spectroscopic and chemometric analysis were collected at early, mid, and late exponential growth phase. Multiple samples were taken to determine whether growth phase was confounding to the analysis.

1.4 Expected results

1. The correlations between abundance and taxonomic composition of terrestrial communities of cyanobacteria and soil salinity level were investigated. We tested the hypothesis that soils with low to moderate salinity have greater diversity and abundance of filamentous cyanobacteria than saline soils. We also predicted that the taxonomic composition of the cyanobacterial community would vary with salinity and that the most saline soils would be inhabited by specialized salt tolerant strains.

2. The effects of light and salinity on pigment accumulation and growth rate for five different strains of filamentous cyanobacteria were investigated to identify the optimal light and salinity conditions for each. We predicted that the optimal salinity for each strain would correlate to the salinity of the soil from which it was isolated, providing evidence that strains of cyanobacteria are genetically adapted to their home soils. We expect that these data will provide a useful basis for optimization of conditions for commercial mass algal culture.

3. The application of ATR-FTIR as a new tool to discriminate soil-born filamentous cyanobacteria will be used as powerful biotechnology method. This study is the first evaluation of *et al* the effectiveness of ATR-FTIR as a tool to discriminate among strains of soil-born filamentous cyanobacteria.. This evaluation is an essential first step to implementation of this technique in phycological research.

CHAPTER II

LITERATURE REVIEW

2.1 Cyanobacteria

Cyanobacteria or blue-green algae are prokaryotic phototrophic organisms, containing photosynthetic pigments and performing oxygen-evolving photosynthesis. The popular blue green algae name comes from the colour of the cell seen under microscope. The photosynthetic pigments in the membranes inside the cell contain chlorophyll *a*, which is green colour, but almost species content phycocyanin, blue coloured pigment, and under some condition form also a red pigment, phycoerythrin (John, Whitton, and Brook, 2000). Blue- green algae are regarded as a type of bacteria because they are prokaryotic; that is, they possess no nuclei membrane and other membrane-bound organelles are absent. Like other non-photosynthetic bacteria, the blue-green algae multiply by binary fission. Consequently, the blue-green algae are recognized also as cyanobacteria (Fogg, 1973). Although, blue green algae are treated by some authors under the conventions of International Code of Botanical Nomenclature, others treat them under International Code of Bacteriological Nomenclature (John, Whitton, and Brook, 2000).

The cyanobacteria appeared about 3 billion years ago and they were the most ancient oxygen-producing photosynthesizers (Linda and Lee, 2000). Vonoye (1961) considered cyanobacteria as the residue of a very ancient vegetation, dating from the epoch in which the atmosphere did not yet contain oxygen.

Blue-green algae or cyanophyceae include about 150 genera, 1400-2000 species; occupying all kind of habitats, their distribution is cosmopolitan. (Pandey and Trivedi, 1994; Jan, 1995). Cyanobacteria are found in almost all environments, including terrestrial, freshwater, and marine environment (Trainor, 1978). In fresh water cyanobacteria occur as phytoplanton or as attached algae in standing or running water. They are commonly found on moist rocks and soil, forming a black coloured crust when dried out (Robert, 1989).

2.1.1 Cell morphology and physiology

Cyanobacteria are unicellular, free living or enclosed within a mucilaginous envelope. Subsequent evolution resulted in the formation of a row of cells called a trichome. When a trichome is surrounded by a sheath, the structure is called a filament**.** It is possible to have more than one trichome in a filament (Carr,1973). Some filament form characteristic cells know as heterocysts, which differentiate from an ordinary vegetative cell, and are the site of nitrogen fixation. The fixed nitrogen can pass through the rest of the filament (John, Whitton, and Brook, 2002). Typically the morphology of the cyanobacteria falls into two categories of thallus organization: Unicellular or colonial and filamentous forms.

2.1.1.1 Unicellular and colonial forms.

The unicellular forms are the simplest of the cyanophyta, and consitute the majority of cyanobacteria (Ennos and Sheffeld, 2000). These single cells are typically spherical, oval or cylindrical and reproduce by binary fission. The non-filamentous forms are mainly coccoid in shape present as either single cells or grouped in palmelloid colonies (Desikachary, 1959). Order *Chroococales* includes all the unicellular or colonial cyanoprokaryotes, which do not form true filament with the

direct physiological interference between the cells (Komarek, 1999). John *et al*. (2002) also stated that unicellular or colonial, sometimes form a pseudofilamentous structure but never form true filament, and chroococcoccales include almost all species incapable of forming hormogonia.

The representative genus *Chroococcus*, which occurs as single cells or colonies of 2, 4, 16 or less frequently, 32 hemispherical cells resulting from adherence of daughter cells after division, and their gelatinous sheath may or may not be noticeable (Graham and Wilcox, 2000). The *Chroococcus* sheath is always distinct, but difficult to distinguish from *Synechococcus* and *Synochocystis* genera because of variability in sheath production (Trainor, 1978).

2.1.1.2 Filamentous forms

The main kind of filamentous forms are the trichomes which was made up of a long series of cells that remain attached after division (Ennos and Sheffield, 2000). The trichome of *Oscillatoria* are the simplest kind of filament (Desikachary, 1959; Pandey and Trivedi, 1994; Jan, 1995) which is made up of a long series of cells placed one over the other to form a "trichome". Cell division in filament of *Oscillatoria* occurs in the same plane, while heterocysts and ankinetes adsent (John, Whitton, and Brook, 2002). The filament sheath of *Oscillatoria* is frequently difficult to distinguish, whereas it can be seen clearly in *Lynbya*, especially at apices of filaments, while *Spirulina* and *Arthrospira* have helical shape of filament (Trainor, 1978). The trichomes of cyanobacteria are commonly unbranched as in *Oscillatoria* and *Lynbya* or may be branched as in *Westiella, Hapalosiphon* etc. Beside the branching types, there are the types known as false branches such as in *Scytonema*,

Plectonema and in *Tylopothrix*, and the types that display true branching as in *Hapalosiphon* and *Stigonema* (Desikaschary,1959).

In the false branching filaments double false branches often occur. This results from the outgrowth from both ends of a filament that has been interrupted by a dead cell or, less commonly, by heterocyst differentiation (Graham and Wicox, 2000). Desikachary (1959) observed that the false branching may be visualized as long hormogones or fragments of trichome which germinate or develop *in situ*, with the ends piercing and growing out of the sheath of the parent filament.

Morphologically, the most complex of thallus in the cyanobacteria is the true branched filament, which can be uniseriate (compose of a single row of cells) as in *Hapalosiphon* or multiseriate (compose of more than one row of cells) as in *Stigonema* (Trainor, 1978 and Carr, 1973). This branching occurs by the formation of true lateral branches, as well as the primary trichome becoming serrate (Carr, 1973). John and Whitton (2002) explained that longitudinal and sometimes oblique cross walls occur in addition to transverse walls, which lead to the formation of true branches. According to Graham and Wicox (2000) branches arise by division of cells in a direction perpendicular to that of the main filament axis, and the branches may be pluriseriate as in *Stigonema* or not pluriseriate as in *Fischerella*.

2.1.2 Cyanobacterial pigments

The absorption of light energy by cyanobacteria is based upon the occurrence of one or two forms of chlorophyll, together with carotenes and phycobilins. The photosynthetic pigments are located in thylakoids, which lie free in cytoplasm (Graham and Wicox, 2000). According to Trainor (1978), cyanobacteria have chlorophyll a, beta carotene, and several xanthophylls. Desikachary (1959) stated that

the commonest xanthophylls in cyanobacteria is called myxoxanthophyll. The photosynthetic pigments of cyanobacteria contain chlorophyll *a*, which gives the green colour, but almost all species can form the blue pigment, phycocyanin, under some conditions and some also form red pigment, phycoerythrin. All these pigments found in the membrane inside the cells (John, Whitton, and Brook, 2002). Edwards, (2003) also confirmed that photosynthetic *Cyanobacteria* have chlorophyll *a* and carotenoids in addition to some unusual accessory pigments named phycobilins**.** The blue pigment, phycocyanin and the red one, phycoerythrin, absorb wavelengths of light for photosynthesis that are missed by chlorophyll and the carotenoids.

2.1.2.1 Chlorophyll

Chlorophyll is a key biochemical component in the molecular apparatus that is responsible for photosynthesis, the critical process in which the energy from sunlight is used to produce life- sustaining oxygen. Because of the presence of chlorophyll *a* is a characteristic that unites all of the photosynthetic algae and plant, and is one of the characters that distinguishes blue-green algae from other photosynthetic bacteria, which is possess a different pigment, bacteriochlorophyll (Pandey, 1994; Linda, 2000). Chlorophyll *a* is the pivotal photosynthetic pigment (Linda, 2000), while others pigment, carotenoids and phycobilins are accessory pigments.

Chlorophyll *b* occurs in some genera such as *Prochloron*, *Prochlorococcus* and *Prochlorothrix*. Chlorophyll *b* is regarded as an accessory pigment because it broadens the range of light that can be used in photosynthesis, and transfers absorbed light energy to chlorophyll *a*. This pigment can be easily formed

from chlorophyll *a* precursor molecule by a single step that involves an O_2 molecule (Linda, 2000).

2.1.2.2 Phycobilin

Cyanobacteria that do not produce chlorophyll *b* typically possess water- soluble phycobilin pigments that extent the range of wavelengths of light that can be harvested for photosynthesis, and transfer captured energy to chlorophyll *a*. Cyanobacterial phycobilin include phycoerythrobilin and phycocyanobilin, which are bound to protein and also collectively called phycobiliprotein (Linda, 2000). Blue-green algae have four phycobiliproteins: C-phycocyanin, allophycocyanin, C-phycoerythrin, and phycoerythrocyanin. All blue-green algae contain the first two, whereas C-phycoerythrin and phycoerythrocyanin occur only in some species (Carr, 1973).

2.1.2.3 Carotenoid

Carotenoids found in cyanobacteria include a variety of xanthophylls, which include oxygen in their molecular structure, and β- carotene, which is lacking oxygen. These pigments are located in thylakoids, alongside chlorophyll *a*. The main carotenoids present in cyanobacteria are β-carotene, nostoxanthin, caloxanthin, echinenone, myxoxanthin and zeaxanthin (Fresnedo, Gomez, and Serru, 1991).

The carotenoids are one of the most important group of natural pigments, because of their wide distribution, structural diversity and numerous functions (Oliver and Palou, 2000). Carotenoids are an important pigment in cyanobacterial cells. Firstly, carotenoids act as accessory pigment to increase the ability of cyanobacteria to harvest blue wavelengths of light that are not directly absorbed by chlorophyll *a.* Carotenoids also provide protection from harmful photooxidation (Linda 2000),

particularly providing protect for chlorophyll against oxidation (Fresnedo *et al*., 1991). Carotenoids have also been successfully used for many years in the treatment of individual suffering from photosensitivity disease such erithropoetic protoporphyria (Oliver and Palou, 2000). β-carotene is the main sources of provitamin *a*, required by animals for the synthesis of the visual pigment and in the regulation of genes involved in limb and skin development (Linda, 2000). In addition to the provitamin *a* activity of some carotenoids, these pigments have recently been implicated in the prevention of or protection against serious human health disorders such as cancer, heart disease, macular degeneration and cataracts (Oliver and Palou, 2000). Del Campo *et al*. (2000) also proposed that carotenoids have been applied as effective preventive agents for a variety of human diseases. Other biological functions of carotenoids are the stimulants of the immune response at different level, enhancers of gap junction communication, and quenchers of free radicals such as singlet oxygen (Oliver and Palou, 2000). β- carotene, lutein and zeaxanthin are proposed to display cancer preventing properties (Del Campo *et al*., 2000). Some research found that in smokers, β- carotene supplementation increases rather than decreases lung cancer incidence and there is the conclusion that diminished retinoid signaling could be a mechanism to enhance lung tumorgenesis after high-dose β- carotene supplementation and exposure to tobacco smoke (Oliver and Palou, 2000). Carotenoids also have application in cosmetic industries, as in epidemiological studies we can find associations between dietary carotenoid components and the incidence of various chronic diseases (Oliver and Palou, 2000; Del Campo *et al*., 2000).

The important carotenoid functions have stimulated research and development of the production and use of carotenoids from microalgae as food additives (Del

Campo *et al*., 2000). Recently, many studies have exclusively focused on provitamin A carotenes or β- carotene and chromatographic methods have been the traditional methods to separate and quantify the carotenoids (Oliver and Palou, 2000).

2.1.3 Microalgae culture

During the last two decades algae and other form of microorganisms have proved exceptionally rich source of useful products and many species of microalgae and cyanobacteria have be induced to accumulate different types of reserves that have been used as sources of pigments, lipids, vitamins or proteins (Roxana *et al*., 2000). Microalgae are now produced on a commercial scale for the production of single-cells protein, polysaccharides, health-food compound such as polyunsaturated fatty acids and vitamins (Janssen *et al*., 1999). To date, only a few microalgal species have been examined extensively and recognized as commercial sources of carotenoids, namely the halophilic green flagellate *Dunaliella sativa*, *Chlorella* sp. and *Haematococus puvialis* (Del Campo *et al*., 2000), and the cyanobacteria *Spirulina* have been produced on large scale, mainly to supply biomass for food and feed (Roxana *et al*., 2000). The research of Janssen *et al*. in 1999 suggested that large-scale cultivation of photo-autotrophic microorganisms is still limited by scale up problems and economical consideration. They also found that light energy often limits reactor. Several studies confirmed that light regimes, salinity , pH, temperature and nitrogen are the factors that affect growth rate and pigment content of microalgae.

2.2 Distribution of cyanobacteria in Northeast Thailand and in Nakhon Ratchasima

The study of Vallisuta (2000) on distribution of cyanobacteria in Thailand found 369 species and concluded that the most abundant region was the north part, where 216 species were found. The next region was the central part where 140 species were found. The second least abundant part was northeast territorial where found 134 species were discovered and the least cyanobacterial abundance was the southern part of Thailand, where only 80 species were located. In the Northeast of Thailand, the occurence of cyanobacteria was quite low in Nakhon Phranom, Mookdaharn, Burirum, Yasothorn and Nongkhai. The dominant species found in Northeast region were *Calothrix* sp. (60%), *Nostoc* sp. (30-59%); *Scytonema* sp and *Anabaena* sp were found in about 30%, whereas *Cylindospermum* sp. and *Fischerella* sp.were quite rare.

Lawan (1990) conducted a study of blue green algae from saline soil in Nakhon Ratchasima province. He was found 8 genera of cyanobacteria distributed in the Nakhon Ratchasima province including *Anabaena*, *Aulosira, Calothrix, Hapalosiphon, Nodularia, Nostoc, Scytonema,* and *Wetiellopsis*. *Calothrix* was the dominant genus with 10 species present in most samples, followed by *Nostoc* genus with 9 species, then *Scytonema* genus with 3 species, whereas the other genera were represented by only one species.

Lawan (1990) also observed that from all the 8 genera found in soil samples, only 5 genera had tolerance to high salinity. These were *Nostoc, Aulosira, Scytonema, Calothrix* and *Hapalosiphon*.

2.3 Saline soil in the northeastern of Thailand

The northeastern area, accounts for one third of the whole country's population area and whereas the total income of the regon is only about 11.2% of the country. This is because farming is the occupation of most of the population, and is why the Northeast region is the poorest region of the country.

Salt-affected soils have been identified as one of the three main hurdles against crop production in northeast region of Thailand. The widespread deforestation of past is considered as the main cause for bringing the salts from lower soil strata up to soil surface, which despite of various control measures, has resulted in rapid expansion of salt-affected areas. (Awadh, 1995). Growing rice plants on the paddy fields in northeast Thailand have been obviously facing some serious obstacles such as drought, poor soil fertility, but the most grave problem has been increasing soil salinity (Srinarong and Panchaban, 2003). According to Arunin (1996), saline paddy fields in northeast alone accounts to 2, 85 million hectares and moderate to extreme salinity conditions compromised 60, 4% of the total productive agricultural area (Awadh, 1995). The results of soil analysis revealed that the soil type can be considered as a poor soil when the concentration of sodium salt (NaCl), exceeds 3,3 ms/m (Srinarong and Panchaban, 2003).

In Nakhon Ratchasima province, there are three types of salinity affected soil (Lavan, 1990):

(1) High terrain areas. These are highland areas with cassava and hemp cultivations, and has low rice production due to the low fertility of the sandy soil. In high terrain areas during the dry season many salt crusts were formed in area of paddy fields.

(2) Low terrain areas. This area is dominated by clay and sandy soils similar to those found in the high terrain area. These areas receive salts from high land and soluble salt in groundwater.

(3) Low basins, contain flexible clay and can be salt- affected by flooding but is generally of low salt concentration

2.4 The effects environmental factors on terrestrial cyanobacteria

2.4.1 Physical factors

2.4.1.1 Light

Light is a very important environmental factor for all photosynthetic organisms. It is essential not only for photosynthesis but it is also involved in the regulation of growth and development. Whitton (1993) showed that most cyanobacteria biomass occurs at the surface, with some cells or filaments can penetrate several centimetres into the soil. He also stated that many cyanobacteria can tolerate high levels of ultra-violet irradiation. Whitton and Pott (2000) observed that cyanobacteria growing on the soil surface often show dark coloration, especially blue-black, red- brown or red and they suggested that the brown colours usually result from the presence of brown sheaths surrounding the photosynthetic trichome. The pigment responsible for the brown coloration is scytonemin, absorbs strongly in the near ultra-violet region of spectrum which appears to be an adaptive strategy for photoprotection against short-wavelength solar irradiation. Jones (1993) stated that many cyanobacteria tolerate high levels of ultra-violet irradiation permitting them to survive at the soil surface.

The UV component of solar radiation is known to cause damage to cyanobacteria and other photosynthetic organisms, therefore, they are avoided death

and severe damage by the migratory patterns. Kruschel and Castenholz (1998) had done the study on the effect of solar UV and visible irradiance on the movements of *Oscillatoria laeteverens* and *Spirulina subsalsa* in microbial mats of hipersaline water to investigate the response of motile cyanobacteria to the UV portion of the solar spectrum. The results of the study showed that targeted motile cyanobacteria upward migrated to or near the surface under low visible light and in complete darkness, while their downward migration occurred in response to visible light over 400 Wm^{-2} and to relatively high levels of UV-A alone. They also investigated that both UV-A and UV-B wavelengths can penetrate deeply enough into mats to potentially act as positioning cues for motile cyanobacteria, and as a consequence allow them to take refuge from higher intensities of damaging radiation. Several study of migratory filamentous cyanobacteria showed that patterns, included upward and downward migration can be closely linked to daily changes in incident light intensity.

High irradiance also influenced the volatile odour compounds composition in the cyanobacterium *Microcystis aeruginosa* and this was interpreted to represent a cellular change during the photooxidation – promoting condition (Walsh, Jones, and Dunstan, 1998). The researches has been reported that UV-B causes deleterious effects in a number of biological systems, such as motility, pigmentation, phycobiliprotein composition and photosynthetic oxygen production in cyanobacteria. Sinha and his colleagues (1993) found that cyanobacteria strains are sensitive to UV and that differentiation of vegetative cells into heterocysts is severely affected by UV irradiation. They observed that heterocysts frequency was almost 50% lower in *Anabaena* sp. and *Scytonema* sp., whereas nearly 70% of heterocysts loss in *Nosto*c occurred when the cells were treated with UV radiation for 1 h. Kale (1972) studied

the effect of light on differentiation of heterocysts in the nitrogen fixing filamentous cyanobacterium *Anabaena ambigua*, and found that the number of heterocysts produced were found to be directly proportional to the light intensity. Other data suggested that growth and survival of the organisms were also severely inhibited after prolonged duration of UV exposure. In addition, the process of nitrogen fixation has also been reported to be depressed during midday when visible and UV irradiance are highest. Babu, Joshi and Viswanathan also stated in 1998 that the effect of UV-B on the growth pattern of *Anabaena*, *Scytonema* and *Nostoc* sp is the same.

2.4.1.2 Salinity/conductivity

Cyanobacteria are an important component of many soils, including saline soil. There is a rich cyanobacterial flora contained in saline and semiarid soils and are usually important components of crusts which is frequently occur at surface of these soils (Whitton and Potts, 2000). Raph (1962) confirmed that most cyanophyta prefer neutral or alkaline soils and they are abundant in salty, arid soils of widely diverse ionic composition. Many species are ubiquitous in soil and, therefore, seem to be tolerant of wide ranges of salt concentrations. Lewin's (1962) studies of soil algae also confirmed that most cyanobacteria prefer neutral or alkaline soils and they are abundant in salty soils.

Enhancement of cyanobacteria growth on saline soil could provide a means of improving soil quality and eventually reversing the trend to increases salinity, for example, *Microcoleus* appear to be the most widespread cyanobacteria on saline surface throughout the world where it can play an important part in stabilization of the underlying soil (Whitton and Potts, 2000). Desikachary (1959) explained the halotolerance of cyanobacteria by characteristics of their protoplasm especially its

plasmolysis and deplamolysis. Resistance to drought, high temperature, and high salt concentration of soil algae including cyanobacteria appear to be related to the characteristics of the wall and underlying protoplasm rather than simply to the osmotic pressure (Raph, 1962). Cyanobateria living at high salinities possess mechanisms to maintain osmotic equilibrium and cell tugor and attempt to maintain a low salt cytoplasm by producing different organic osmolytes such as disaccharides sucrose and trehalose, and glycine betaine (Whitton and Pott, 2000). Gideon *et al*. (1996) found that the key feature of microbial crusts present on surface of soils throughout the world including arid zones is the abundance of filamentous sheath-forming and polysaccharide-excreting cyanobacteria.

2.4.1.3 pH

One of the major factor influencing the occurrence of cyanobacteria is pH. Most soil algae are found over a fairly wide pH range. According to some reports cyanobacteria are infrequent below pH 6.0 in most temperate soils and entirely absent pH below 4.0 (Jones, 1993). Sreenivasan (1972) studied the ecology of cyanobacteria in tropical inland water also confirm that water with pH below 7.0 did not have any cyanobacteria. The report of Lawan (1990) of the soils around Nakhon Ratchisima stated that in the high and low acid soil samples, there is low population of cyanobacteria, while they are highly abundant in neutral and alkaline soil. He also found that *Nostoc* and *Anabaena* were the most common genera and show the greatest pH tolerance. On the other hand, cyanobacteria may also influence the pH of soils due to their metabolic activities. The study of Nalewajko, Colman and Olaveson (1997) about effect of pH on growth, photosynthesis, respiration, and copper tolerance of algae found that the least acid-tolerant strains did not grow at all at pH 4.5 or 4.8 and

had significantly lower growth rates at less acidic pH values. They also showed that rates of photosynthesis declined with decreasing pH.

2.4.2 Biological factors, symbionts

Cyanobacteria contribute to the fertility of many agricultural ecosystems, either as free-living organisms or in symbiotic association with other living organisms such as water-fern *Azolla*, other crop plant such as *Gunnera*, and lichens (Jones, 1993; Hilda and John 1995). Research of Miao *et al*. in 2001 found that cyanobacteria produce many bioactive secondary metabolites and there is an example of a patented antifungal compound that is produced by a strain of *Nostoc* isolated from a lichen. Cyanobacteria play an agricultural importance by their ability to fix atmospheric nitrogen, contributed to enhance crop fertility (Jones, 1993). The first association form of cyanophytes and plants is the symbiosis of *Nostoc* and *Gunnera*. *Gunera* is a genus of perennial herbaceous flowering plant. *Nostoc* living in the soil infect young *Gunera* plant by moving from the soil into glands on the stem of *Gunera* via copious mucilage release by these glands and finally it is incorporated within living *Gunera* cells. When *Nostoc* is living in the *Gunera*, most of the cells are like heterocysts which can actively fix nitrogen. Certainly, *Gunera* obtain almost all their nitrogen from *Nostoc* and it speculated that the *Nostoc* may obtain carbon from *Gunera* (Hilda and John 1995). The association of *Hapalosiphon* with submerged growths of *Sphagnum* has been widely reported in the literature, and it has been observed that growths of *Hapalosiphon* are frequently associated with *Sphagnum cuspidatum* in the edge of small pools in the 'flow country' of N-E Scotland (Whitton and Potts, 2000). A species of *Anabaena* lives symbiotically with the small aquatic fern *Azolla*. *Azolla* grows on the surface of water, the filaments of *Anabaena* live in a special cavity in

the fern's leaf. The association between *Anabaena* and *Azolla* is similar to that between *Nostoc* and *Gunera* in that the cyanobacteria can fix nitrogen and are rich in heterocysts. The fern can obtain all of the nitrogen it needs from *Anabaena* and in turn can be used as a green manure rich in nitrogen. In China and Vietnam *Azolla* has been used as a fertilizer in the cultivation of rice for a long time (Hilda and John, 1995). In India, the practice of utilizing cyanobacteria as an efficient source of biofertilizers for rice also have been advocated and adopted (Gopalaswamy and Kannaiyan, 2002). Several articles published about endosymbiosis which is a vital link between prockaryotic and eukaryotic forms. Geoffrey (2001) explained the plastids, the photosynthestic organelle common to all algal groups, is ultimately derived through endosymbiosis of a cyanobacterial-like organism. A broad consensus has developed that the plastids of algae arose from the same primary, cyanobactrial endosymbiosis (Palmer, 2003). Cyanobacteria contribute the majority of nitrogen in the Arctic by fixing up to 82% of all nitrogen introduced into the ecosystem. (Nigel and Trevor, 2002).

Considering the economic importance of cyanobacteria is very large in both negative and positive senses. In negative side, many cyanobacterial strains appear to be toxic blooms, cause harmful significance to livestock and human life. In contrast, cyanobacteria are also having an increasingly positive economic importance, forming the basis of new food industries and their widely use as biofertilizers in agriculture (Kansiz *et al*., 1999). Certainly, the development of a rapid, inexpensive, accurate and non-destructive techniques to discriminate and to monitor environmental control is needed and was found useful. ATR/FT-IR spectroscopy was the new powerful approach has developed in this study.

2.5 FT-IR microspectroscopic study on cyanbacteria

2.5.1 Fourier Transform Infrared (FT-IR)

The infrared spectrometer is an instrument which uses the infrared radiation to determine molecular composition of samples. An infrared spectrum produced by the spectrometer will measure absorption or transmission of IR photons versus frequency (usually expressed as wavenumber in cm^{-1}). Infrared radiation can be used to determine how much of each substance is in mixture and it does this by exciting the vibrational energy of bonds between atoms. Only molecular bonds with a changing dipole moment (so-called polar functional groups) will absorb IR photons and be registered in the IR spectrum. Each molecular bond will absorb a very specific energy. By looking at the absorption spectrum the different kind of bond present can be determined exactly, and this is then used to determine the types of molecules present. For example, spectral band from methyl groups of lipids found in 1740 and 2850-2970 wave number, the stretching band of Amide I is in 1650 wavenumber and Amide II is in 1540 wavenumber, while band from phosphate group of DNA and RNA found in 1240 wave number and the band of polysaccharides found in 1200-900 wavenumber (Figure 4.43.)

FT-IR spectroscopy involves the use of an interferometer to create the IR spectrum and has a much superior signal to noise performance compared to earlier dispersive instruments (Schure and Baker, 1992).

Infrared spectroscopy has been used as method for chemical analysis since the 1930s but became widely used in the chemical sciences in the last 20 years (Katon, 1996) due to the advent of FT-IR spectroscopy. FT-IR spectroscopy has been used in many ways in industry and research. In industry it can be used to analyze the

ratios for varying mixtures in paints, plastics, films polyester fibers, additive in food etc.. In research FT-IR has been used to analyze mechanisms by using isotopes to tag various atoms in the molecules structure (Schure and Baker, 1992).

In the past four decades several researchers have demonstrated that infrared spectra from bacteria can be used for identification and differentiation (Helm and Naumann, 1995). In the past 5 years the application of the spectroscopic technique to characterize tissue specimens has been tested and showed that infrared signature are potentially suitable for characterization of these samples (Lasch *et al*., 2002). Consequently, during the last years the use of FT-IR spectroscopy to determine the structure of biological macrolmolecules has dramatically expanded (Jabs, 1999). Recently, microscopic FT-IR is widely used in the field of Biology and Medicine. FT-IR can detect biomolecular change in the cells and tissues responsible or various disorders (Ramesh *et al*., 2001). Examples of application FT-IR in medical research are the research of Reno *et al*. in 2003 who applied FT-IR to vascular biology, analyzing the chemical composition of vessel wall layer which could be responsible for the morphological modification observed in saphenous vein after grafting. Another application was the research of Mordechai *et al*. (2003) who used FT-IR for the follow-up of childhood leukemia chemotherapy to characterizie lymphocytes isolated from treated children. Infrared spectroscopy and infrared microspectroscopy have used also intensively in the past decade as a diagnostic tool to distinguish normal form diseased cell and tissue (Pacifico *et al*., 2003).

Nauman *et al*. (1988) were studied the differentiation and identification of pathogenic bacteria using FT-IR and multivariate statistical analysis. Their

correspondence analysis proved that the various different bacterial species were clustering in distinct regions of the correspondence maps suggesting that there do exist correlation between spectral data and biochemical/microbiological classification. Helm and Naumann (1995) also stated that FT-IR spectroscopy can be used to detect and identify particular cell constituents such as capsules, endospores or storage materials in bacteria. The research of Kirschner and Naumann in 2003 demonstrated that FT-IR spectroscopy can be used as novel approach for microbial characterization and identification. Mariey *et al*. (2001) also used FT-IR spectra of intact cells to discriminate, classify and identify microorganisms. FT-IR has also used to detect the significant spectral differences between bacterial and fungal samples. The study of Kanzis *et al*. in 1999 demonstrated that FT-IR spectroscopy in conjunction with chemometrics methods can consistently discriminate between cyanobacterial strains.

It has been demonstrated that FT-IR spectroscopy has an advantage over other microbiological identification methods, as it require minimal sample preparation, can be performed rapidly, is easy to use, and provides precise discrimination (Orsini *et al*., 2000). The FT-IR spectroscopic method is now considered to be a comprehensive and sensitive method for detection of molecular changes in cells and for the discrimination of cellular samples; the FT-IR technique could be used for rapid discrimination between bacterial and fungal infection and contamination and may be usable for future rapid identification of viral and bacterial pathogens (Erukhinovitch *et al*., 2004). FT-IR spectroscopyis also a reliable identification technique; it is not only provide a competitive and rapid identification method but appear to be a very promising tool for the study of

microbial metabolism, antibiotic susceptibility and other interactions with drugs (Orsini *et al*., 2000). The research at the Center for Biospectroscopy of Monash University in Melbourne, Australia has pioneered the use of FT-IR spectroscopy to discriminate cyanobacteria. This work demonstrated that FT-IR spectroscopic technique was sensitive enough to discriminate cyanobacteria even at the strain level. Moreover, they demonstrated that FT-IR microspectroscopy apart from being discrimination method, the technique may provide information on the phylogeny of cyanobacteria (Kanzis *et al*., 1999).

With the aim to extend the research of Kansiz *et al*. the new, inexpensive method has been developed in this research, which was used attenuated total reflectance (ATR) coupled with FT-IR spectroscopy to acquire spectra of the samples and to discriminate cyanobacterial strains. This approach has an advantage as more rapid and a spectroscopic microscope is not needed.

2.5.2 Attenuated Total Reflection (ATR).

ATR is the reflection technique which is based on the phenomenon known as total internal reflection. When radiation traveling in a medium of high refractive index strikes an interface with a lower refractive index material the radiation is partly reflected and partly refracted from the interface. If the radiation is infrared radiation and the higher index material is transparent while the lower index material absorbs certain frequencies, the infrared radiation will partly absorbed at these frequencies upon reflection. The totally reflected radiation will then be attenuated at the frequencies at which the lower index material absorbs. This results in an infrared spectrum of the material with the lower refractive index. The high index material used is generally KRS-5 (a mixture of thallium bromide and thallium

iodide), ZnSe (zinc selenide, which is used in this study), germanium, and diamond. The method has been widely used to quickly obtain an infrared spectrum of difficult materials such as linoleum, toothpaste, plastics (Katon, 1996) and living specimens as cyanobacteria. In this research crystal diamond had been widely used as the development of diamond as an ATR material has opened up a number of sampling opportunities in the mid infrared. Reproducibility is also easier than with solids sampling using the bulkier classical 6 reflection ZnSe type ATR units (Dave Coombs, 2004). Zine Selenide is the common used for ATR sampling and it is ideal for analysing liquids and non- abrasive pastes and gels

Nowadays, ATR-FTIR is increasingly using in the field of bacterial research. Nadtochenko (2005) was used ATR-FTIR spectroscopy successfully in the identification E. Coli, LPS, PE, and PGN as photocatalytic peroxidn.products. ATR-FTIR is also used to monitor bacterial biofilm which have a profound impact on industrial, food processing, and medical settings creating significant problems (Gulley and Bullen, 2005). Whittaker (2003) was explored his experiment on identification of foodborne bacteria by infrared spectroscopy using cellular fatty acid methyl esters. Foodborne bacterial mixtures of FAMEs were measured by ATR-FTIR spectroscopic procedure and discrimitated by multivariate analysis. Results showed that the Enterobacteriaceae could be discriminated from the vibrios and the results also suggested that this infrared strategy could be used to identify foodborne pathogens. Concerning about the presence of microorganisms in food products has important ramification for safety, quality, regulations and public health. Yang and Seo (2005) were developed a required rapid and reliable methods to detect of microorganism, especially foodborne pathogens by using ATR-FTIR

and chemometrics. Their results indicated that FTIR spectroscopy could be used as a rapid method for the identification and numeration of bacteria in food. ATR-FTIR spectroscopy, the sensitivity techniques could also be used to reveal the universality of the functional group chemistry of bacterial cell surface. In investigation of interaction between antimicrobial agents and bacterial biofilms using ATR-FTIR spectroscopy, Suci *et al.* (1998) also confirmed that the analytical capabilities of this technique for providing information on both transport of an antimicrobial agent to bacteria embedded in the biofilm and interactions between an antimicrobial agent and biofilm component are described. Jiang *et al*. (2004) were used ATR-FTIR spectra of bacterial cell surfaces exhibit carboxyl, amide, phosphate, and carbohydrate related features, and these are identical for both Gram-negative and Gram-positive cells. In the interests of ATR-FTIR spectroscopy, structural changes occurring in the cells of several bacteria during their growth curves could be investigated. Frederick (2004) also was studied about using ATR-FTIR in investigation of structural changes in the cells of bacteria during population growth..The results showed that all species had significance of spectral changes during their growth curves, indicating structural change in the cells during increases in cells numbers. The study of Ede *et al*. (2004) showed that IR spectra reflect all of the components of the cells, including the cell walls, cell membranes, internal structures, and the cytoplasm. Boualam *et al*. (2002) were used ATR-FTIR spectroscopy, as an in situ rion- destructive method to detect the effect of organic matter of different origins on the development of the biofilm and to evaluate the usefulness of the method as an early warning signal for changes in the nutritive status of drinking water. Sockalingum *et al*. (1998) were described that ATR-FTIR

spectroscopy as a new emerging methods which could be helped in differentiating between bacterial and yeast strains. Lynch (1993) also studied the differentiation of living bacteria by ATR-FTIR spectroscopy and demonstrated that this technique makes it possible to classify and differentiate between microorganisms in vivo. ATR-FTIR could also be used to investigate the interaction of a bacterial endotoxin with different surfaces. Reiter *et al*. (2002) demonstrated distinct interactions of the negative charged lipopolysaccharide (LPS) with positive charged surfaces and the ability of LPS to remove positive molecules from a biomembrane.

CHAPTER III

MATERIALS AND METHODS

3.1 Materials and chemicals

3.1.1 Organisms

Composite of cyanobacteria living in saline soil of Nakhon Ratchasima province, Thailand.

3.1.2 Instrumentations:

1. Inverted light microscope (CETI)

2. Polarizing microscope (Olympus BX51) with automatic photographic system

3. Microscope digital camera (Olympus SEX9)

4. Growth cabinet (Canvion)

5. Vacuum filtration apparatus with glass fiber filter

6. Freezer

7. Orbital shaker (Gallenkamp)

8. Air pump

9. UV-Visible spectrophotometer (Cary 1E)

Varian Cary 1E UV-Visible Spectrophotometer is used for colorimetric determination of concentration of chlorophyll a. This is a double beam, ratio recording spectrophotometer, which is controlled by a PC. It is capable of scan

overlay; spectral addition, subtraction, division and averaging; spectral; derivative and smoothing selection; and making concentration measurement (Tissue, 2000)

10. High performance liquid chromatography (HPLC) SP1100, (Hewlet Packard). Chromatography is a separations method that relies on differences in partitioning behavior between a flowing mobile phase and a stationary phase to separate the component s in a mixture. High Performance Liquid Chromatography is a form of chromatography to separate compounds that are dissolved in solution. HPLC instruments consists of a reservoir of mobile phase, a pump, an injector, a separation column, and a detector. Compounds are separated by injecting a plug of the sample ,mixture onto the column. The different components in the mixture pass through the column at different rates due to differences in their partitioning behavior between the mobile liquid phase and the stationary phase.

11. Fourier transform infrared (FTIR) microspectroscopy with attenuated total reflectance (ATR).

Fourier Transform InfraRed spectroscopy is a widely accepted method for performing compositional analysis in solids, liquids and gases. It is useful tool for identifying both organic and inorganic chemicals. This is a non destructive technique used to collect infrared wavelength data of material to identifying their composition. The collected data by spectrometer is sent to computer as an interferogram then computer determines the frequency and identify of each component wave of the interferogram by performing a Fourier Transform of the data. These frequencies and intensities make up the peak of an Infrared (IR) spectrum. Infrared spectroscopy focused on electromagnetic radiation in the frequency range 400-4000cm^{-1},where cm^{-1} is known as wave number which is equivalent to frequency. When a molecule is

exposed to infrared radiation, it absorbs specific frequencies of radiation, and the frequencies which are absorbed are dependent upon the functional group within molecule and the symmetry of the molecule. For example , a carbonyl group, C=O, always absorbs infrared light at 1670 – 1780 cm^{-1}, where is caused the carbonyl bond to stretch. Therefore , to identify molecule using IR spectroscopy, often the strength of the bond within the molecule can be estimated by comparing their stretching frequency (Levin, 2004)

An attenuated total reflection accessory operates by measuring the change that occur in a totally internally reflected infrared beam when the beam comes into contact with a sample. This internal reflectance creates an evanescent wave that extends beyond the surface of the crystal into the sample held in contact with the crystal. In regions of the infrared spectrum where the sample absorbs energy, the evanescent wave will be attenuated. The detector records the attenuated IR beam as an interferogram signal, which can then be used to generate an infrared spectrum (Levin, 2004).

12. Glasses and equipments using for the microbiological laboratory

3.1.3 Chemicals and standards

Sodium carbonate, Na_2CO_3 (Carlo Erba

Aluminum Oxide, (nutral for chromatography) (RS, Carlo Erba)

Sodium hydroxid, NaOH anhydrous pellets (RPE Carlo Erba)

Cobalt (III) nitrate, $Co(NO_3)_2$ (RPE Carlo Erba)

Potassium di hydrogen phosphate, KH_2PO_4 (RPE Carlo Erba)

Manganese (II) chloride, $MnC_{l2}. 4H_2O$ (RPE Carlo Erba)

Sodium chloride for analysis, NaCl (RPE Carlo Erba)

Boric acid, H_3BO_3 ACS-ISO (RPE Carlo Erba)

Sodium molybdate, $NaZnMoO_4$. $2H_2O$ (RPE Carlo Erba)

Copper (II) sulphate 5 hydrate, $CuSO_4$. $5H_2O$ (BDH AnalaR)

Iron (II) sulphate 7-hydrate, $FeSO_4$. $7H_2O$ (BDH AnalaR)

Di Potassium hydrogen orthophosphate, K_2HPO_4 (BDH AnalaR)

Ethylenediaminetetraacetic acid disodium salt, $C_{10}H_{14}N_2Na_2O_8$ (BDH AnalaR)

Zink sulphate, $ZnSO_4$. $7H_2O$ (BDH AnalaR)

Sodium nitrate, $NaNO_3$ (BDH AnalaR)

Calcium chloride 2-hydrate, $CaCl_2.2H_2O$ (BDH AnalaR)

Magnesium sulphate 7-hydrate, $MgSO_4$. $7H_2O$ (BDH AnalaR)

Ammonium Iron (III) citrate, C_6H_8x Fe x H_3N (DAC MERCK)

Citric acid, C6H8O7 (DAC MERCK)

Agar - Agar purified and free from inhibitors for microbiology, (DAC MERCK)

Potassium iodine, KI (A.R., Univar)

Iodine I_2 (A.R., Univar)

2,6-Di-tert-butyl-4-methylphenol 99% organics (ACROS)

Methanol, CH_3OH (HiperSolv for HPLC, BDH)

Acetonitrile, CH_3CN (HiperSolv for HPLC, BDH)

Acetone, CH_3COCH_3 (HiperSOlv for HPLC, BDH)

Dichloromethane, CH_2Cl_2 (for HPLC, Carlo Erba)

Acetone, CH_3COCH_3 (ACS-ISO for analysis RPE Carlo Erba)

Cycloheximide, $C_{15}H_{23}NO_4$ (A.R., ACROS)

Beta carotene standard, (origins, Fluka)

3.2 Methods

3.2.1 Biological methods

3.2.1.1 Sampling of soil samples

Soil samples were collected from villages at 10 random locations, according to the map of saline distribution in the Nakhon Ratchasima province, northeast of Thailand. Composite soil samples of surface soils from the top about 0.5 cm were collected with plastic tube, 5 cm in diameter for analysis the properties of soil particularly salinity, electro-conductivity and pH. Sampling points were taken at 3 different locations; namely, rice field, plantation and non-plantation areas in each village.

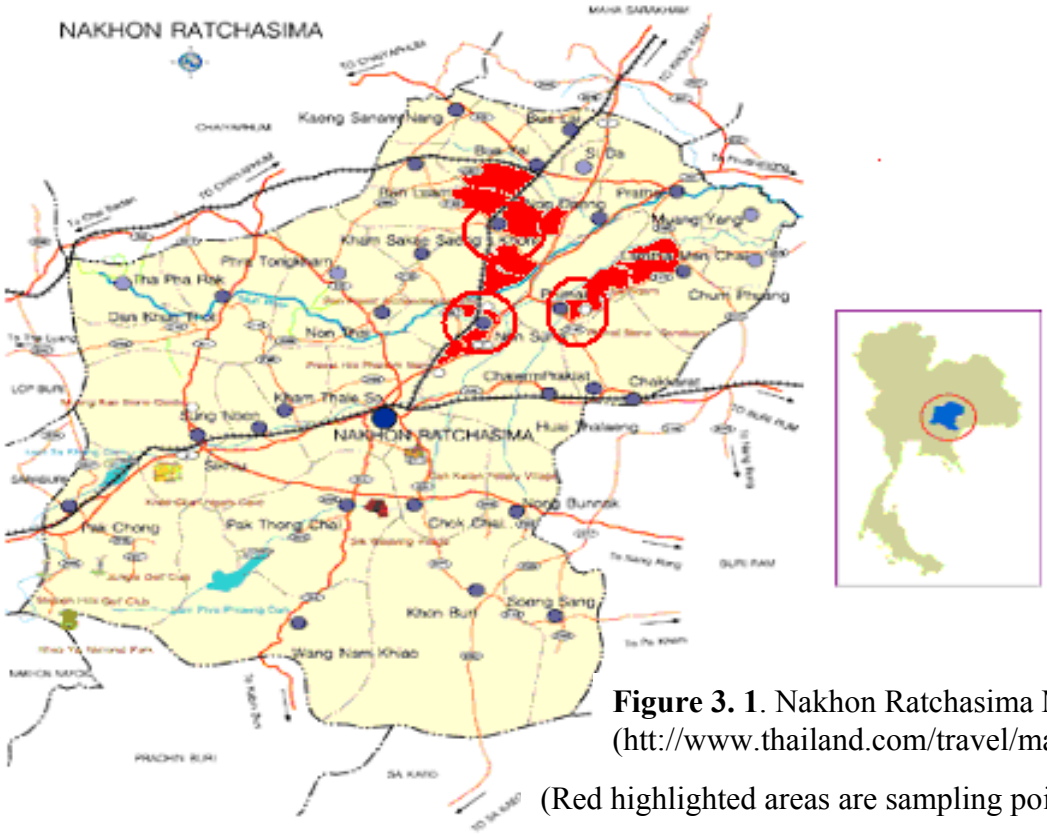


Figure 3. 1. Nakhon Ratchasima Map (htt://www.thailand.com/travel/map)

(Red highlighted areas are sampling points)

Table 3.1 Location of soil samples

Sample No.	**Location**	
	Villages	**District/Ampur**
1	Ban Khok Phet	Khong
2	Ban Rai	Khong
3	Ban Non Thong Larng	Khong
4	Ban Mun/ Reservoir Ta Kro	Khong
5	Ban Nong Bua Tung	Pi Mai
6	Ban Ta Ko Tung	Pi Mai
7	Ban Ta Lat Khae	Pi Mai
8	Ban Chat	Non Sung
9	Ban Done Ri	Non Sung
10	Ban Ta Not	Non Sung

3.2.1.2 Analysis of soil properties

30 soil samples from 10 villages of 3 Ampoes were analysed for 6 properties:

1). pH value determination

10 g of air-dried soil of each sample was put in 100 mL beaker and added 50 mL distilled water . The suspensions were stirred three times and the pH of suspensions measured after 30 minutes of equilibration by pH Metter.

2). Electro conductivity and salinity determination

50 g of air-dried soil of each sample was put in 300 mL beaker and added 250 mL distilled water. The suspensions were stirred and then filtered by What man No. 42. The electro conductivity and salinity of filtrates were measured by Conductivity Meter.

3). Total nitrogen determination

8 g of air-dried of soil sample was put in 500 mL conical flask and were added 20 g of Catalyst mixture (anhydrous Na_2SO_4, $CuSO_4$ and Se metal in 100:10:1) and 35mL H_2SO_4 conc. and then boiled until the mixture will be digested to be clear mixture. After leaving for cold down, the mixture were added 200 mL distilled water and balanced the base pH by adding 40% of sodium hydroxide. The mixture then filtered through What man No. 42. The extraction solution were then titrated to determinate total nitrogen in the soil sample.

4). Total phosphorous determination

1 g of air-dried soil put in 50 mL conical flask and added 20mL of the extracting solution (consisted 0,1 N HCl and 0,03 N NH_4F), stepped the bottle and shake for 30 minutes. The mixture then filtered through filter paper What man No. 42. The total phosphorous were measured by spectrophotometer at 882 nm. Value of

spectrophotometric measurement and standard phosphate concentration were used for sample calculation.

5). Available potassium determination

Weigh 5 g of soil sample and put in 100 mL plastic tube, then added 50 mL of 1N NH_4 OAc (mixture of 114 mg of glacial acetic acid dissolve in 1,000mL distilled water and 138 mL of NH_4OH conc., diluted into 1,980 mL distilled water and balanced pH 7,0 by using acetic acid or NH_4OH, added water into volume to be 2 liters), then shake for 30 minutes. After that the mixtures were filtrated and measured the available potassium by spectrophotometer.

6). Soil texture analysis

An amount of 50 g of air dried soil put in 500 mL beaker and were wet in 50 mL of 6% H_2O_2 Calgon (dissolve 50 g of Sodium hexa-metaphosphate $[(NaPO_3)_6]$ in 1 liter of distilled water) dried the soil in oven at 105^oC for 24 h. and then put in dessicator. After that 150 mL of distilled water and 65 mL of 0,5 N $[(NaPO_3)_6]$ were added, then stirred manually for 10 minute. Put soil in Bouyoucus cylinder with 300 mesh and was soil particles from dispersion cup by distilled water and then transferred soil particles from sieve to Petri dish and put in oven with 105^oC until the weight of soil will be constant. The resultant is sand.

Put the soil suspension in volumetric cylinder, and hung the hydrometer over the soil the filled distilled water into the cylinder until it was 1,000 mL by volume.

Blank cylinder was prepared by 65 mL of 6% Calgon solution and distilled water. The sedimentation cylinder of blank and samples were put in water bath for temperature control, then stirred the suspension by plunger until it was

homogenous suspension. Read the value from hydrometer at 0.5 and 3 minutes. Taking gently hydrometer out of the suspension and measured again at 10, 20 and 40 minutes and 1, 1,5, 2, 4, 8, 16 and 24 h. by pulling the hydrometer down to the suspension about 10 minutes before the time of measurement. The textural class of the soil used for particle size analysis can be derived from the triangle diagram of the Soil Survey Staff of the U.S.D.A.

3.2.1.3 Sampling of terrestrial cyanobacteria.

Terrestrial cyanobacteria were also collected from the 10 random villages with three sampling points the same as for soil samples. Composite sub-samples of different populations were collected by scraping cyanobacterial mats carefully off the surface of soil with a knife. In each sampling point, 3 different cluster samples were taken, each covering 2-3 cm^2 . The cyanobacterial samples were put in plastic bag that had a zipper, 8 x 10 cm in size, then some drops of BG 11 medium added and stored at the cool container kept cool to maintain all the collected cyanobacteria for the next continuously experiments until transported in the laboratory condition.

Figure 3.2. Sampling of terrestrial cyanobacteria

3.2.1.4 Isolation and purification of cyanobacterial strains

1). Isolation procedures.

The cyanobacteria population were enriched by adding 5mL BG 11 medium and growing them at 30^{o}C room temperature under continuously illumination provided by 4 daylight fluorescent illumination with photon flux 30 µmol quanta m^{-2} s^{-2}. After growth of cyanobacteria, composite of algae were cultured in agar medium by streaking on agar medium in petri dishes. Different strains of algae were isolated and made axenic by numerous restreaking isolations on sterilized BG 11 agar medium in Petri dish until the culture was unialgal strains.

2). Purification of the strains

Several purification methods were used to purify the algal strains, according to the type of contamination appearance of the culture. If the strain was only slightly contaminated, with other bacteria then standard plating procedure on Petri dish and micromanipulation of the colonies was carried out to obtain a pure

cultures. In some cases, bacterial contamination of cultures were removed by numerous equilibrium centrifugations with using the vortex mixture to separate bacteria off the algae; and any eukaryote organisms contaminating the cultures (e.g. Microalgae) were removed by growing the cultures on 0.8% agar with sterilized BG 11 medium (CITE medium) in Petri dishes, which was supplemented with cycloheximide at a concentration of 50 mg/liter (which prevents eukayotic cell division, but does not affect prokaryotes).

3.2.1.5 Culture of purified cyanobacterial strains

The purified algae strains from the Petri dishes were transferred into sterilized liquid BG11 medium in 5 mL Erlenmyer flasks. All the algal cultures were grown at room temperature (20ºC) under continuous fluorescent light illumination with photon flux of 30 μmol quanta m^2s^{-2} .

Once purified, cultures established in the smaller vessels, were transferred to Erlenmeyer flasks of 1L volume, then continued to grow with different selected factors in preparation for the experiments involving Biospectroscopy and pigment analysis.

Table 3.2 Cyanobacterial strains used for pigment experiments

No.	Cyanobacterial strain	Location
1	*Oscillatoria limosa*	Takhro reservoir (Khong)
2	*Phormidium* sp.	Non Thong Lang (Khong)
3	*Phormidium laminosum*	Talatkhae (Pimai)
4	*Scytonem javanicum*	Ban Chat (NonSung)
5	*Nostoc punctiforme*	Ban Done Ri (Non Sung)

3 2.1.6 Conventional identification and classification

Cyanobacterial strains collected from saline soil of Nakhon Ratchasima were identified into genera and species levels by taxonomic principles of Desikachary (1959), Prescott (1954), Kants and Bold (1969), Komarek and Anagnastidis (1999) Peerapornpisal (1996), and Whitton and Brook *et al*. (2002). The Image lite software (CITE reference) was used for study the morphology of organisms.

3.2.1.7 Growth measurement

The objective of this experiment is to estimate how the growth rate of the cyanobacterial strains are effected by different photon fluxes and salt concentrations by using cell numbers and chlorophyll-a as an indicator of biomass.

1). Growth curve measurement.

Five cyanobacterial strains were grown in growth cabinet at temperature 28^{o}C, illuminated at 20, 40, 60 and 80 µmol photon quanta $m^{-2}s^{-1}$ provided by cool-white fluorescent tube lamps to study the effect of photon flux on growth rate. The same cyanobacterial strains were grown at 40 µmol photon quanta $m^{-2}s^{-1}$ with sodium chloride added to the culture medium at 0, 0.5, 1, 1.5 and 2.5 g per 10 mL to study the effect of salinity on growth rate. There were 3 replicates for each level of photon flux and each different salinity concentration. 5 mL aliquots of cell culture were taken every two days starting from the lag phase until the late stationary phase of growth, transferred to sterile test tubes and agitated for one hour to disrupt the cluster of cells. The cell culture were then transferred into test tube and measured the optical density of suspension at 700 nm measured by UV- Visible spectrophotometer.

2). Chlorophyll -a measurement

For chlorophyll -a measurement, 20 mL aliquot of the cell culture grown as outlined in 3.2.1.4 were taken every two days continuously starting two days after inoculation until late exponential to early stationary growth phases. The cell culture were filtered through 47 mm Whatman GF/C paper under low vacuum (250 mmHg below atmospheric), and the filter containing cyanobacteria were rolled in an aluminium foil and kept frozen at minus 20^{o}C until the culture will be extracted for pigment measurement by UV-Visible spectrophotometer. The procedure of chlorophyll-a extraction and measurement was followed by Wright, Jeffrey and Mantoura (UNESCO, 1997). 20 mL culture aliquots were taken then were filtered through 47 mm What man GF/C under low vacuum (250 mmHg below atmospheric). The filter with algal sample was rolled in aluminum foil and immediately frozen in minus 20^{o}C. and kept before pigment will be extracted. The frozen filter containing cyanobacterial sample was cut into small slices (several mm x 1cm) and ground for 30 second with alumina in motar and then was extracted with 100% acetone. The combined extracts were transferred to 10 mL opaque volumetric tubes which were diluted by distilled water to made up to exactly 10 mL with 90% acetone. The extraction were measured concentration of chlorophyll a by UV-Visible spectrophotometer Cary 1E at 700 nm absorbance.

3.2.1.8 β carotene extraction and measurement

1). Culture harvest

Culture for the HPLC trials were grown as outlined in 3.2.1.4. Cells were harvested during the exponential growth phase and 20 mL culture aliquots were taken for β carotene analysis and 20 mL culture were also taken for dry weight

measurement. The culture then were filtered through 47 mm What man GF/C under low vacuum (250 mmHg below atmospheric). The filter with algal sample was immediately frozen in minus 20^{o}C.

2). Pigment extraction

The frozen filter containing cyanobacterial sample was cut into small slices (several mm x 1cm) and ground for 30 second with alumina in motar and then was extracted with 90% Methanol warming 78^{0}C in water bath. The combined extracts were transferred to 10 mL opaque volumetric tubes which were made up to exactly 10 mL with 90% Methanol.

3). Determination of β-carotene by HPLC.

An aliquot of the pigment extraction (about 25µl) was injected immediately into the chromatography column for pigment analysis. Extracts were quantitative analysed by chromatographic method described by Jeffrey, Mantoura and Wight (1997). The standard solution used for β-carotene detection were obtained by dissolving pure original compound of β-carotene in tetrahydrofuran containing butylated hydroxytoluene (BHT 1%w/v) and store in opaque flask at 4^{o}C. The use of the antioxidant BHT compound as was aimed to prevent oxidation during the extraction and sample treatment (Oliver and Palou, 2000).

3.2.2 Fourier Transform Infrared Microspectroscopy and Chemometrics Methods

In this research the biospetroscopic techniques was used to discriminate chemical composition of filamentous cyanobacteria during the growth phases and to investigate whether FTIR microspectroscopy combined with chemometric techniques has the potential to correctly classify soil-borne cyanobacteria as has been previously

demonstrated with cyanobacteria isolated from aqueous environments (Kansiz *et al*., 1999).

3.2.2.1 Cell culture

Seven cyanobacterial strains such as *Oscillatoria limosa*, *Arthrospira platensis, Phormidium laminosum, Phormidium* sp., *Scytonema javanicum, Nostoc punctiforme* and *Phormidium angustissimum* growing in saline soil of Nakhon Ratchasima Province, Thailand, were isolated as described in section 3.1 and used as subjects for IR spectroscopy. The strains used are summarized in Table 2. These strains are abundant in almost sites of the salinity areas of this study and these strains were chosen by their some representative characteristics, particularly *Oscillatoria limosa*, *Arthrospira platensis*, and three species of *Phormidium* are the species non heterocystous unbranched with different size in their filaments, while *Scytonema javanicum* is represent of the branched filamentous cyanobacteria and heterocystous the same as and *Nostoc punctiforme*.

Table 3.3 Cyanobacterial strains used for FTIR method

No.	Cyanobacterial strain	Location
1	*Oscillatoria limosa*	Takhro reservoir (Khong)
2	*Arthrospira platensis*	Nong Bua Tung (Pimai)
3	*Phormidium* sp.	Non Thong Lang (Khong)
4	*Phormidium laminosum*	Talatkhae (Pimai)
5	*Scytonem javanicum*	Ban Sart (NonSung)
6	*Nostoc punctiforme*	Ban Done Ree (Non Sung)
7	*Phormidium angustissimum*	Khok Phet (Khong)

3.2.2.2 Strains and growth conditions

All the seven selected cultures were grown in three replicates in 1 L Erlenmeyer flasks with BG 11 medium at 28^{o}C in 15 h. light/9 h. dark cycle of illumination with a photon flux of 30 μmol photons quanta $m^{-2}s^{-1}$ provided by cool-white fluorescent tube lamps. The flasks were stopped with sterile cotton and were agitated regularly 2 times a day at 12 hours intervals.

3.2.2.3 Sample preparation

The spectral acquisition were performed 3 times during early, mid- and late exponential growth phase. The cultures of 3 replicates of each species were vigorously agitated and aliquots were removed for spectral acquisition. 10 mL of solution of each culture were transferred to 15mL centrifuge tubes and centrifuged at 3000-3500 rpm for about 5-8 minutes, as for the *Scytonema* sp., *Oscillatoria* sp. Were centrifuged at 10000 rpm for 8 minutes to wash the cells. The supernatants were decanted off and the resultant pellet resuspended in distilled water. This process of washing the cells was repeated twice using vortex stirrer mixture to thoroughly wash

the cells of growth medium each time. The final wash was performed in with ultra pure Milli Q water to prevent any interferences resulting from components of the growth medium that might be revealed in the spectra. The samples 0,05mL of volume were then deposited on ATR stage using an auto pipette and air dried before spectra were acquisition.

3.2.2.4 Spectroscopic measurements

Microspectroscopic measurements were carried out using PerkinElmer Fourier Transform Infrared Spectroscopy coupled with Attenuated Total Reflectance accessory. ATR cell was equipped with ZnSe trough plate. FT-IR system was controlled by an IBM-compatible PC running Spectrum FT-IR software version 3.1. Absorbance spectra were collected over the wavenumber range of 3500-800 cm^{-1} at a resolution of 8 cm^{-1}. to enhance the signal-to-noise ratio, 64 scans were co-added and signal averaged. The reference background was collected with an open beam in the absence of any test sample.

IR spectra of samples were collected three times, at early; mid; and late exponential growth phases. 30 IR spectra were acquired from three replicate bottles at each growth phase and with all seven species.

3.2.2.5 Spectral data treatment

The acquired ATR-FTIR spectra were imported into the multivariate statistics program UNSCRAMBLER 9.1. To minimize differences between absorbance spectra, such as baseline shifts, all the measured spectra were manipulated using ‘Rubber Band Correction which baseline corrected and normalized to the most intense band in the spectrum in the OPUS 2.2 software. All the spectra were then exported as JCAMP-DX files and imported into Unscrambler 9.1 Data analysis and

investigation of clustering was accomplished using Principal Component Analysis (PCA) and soft Independent Modeling of Class Analogy (SIMCA). These two classification methods were performed using Unscrambler 9.1 software.

3.2.2.6 Chemometric methods

Chemometrics is the application of mathematical and statistical methods to improve chemical measurement process and to extract more useful chemical and physical measurement dat (Kansiz *et al*., 1999). Chemometrics such as principal component analysis and soft modeling by class analogy were applied to the analysis of infrared spectra in this study.

a. Principal Component Analysis (PCA)

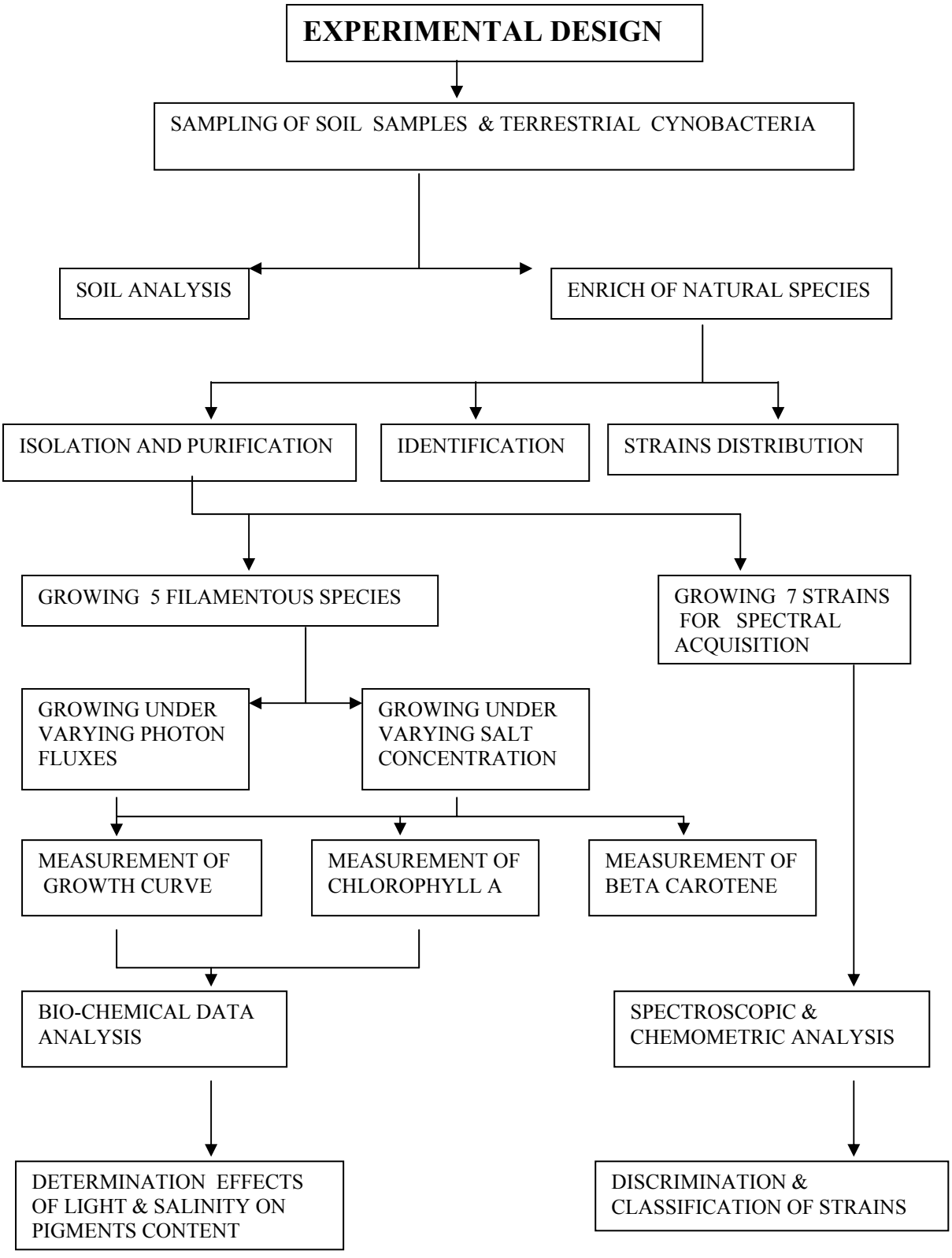
PCA is a multivariate data reduction method in which original variables are transformed into new orthogonal ones referred to as principal components, or PCs. Each PCs is a linear combination of the original variables, and each object is characterized on each PC by score, and similarly every variable has a value on each PC-the loading. PC1 accounts for the largest amount of data variance, and so on. A PC scores plot is a common method for exploratory visualisation of the data structure, and focuses on the relationships between objects, while a loading plot provides a means of identifying variables which contribute strongly to each PC (Deborah *et al*., 2005).

b. Soft Independent Modeling by Class Analogy (SIMCA)

SIMCA is a well known pattern recognition method which describe each class separately in a principal components (PC) space. The original SIMCA and modifications using different distance measures were compared with respect to classification and their robustness towards the number of PCs selected to describe the

different classes (Maesschalk *et al*., 1998). The SIMCA method bults principla components models for each class in the training set. The classification of a test sample is achieved by determining which space the sample occupies and if it can be a member of one, more than one or none of the class (Molfetta *et al*., 2004). Kansiz *et al*. (1999) described SIMCA involves the construction of separate PCA models for each class to describe and model the variation. The scores are also used to build up a measure of the samples distance to model center, called leverage. Finally both object-to- model distance and leverage are taken into account to decide to which class the sample belongs.

3.2.2.7 Experimental design



CHAPTER IV

RESULTS AND DISCUSSION

4.1 Soil analysis

Seven properties such as pH, electro-conductivity, total nitrogen, phosphorous, potassium, particle size of soil and soil texture class of each soil samples were analysed to investigate the relationship between soil condition and the occurrence of filamentous cyanobacteria as shows in table 4.1

Tablbe 4.1 Soil properties and cyanobacteria distribution

Village	pH	EC	N	P	K	Strain	(%)strain
1. Ban Khokphet	7.000	0.120	7.500	19.033	92.667	9	45
2. Ban Rai	7.500	0.237	0.156	15.897	202.000	3	15
3. Ban NonThongLang	6.500	0.150	0.075	27.007	170.500	12	60
4. Ban Takhro	7.500	0.140	0.100	8.863	106.833	8	40
5. Ban NongBuaTung	6.300	0.310	0.071	13.317	146.833	11	55
6. Ban TaKoTung	7.200	0.380	0.105	82.837	225.833	9	45
7. Ban TaLatKhae	5.700	0.39	0.158	17.640	127.333	6	30
8. Ban Chat	7.600	0.170	0.053	24.340	162.500	5	25
9. Ban Done Ri	7.200	1.560	0.119	59.150	84.667	3	15
10. Ban TaNot	8.000	0.357	0.051	13.743	101.833	2	10

4.1.1 pH

Soil pH is an indication of the acidity or alkalinity of soil and is measured in pH units on a scale from 0 to 14. pH is defined as the negative logarithm of the

hydrogen ion concentration. For the 30 soil samples analysed in this study, soil pH were assigned to 7 classes: 4 pH level: 4,0-5.0 (very strongly acid); 5.1-5.5 (strong acid); 5.6-6.0 moderately acid) 6.1-6.5 (slightly acid); 6.6-7.3 (neutral); 7.4-7.8 (slightly alkaline) and 7.9-8.4 (moderately alkaline) as shown in table 4.1

The pH range from all the sampling sites was 5.3-8.0 and the average pH was 6.6. The highest pH levels were recorded in Ban Chat, Ban Don Ri, Ban Rai and mostly in Ban Takhro. pH values reached 7.0-8.0 in all three sites. The Lowest pH value found in the Ban Non Thong Lang from a sample collected from the road side. The results show that soil samples collected from rice field site of all the 10 villages (No.1 in each village) have pH values ranging from neutral to slightly alkaline (pH up to 7.0). The Ph of soil samples collected from road site and non-plantation field varied in all 10 villages.

In Khok Phet village, pH was slightly alkaline in the rice field but on the road side and non-plantation sites soil pH values were in low acidity to neutral range. Soils in Ban Rai had quite high pH values, (7.3-7.7), especially from sites found high along the road side. At Ban Non Thong Lang, soil pH was highest on the rice field and lowest on the road side and around neutral in the non- plantation site. Soil sample from Ban Takhro were collected around the Reservoir Takhro and pH value was quite similar in all three sites (7.4-7.5). Ban Nong Bua Tung had also quite similar pH values for all three sites with pH ranging from neutral to slightly acid: 6.3-6.4. In Ban Tako Tung soil pH was in the range from neutral to slightly alkaline. Ban Talat Khae, the pH of sample sites was low, varying from strongly acid to slightly acid (5.0-6.5). At Ban Chat, Ban Done Ri and Ban Tanot, pH was neutral to moderately alkaline, (7.2-8.0).

4.1.1.1 The correlation between soil pH and occurrence of cyanobacteria.

Examination of the number of filamentous cyanobacteria strains found in each village indicated that soil pH affected the abundance of filamentous cyanobacteria strains that were isolated fro. The result in the table 4.1 show that in Ban Khok Phet, Non Thong Lang, Nong Bua Tung, and Tako Tung where pH value are slightly acid to neutral, which is usually regarded as the appropriate pH value for growth of cyanobacteria, cyanobacterial strains were found in high abundance (9-12 species in each sample sampling site). Around 60% of total number of filamentous cyanobacteria strains identified in this study were collected from the Ban Non Thong Lang field site, 55% from Ban NongBuaTung and 45%. From Ban Khok Phet. The lowest strain diversity was in Ban Rai, DonRi (15% of the total strains identified)and Tanot (10% of the total number).

Phormidium laminosum and *Lynbya* sp (1) were found in almost every village, the next most common were *Anabaena sphaerica*, *Arthrospira platensis* and *Lynbya* sp (2), whereas *Scytonema chiatum*, *Anabaena torulosa* and *Phormidium ucinatum* were only rarely found in this region.

The present results are in agreement of with Gopalawamy and Kannaiyan (2002) showed the distribution of cyanobacteria favours neutral to slightly alkaline conditions.

4.1.2 Electro-conductivity

Soil electrical conductivity measures the ease with which electricity can travel through soil water. Electrical conductivity is influenced by soil properties such as water and clay content, soil organic matter levels, and particularly salt content.

4.1.2.1 The relationship between electro conductivity of soil and occurrence of filamentous cyanobacteria

Soil electrical conductivity measures the ease with which electricity can travel through soil water. Electrical conductivity is influenced by soil properties such as water and clay content, soil organic matter levels, and particularly salt content.

The analyzed soil samples are showed in the figure 4.1 below. The electro-conductivity levels were between 0.03-2.4 µs/cm. The relationship between distribution of cyanobacteria and electro-conductivity is shown in Figure 4.1 The electroconductivity was found to be highest in Ban Nonthonglang (3) with 2.1-2.4 µs/cm and lowest Ban Tanot (10) with 0.81 µs/cm, which indicates a low level of salinity in this area .

The distribution of cyanobacteria in correlation with electroconductivity is shown in the table 4.1 The most most number of cyanobacterial strains were found in the villages: Nonthonglang, Khokpet, Nongbuatung, and Takotung where electroconductivity was lower than 0,2 µs/cm which would indicate that more cyanobacteria strains are found where salinity levels are low.

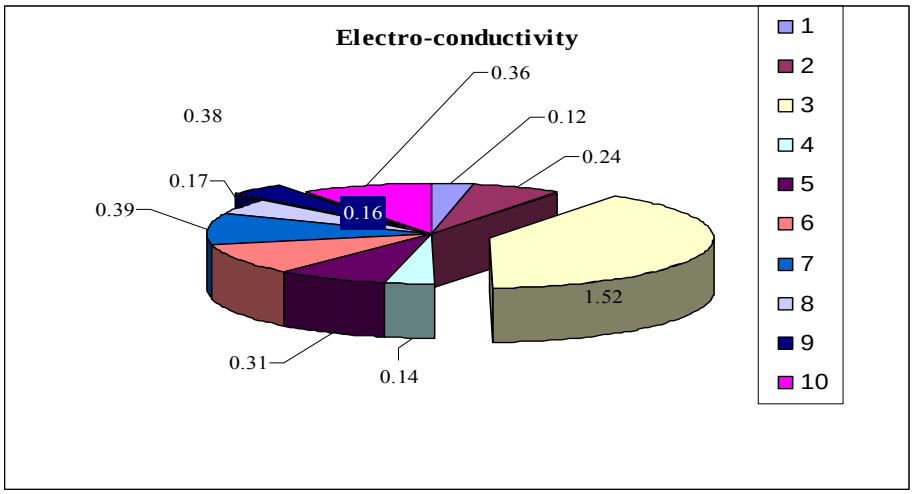


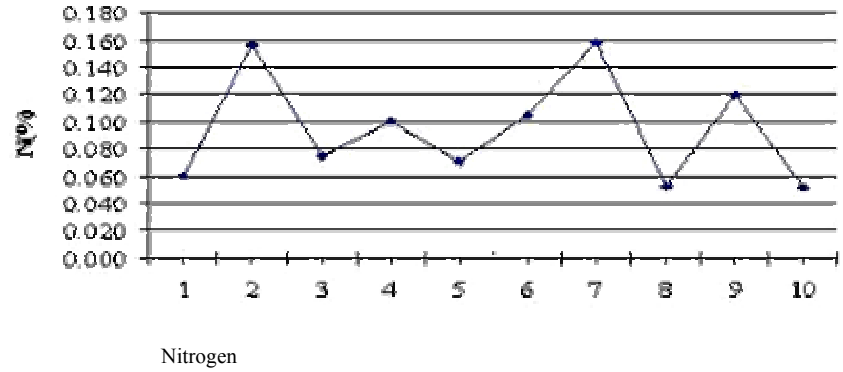
Figure 4.1 Electro-conductivity data in the sampling sites

4.1.3 Organic matter

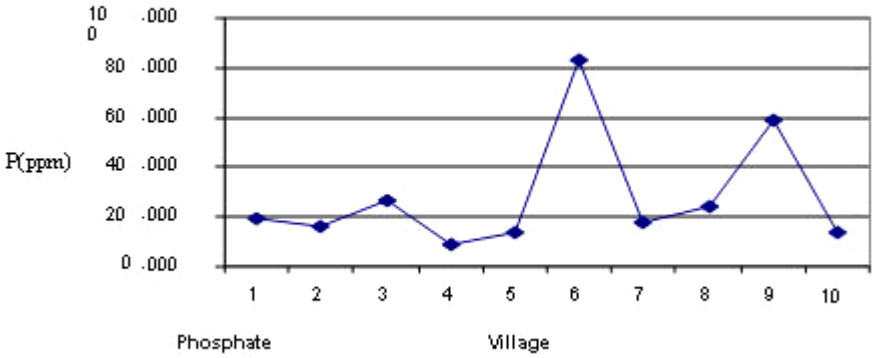
The results of organic content obtained from the sample sites are shown in Table 4.1

Average total nitrogen was highest at Ban Talatkhae at 0,156%, and at high levels at Ban Donri, and Ban Rai. Total nitrogen was low at Nongbuatung aNonthonglang , and Ban Khokphet.with the lowest level measured at Ban Chat.

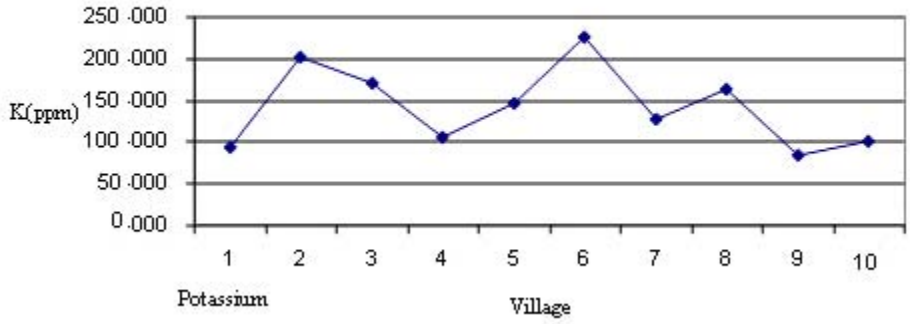
4.1.3.1 The available nitrogen, phosphorous and potassium



(a)



(b)



(c)

Figure 4.2 The value of organic matter, total nitrogen (a) available phosphorous (b) and available potassium (c) in the soil sample.

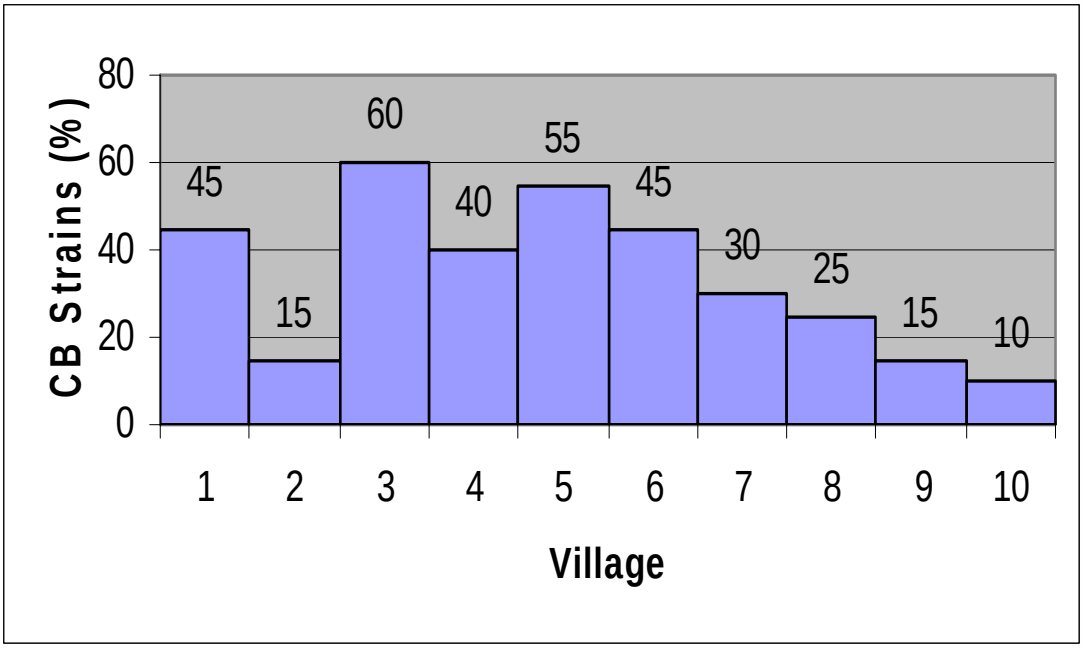
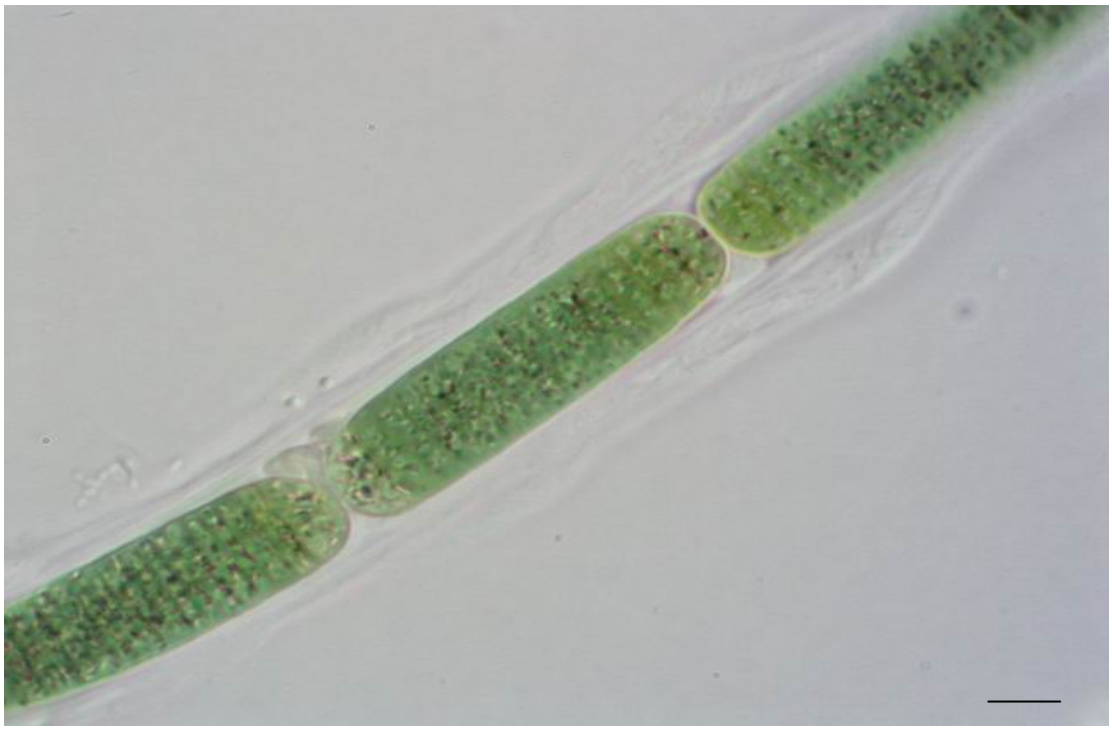
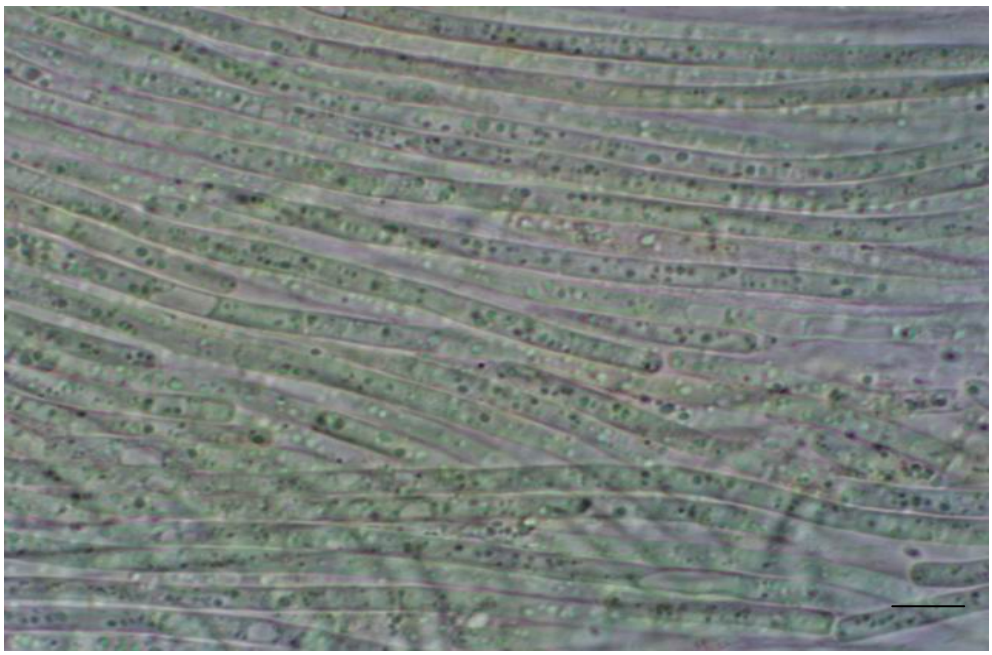


Figure 4.3 Distribution of filamentous cyanobacteria in 10 villages (%)



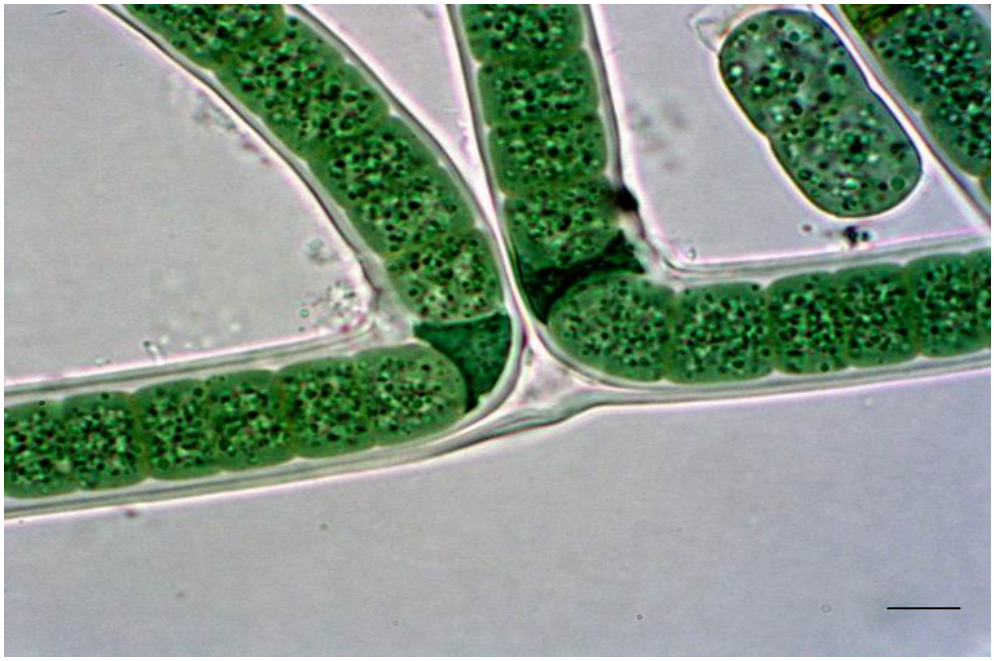
Scale bar = 10 µm

Figure 4.4 Microimage of *Oscillatoria limosa* Ag.ex Gomont



Scale bar = 10 μm

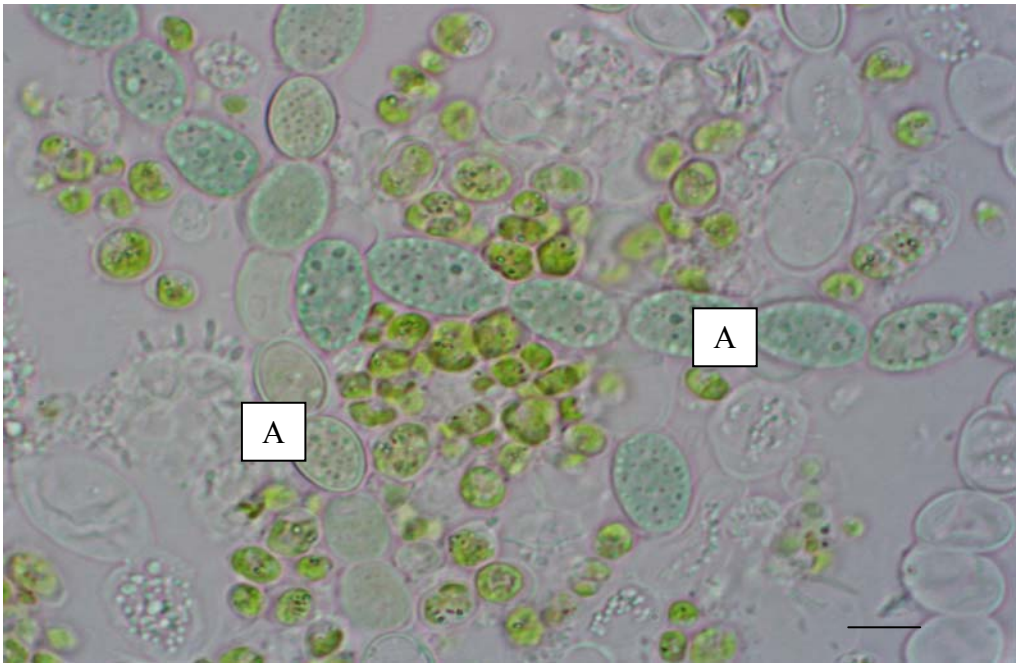
Figure 4.5 Micro image of *Phormidium laminosum* Gomont



Scale bar = 10 μm

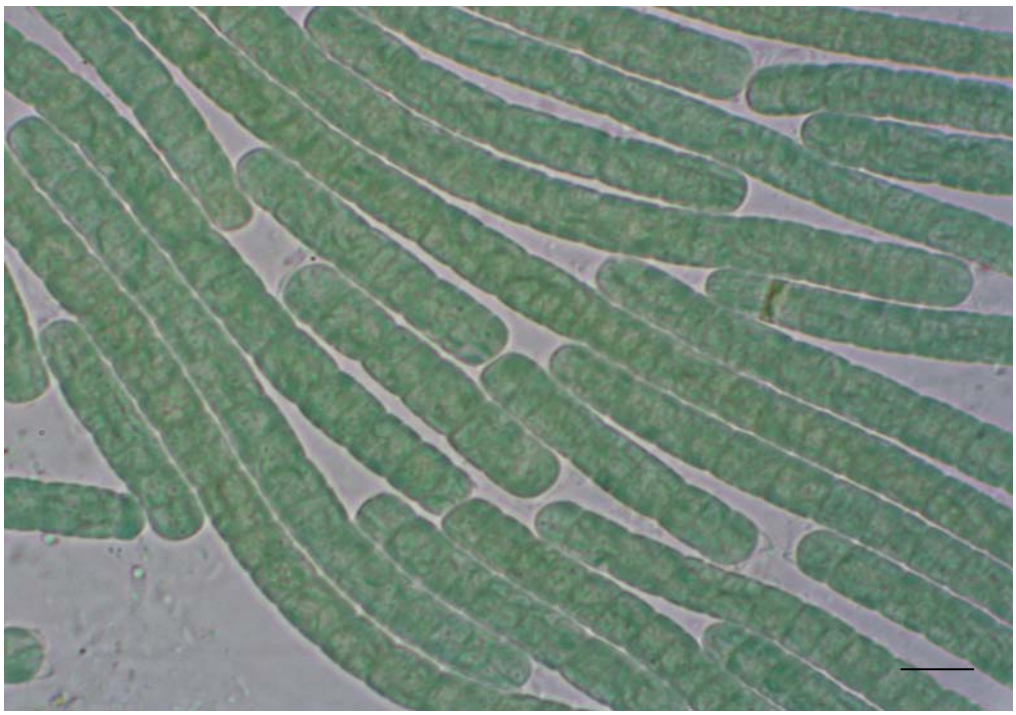
Figure 4.6 Micro image of *Scytonema chiatum*

Figure 4.7 Micro image of Scytonema javanicum Scale bar = 10 μm



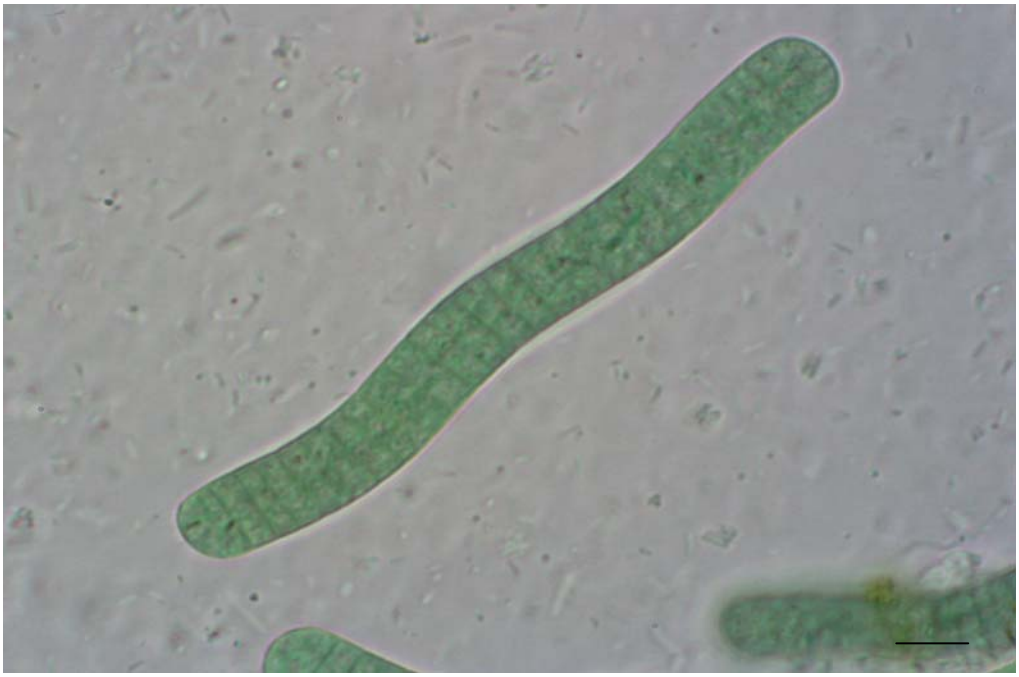
Scale bar = 10 μm

Figure 4.8 (A) Micro image of *Anabaena variabilis* var. *elipsospora* Fritsch



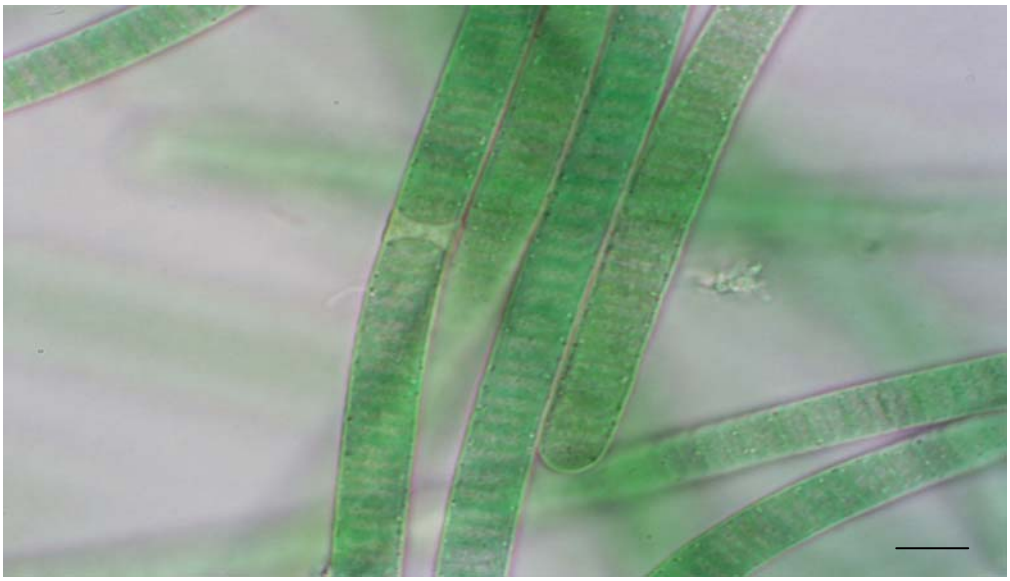
Scale bar = 10 μm

Figure 4.9 Micro image of *Phormidium* sp**.**



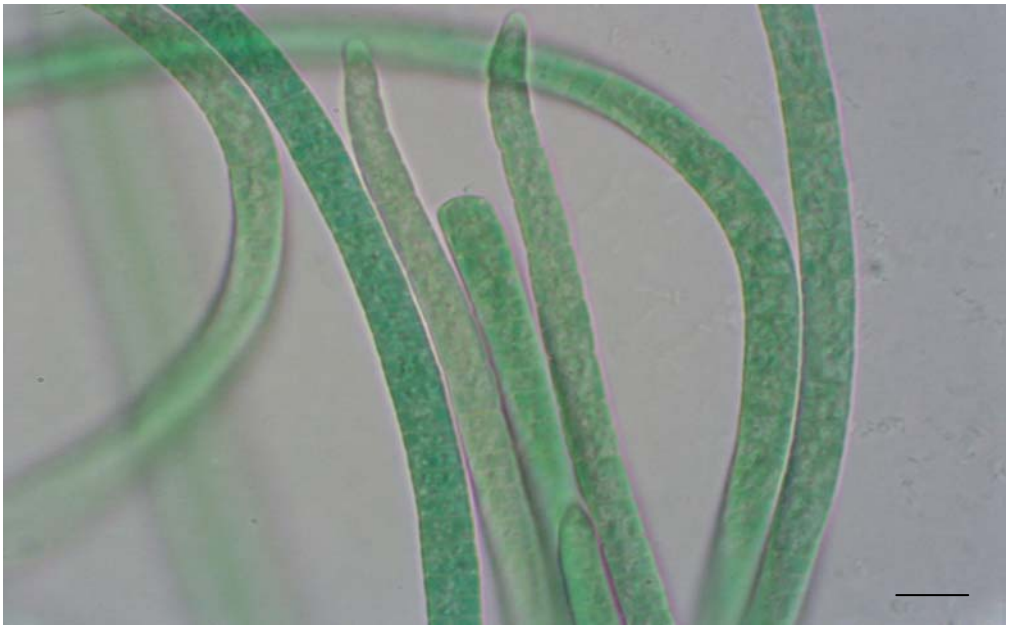
Scale bar = 10 μm

Figure 4.10 Micro image of Arthrospira platensis (Nordst) Gomont



Scale bar = 10 μm

Figure 4.11 Micro image of Oscillatoria sp.



Scale bar = 10 μm

Figure 4.12 Micro image of *Phormidium* sp.

4.2 Effect of Light

Five soil born filamentous cyanobacteria were grown under four photon fluxes densities namely, 20, 40, 60, 80 µmol photon quanta $m^{-2}s^{-1}$, to investigate the effect of light on growth rate and pigment accumulation. The results obtained are presented as in following section.

4.2.1 Effect of light on growth of filamentous cyanobacteria

Light effected on the growth of cyanobacteria, and each strain can be adapted to varying light intensity as showed in the growth curves.

4.2.1.1. *Oscillatoria limosa* Ag.ex Gomont

The result showed that at lag phase the growth rate was not different between four levels of light. The growth rate was slightly different, in particular with 80 µmol photon quanta $m^{-2}s^{-1}$, while other light levels were in similar growth rate. However at the mid to late exponential growth phase the curve of level at 40 µmol photon quanta $m^{-2}s^{-1}$ was increased significantly to compare with other levels. The data suggested that *Oscillatoria* strain was enable to survive under high irradiance but the appropriated light condition is low, at around 40 mol photon quanta $m^{-2}s^{-1}$.

4.2.1.2 *Phormidium laminosum* Gomont

Phormidium laminosum was showed the high light adapted strain. The growth rate was not different under all four light level during lag phase to early-exponential growth phase the same as and in *Oscillatoria limosa*, and the growth curve of the third light level was increased higher than other, while the growth curve of the highest level was dropped, but the significance was low. The results can be showed that *Phormidium laminosum* can grow good under photon fluxes from 20 to 60 µmol photon quanta $m^{-2}s^{-1}$.

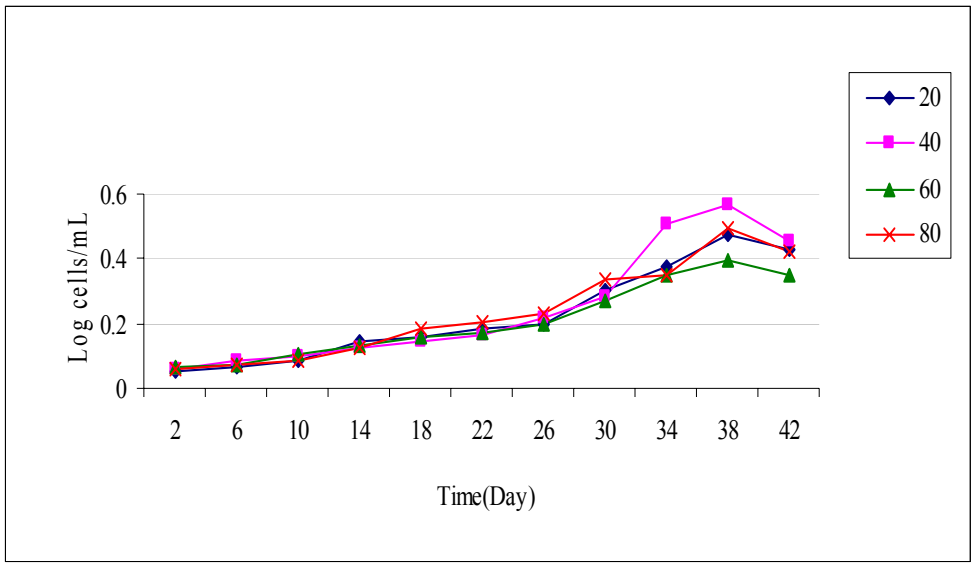


Figure 4.13 Effect of light on growth rate of *Oscillatotria limosa*

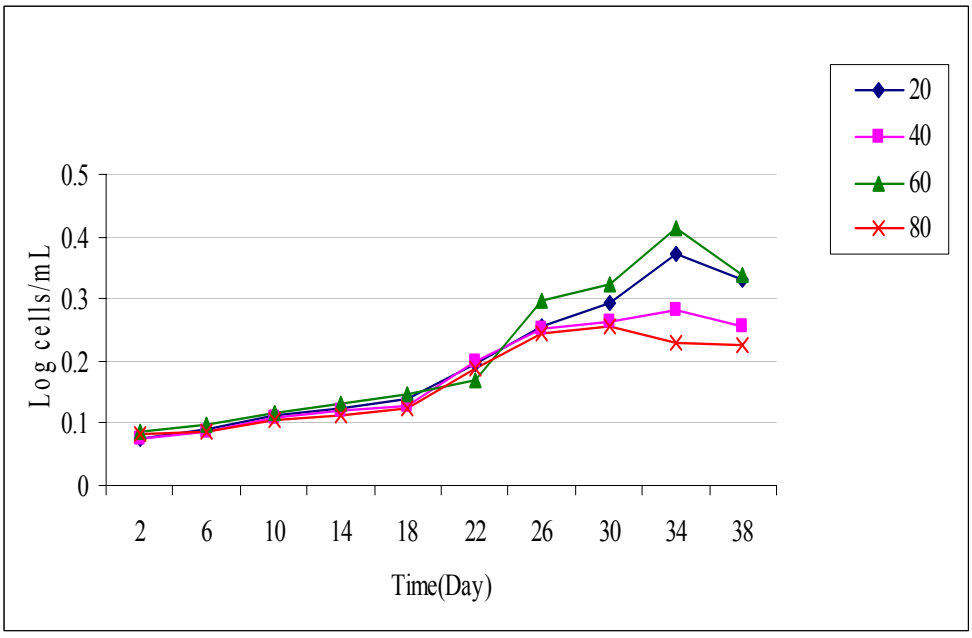


Figure 4.14 Effect of light on growth of *Phormidium laminosum*

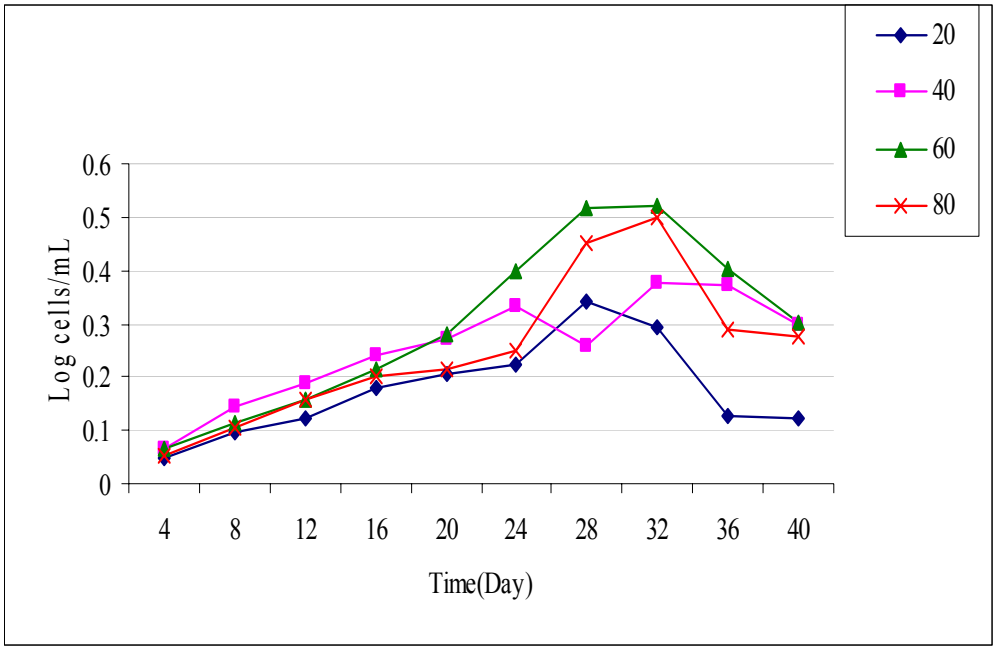


Figure 4.15 Effect of light on growth of *Phormidium* sp.

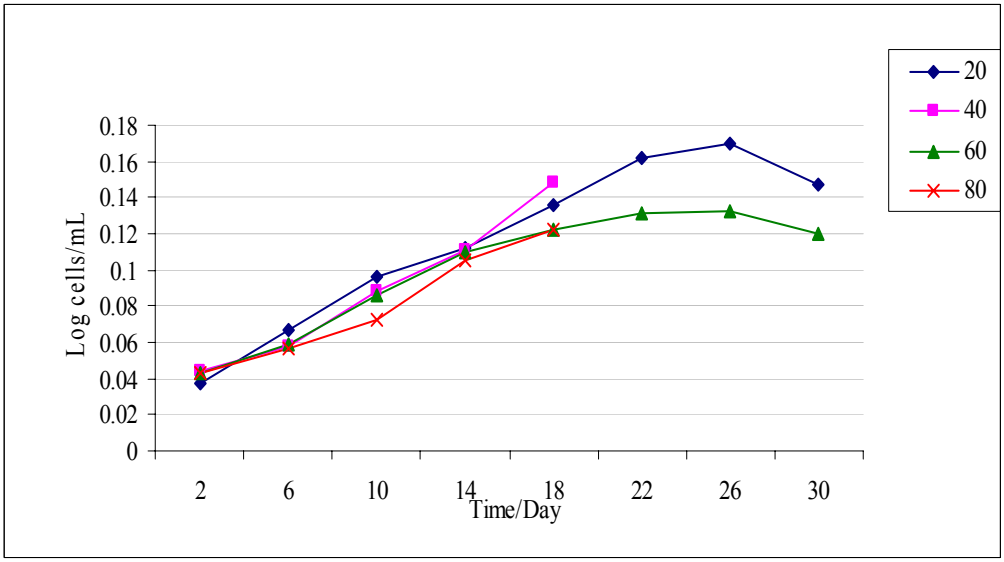


Figure 4.16 Effect of light on growth rate of *Scytonema javanicum*

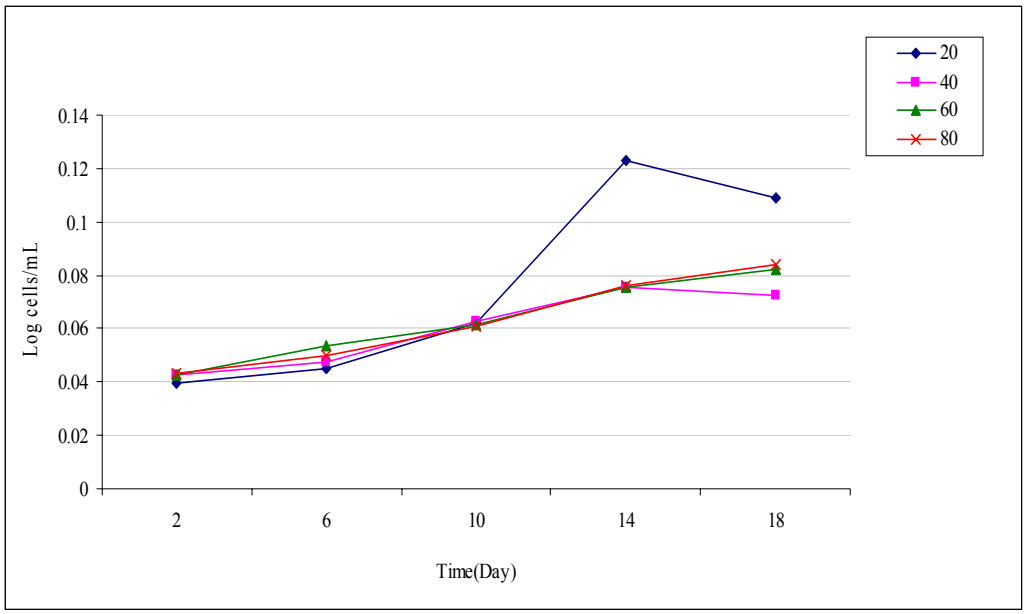


Figure 4.17 Effect of light on growth rate of *Nostoc punctiforme*

4.2.1.3 *Phormidium* sp

The growth curve of *Phormidium* sp showed that the specific growth rate measured of this strain was sufficient for high light intensity, around 60 µmol photon quanta $m^{-2}s^{-1}$ and even at 80 µmol photon quanta $m^{-2}s^{-1}$ still gown well to compare with others lower light intensities such as 20 and 40 µmol photon quanta $m^{-2}s^{-1}$. The results also indicated with photon fluxes at 20 µmol photon quanta $m^{-2}s^{-1}$ was not sufficed for the maximum growth rate of Phormidium sp.

4.2.1.4 *Scytonema javanicum* (Kürtz.) Bornet ex Born.et Flah

Scytonema javanicum was the only one branching cyanobacteria selected for the experiment and the result suggested that this strain was not able to grow in the high light intensity. The growth curve showed that light intensity appropriated for growth was no higher than 40 µmol photon quanta $m^{-2}s^{-1}$. The treatments with 60 and

80 µmol photon quanta $m^{-2}s^{-1}$ were broken down after two weeks exposure to high light intensities. In particular, the color was became yellowish and the growth was down.

4.2.1.4 *Nostoc punctiforme* (Kürtz.) Hariot

Nostoc punctiforme was not also able to acclimate to high light intensity. The experiment was failed after exposing to illumination around nearly two weeks. Therefore the experiment for determination growth curve of Nostoc punctiforme was not successful with these conditions.

In over all, there were a large variations occurred in the growth rate measurements of fives filamentous cyanobacteria strains, according to their life forms. In particular, *Oscillatoria limosa*, *Phormidium* sp, and *Nostoc punctiforme* were in strong cluster, difficult to disintergrate and to take average of volume needed for measurement. Although, the results of experiments showed that growth rate decreased proportionally to the light intensities and proportionally to the time of CB were exposed to light. Presumable that at the high light intensity leading to photoinhibition and in this case cyanobacteria reacts quite differently to light intensity,. This result was agreed with the conclusion of Janssen (1998).

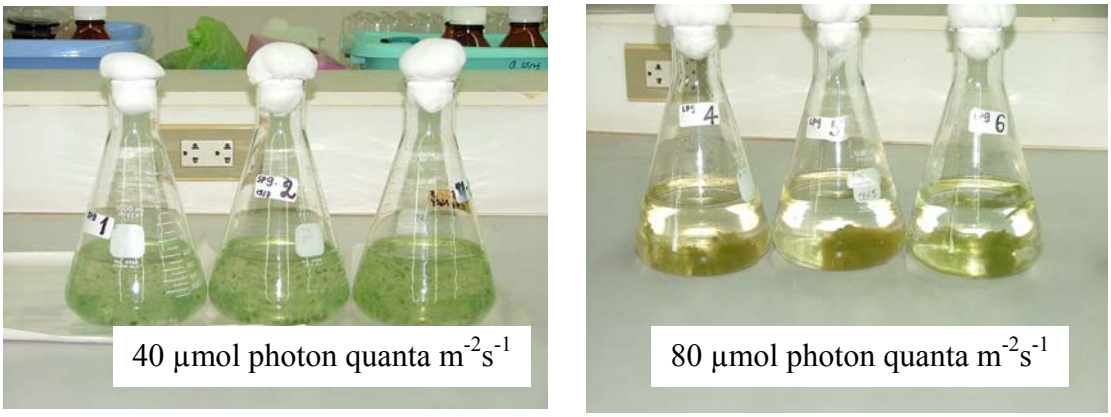


Figure 4.18 *Scytonema* culture at different photon fluxes intensities

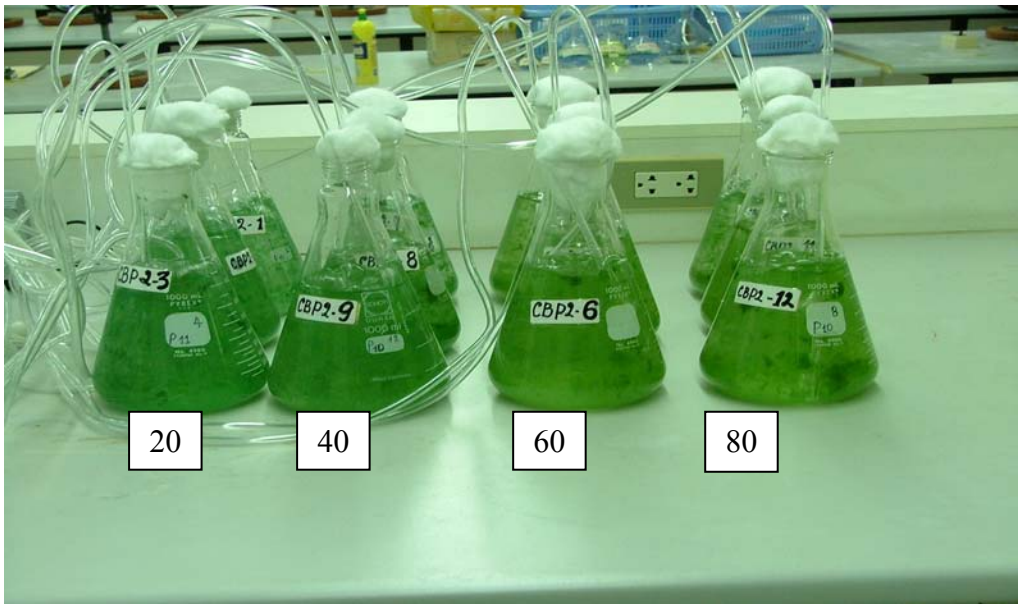


Figure 4.19 *Oscillatoria limosa* culture at different photon fluxes

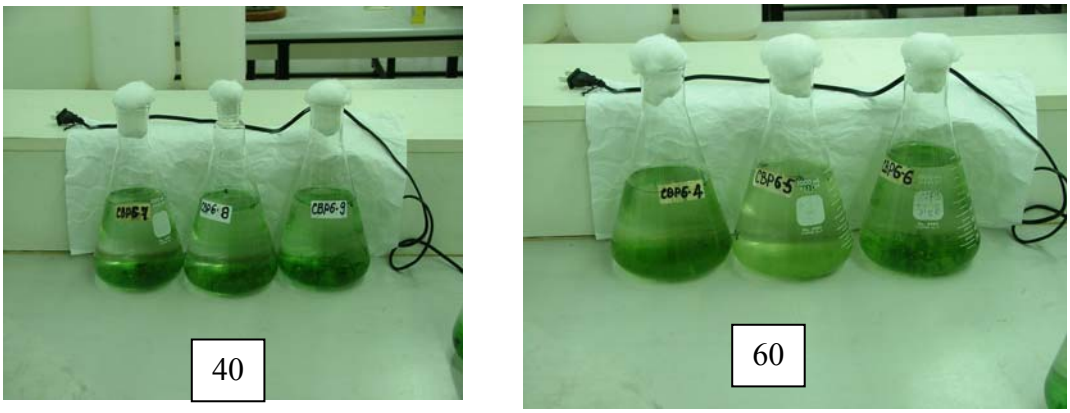


Figure 4.20 *Phormidium* sp culture at 40 and 60 µmol photon quanta $m^{-2}s^{-1}$

4.2.1 Effect of salinity on growth of filamentous cyanobacteria

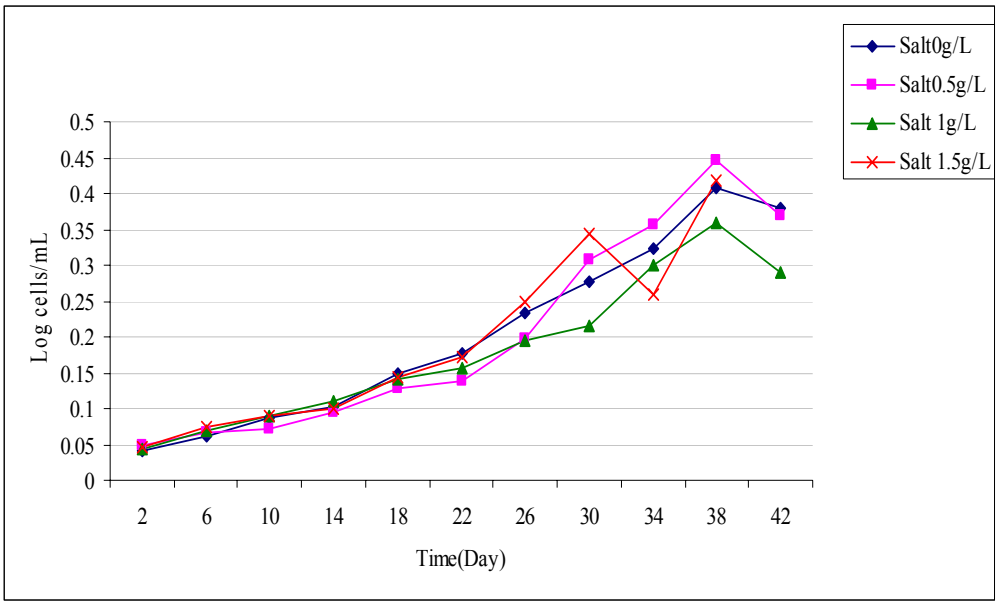


Figure 4.21 Effect of salinity on growth of *Oscillatoria limosa*

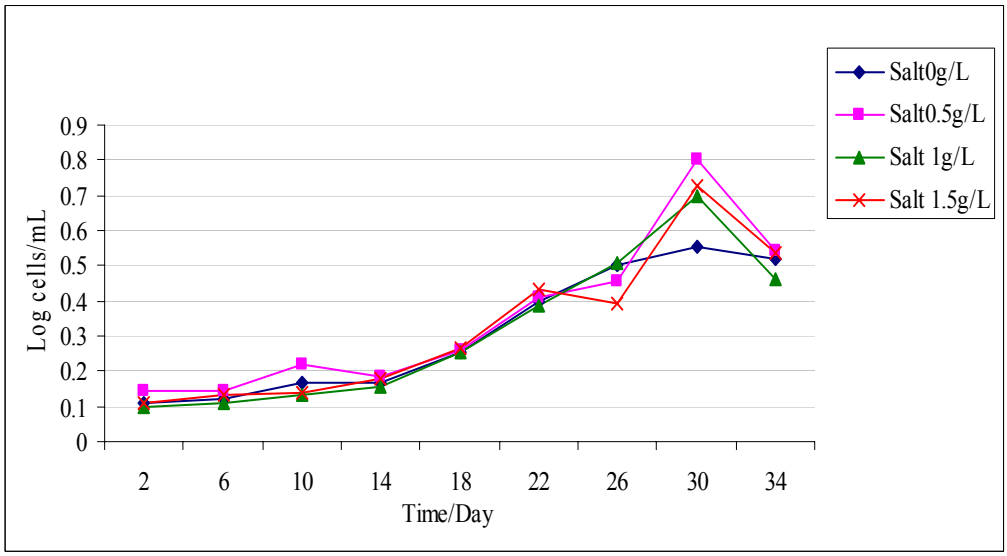


Figure 4.22 Effect of salinity on growth of *Phormidium laminosum*

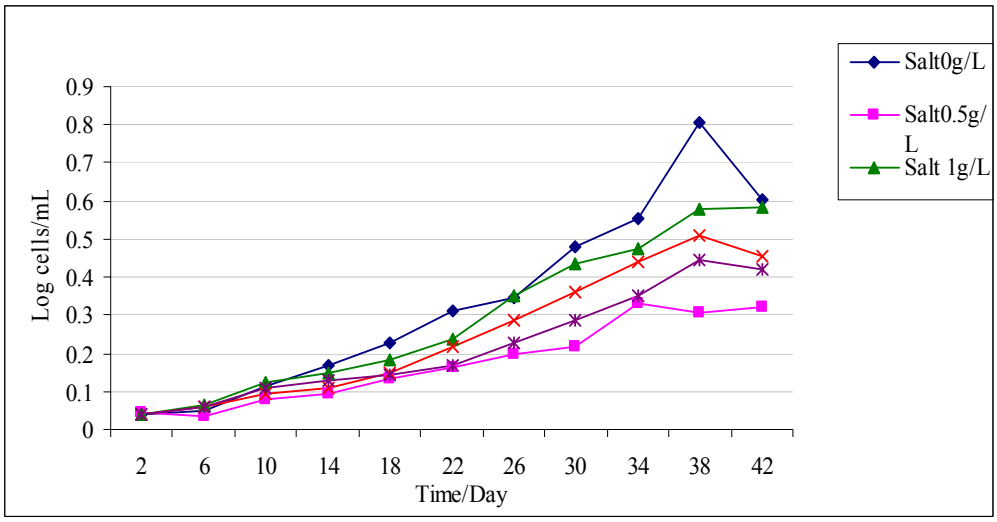


Figure 4.23 Effect of salinity on growth of *Phormidium* sp

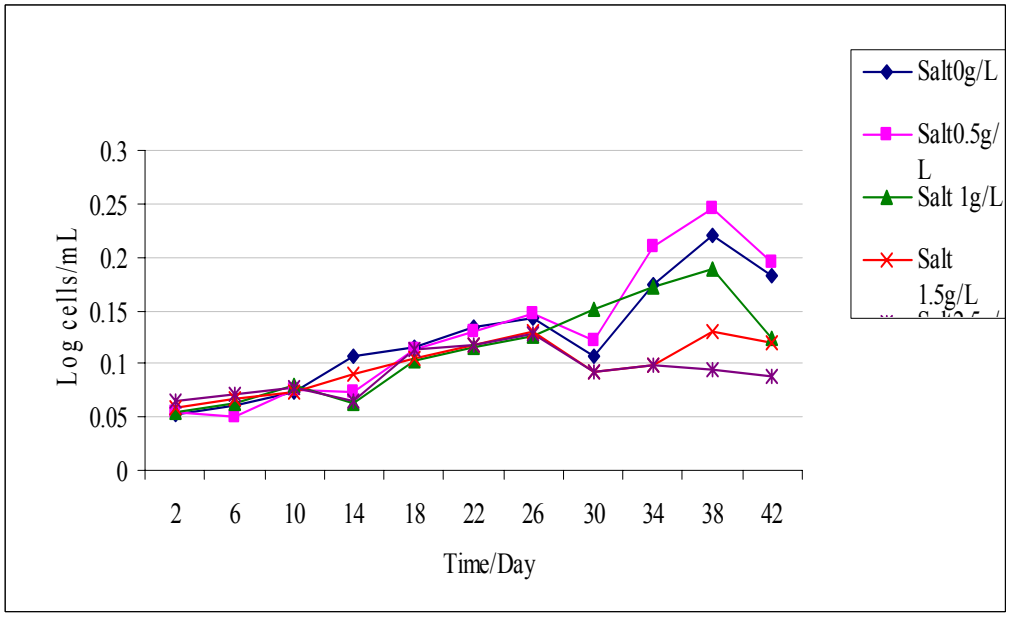


Figure 4.24 Effect of salinity on growth of *Scytonema javanicum*

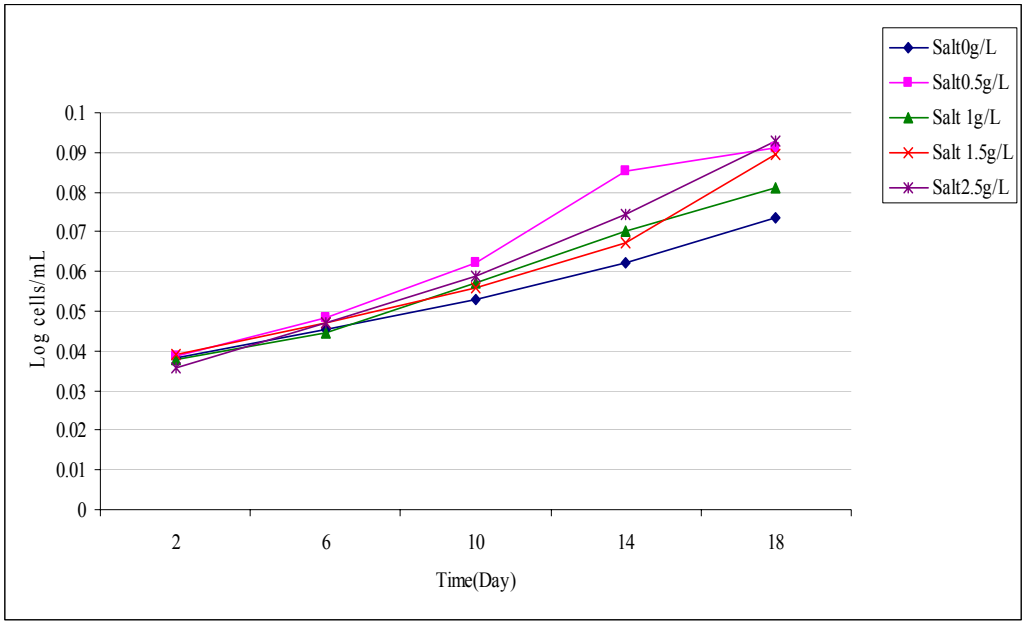


Figure 4.25 Effect of salinity on growth of *Nostoc punctiforme*

4.2.2 Effect of light on chlorophyll *a*

Cultures of fives strains, growing in the BG 11 medium and exposed to varying light intensities. In particular, exposed to 20, 40, 60, 80 40 µmol photon quanta $m^{-2}s^{-1}$ Figures bellow showed the pigment a content of cell obtained from the early to late exponential growth phases. The results were slightly typical for each strains as in follows:

4.2.2.1 *Oscillatoria limosa*

When cells growing exponentially with medium for blue green algae, the color of cell cultures exposed to 20, 40 µmol photon quanta $m^{-2}s^{-1}$ were greenish, while the treatments with 60 and 80 µmol photon quanta $m^{-2}s^{-1}$ were changed from greenish to green-yellowish color. As showed in photo…below. The result of chlorohyll *a* indicated that amount of pigment in the cell increased by the exponential

growth phases, particularly the content was highest at the mid to late exponential phase. In other hand, noted from the result that the amount of chlorophyll *a* increased proportionally with biomass or growth curve of culture. As for the *Oscillatoria limosa* the intensities of light from 20 to 80 µmol photon quanta $m^{-2}s^{-1}$ were not affect to accumulation of chlorophyll *a.* The statistic analysis had no significant between treatments.

4.2.2.2 *Phormidium laminosum*

Light intensity affected to chlorophyll a content in *Phormidium laminosum* during the exponential growth phases. Photon fluxes at 40µmol photon quanta $m^{-2}s^{-1}$ was the most appropriated light condition for accumulation of chlorophyll *a.* The curve showed that amount of chlorophyll *a* increased sharply at mi exponential growth phase and slightly decrease by the late exponential to stationary phase.

4.2.2.3 *Phormidium* sp.

Light intensity affected also to chlorophyll *a* content in cell culture of *Phormidium* sp which is slightly in the same trend of *Phormidium laminosum*. As for the *Phormidium* sp. photon fluxes at 20-40 µmol photon quanta $m^{-2}s^{-1}$ seemed to be optimal light condition for growth and chlorophyll *a* accumulation, while photon fluxes intensity at 60-80 µmol photon quanta $m^{-2}s^{-1}$ showed the photoinhibition, especially at the late exponential phase to stationary phase, the amount of chlorophyll *a* decreased more quickly to compare with other treatments.

4.2.2.4 *Scytonema javanicum*

High light illumination affected to coloration of *Scytonema javanicum*. During the experiment the coloration of the treatment of 60 and 80 µmol photon quanta $m^{-2}s^{-1}$ were changed continuously from greenish to green-yellowish or yellowish started from mid exponential phase. The amount of chlorophyll were dropped more quickly to compare with lower light level, for example at around 20-24 days, while the treatment of lower light illumination were dropped at 28 to 30 days of growth.

4.2.2.5 *Nostoc punctiforme*

As for the *Nostoc punctiforme*, the result of chlorophyll *a* showed in similar significance of growth curve. The experiment were not successful for measurement of chlorophyll *a* content for almost four treatments. The intent increased of curve at around two weeks after exposition to light was the error of volume taken for pigment analysis. *Nostoc punctiforme* experiment suggested that this method was difficult to perform, because of strong cluster of the cells and this is low light grow species.

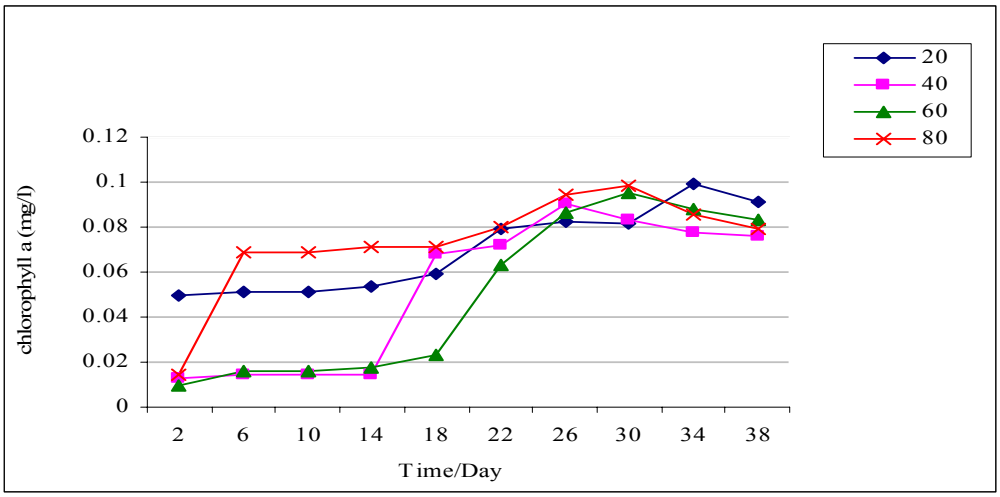


Figure 4.26 Effect of light on chlorophyll *a* content of *Oscillatoria limosa*

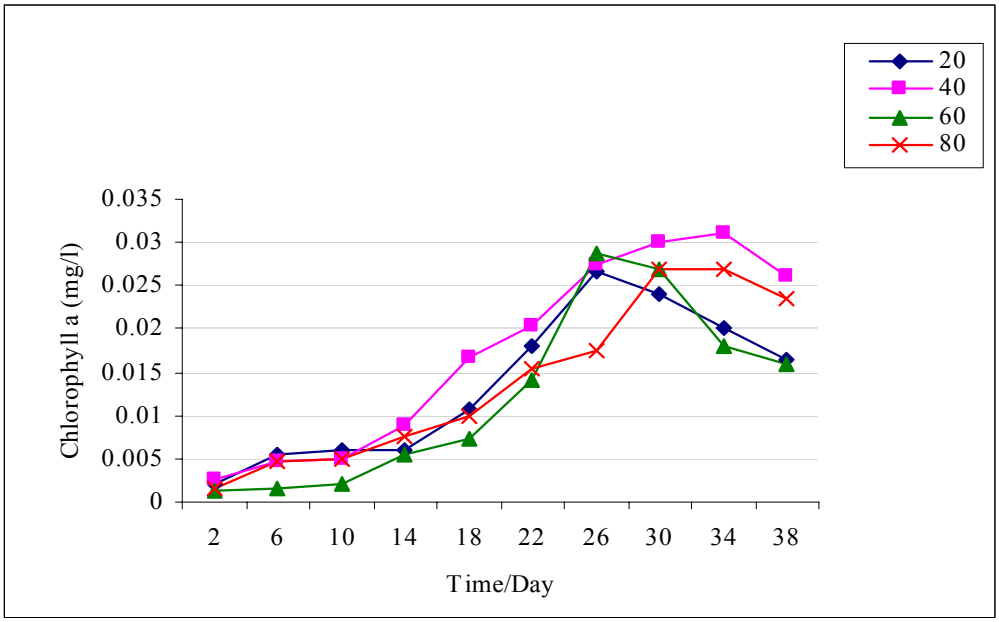


Figure 4.27 Effect of light on chlorophyll *a* of *Phormidium laminosum*

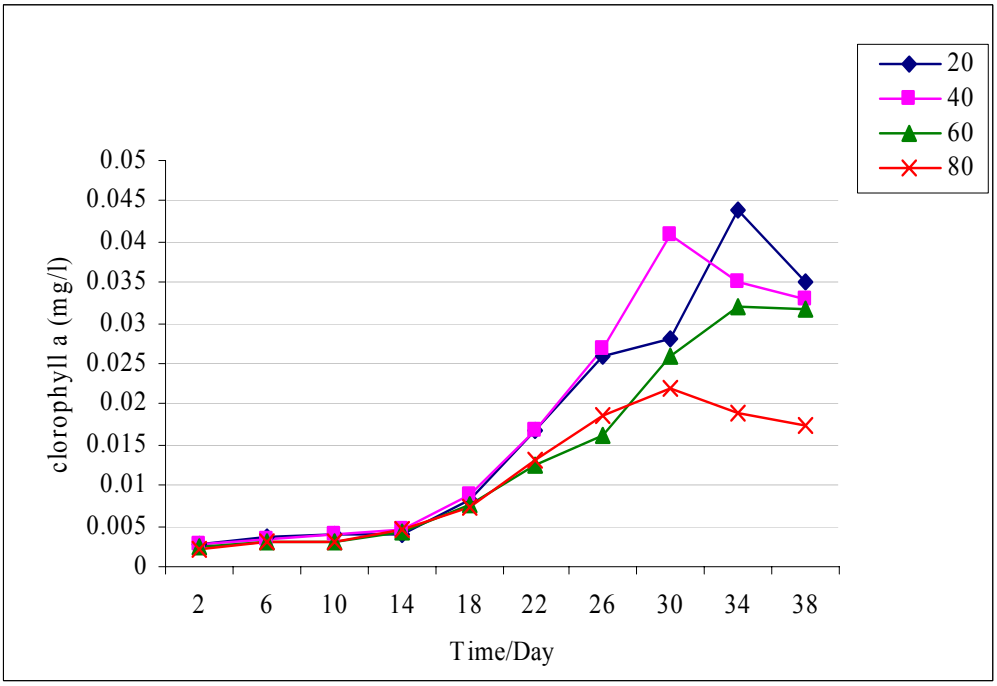


Figure 4.28 Effect of light on chlorophyll *a* content of *Phormidium* sp

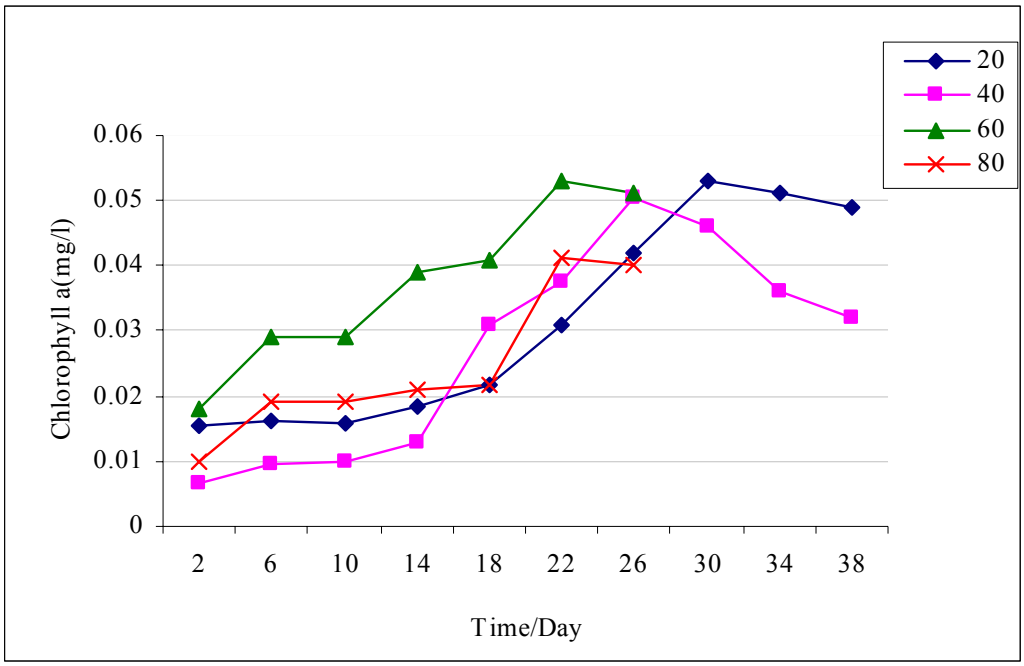


Figure 4.29 Effect of light on chlorophyll *a* content of *Scytonema javanicum*

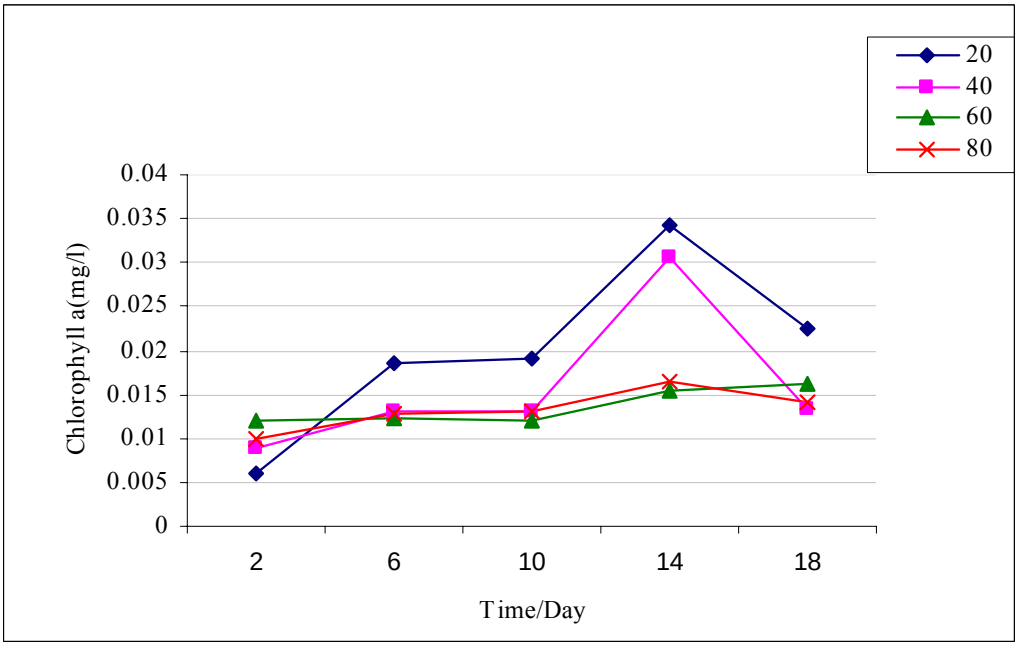


Figure 4.30 Effect of light on chlorophyll *a* content of *Nostoc punctiforme*.

4.2.3 Effect of light on beta-carotene.

Culture of five filamentous cyanobacteria were taken at mid-exponential growth phase for β-carotene measurement by HPLC. The results of β-carotene in each strain showed in the figure 4.31 bellow. *Scytonema javanicum* was the most high β-carotene content strain and measured highest at photon fluxes 40 μmol photon quanta $m^{-2}s^{-1}$ with 28.01 mg per liter, and the control also measured high amount with 23,85 mg per liter. Next high β-carotene content strain was *Phormidium laminnosum* , measured highest at 0.5 g per liter and it was in similar amount at 40 and 60 μmol photon quanta $m^{-2}s^{-1}$ and 8080 μmol photon quanta $m^{-2}s^{-1}$ with 18-19 mg per liter. As for *Phormidium* sp. contained β-carotene highest at 40 μmol photon quanta $m^{-2}s^{-1}$ and decreased in around 20% to compare with the maximum amount of β-carotene. *Oscillatoria limosa* contained β-carotene in a similar amount with *Phormidium* sp with 12, 14 , and 17 mg per liter respectively by levels of light intensities and also found that β-carotene in *Oscillatoria limosa* still increased in the at photon fluxes 80 μmol photon quanta $m^{-2}s^{-1}$. The result of β-carotene of *Oscillatoria* was correlated with result of growth curve and curve of chlorophyll *a* to confirmed one more that this strain was high light acclimatized strain. The lowest β-carotene content species was *Nostoc punctiforme* with which found β-carotene no higher 4.5 mg per liter at 20 μmol photon quanta $m^{-2}s^{-1}$. The result was in similar amount in almost the treatments.

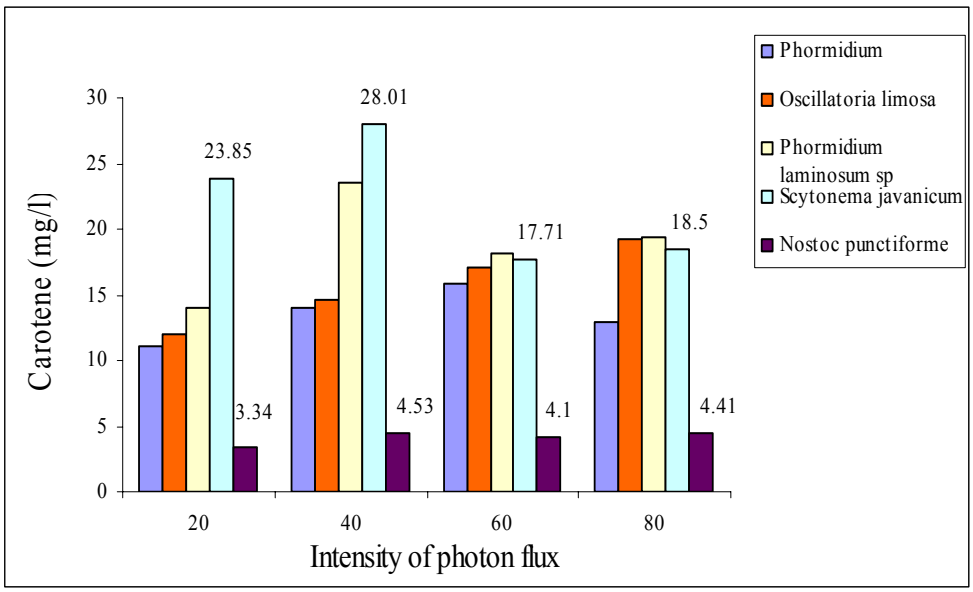


Figure 4.31 Effect of light on content β-carotene of 5 cyanobacterial strains

4.3 Effect of Salinity

The effects of various salt concentrations in the growth medium on growth, chlorophyll *a* content and β-carotene had been performed continuously by the effect of light. The results obtained are presented and discussed as in follows.

4.3.1 Effect of salinity on growth

4.3.1.1 *Oscillatoria limosa*

The results of effect of various salt concentration (0.5 g/Leter; 1 g/Liter; 1.5 g/Liter) on growth rate of *Oscillatoria limosa* was slightly in the same rate in almost three growth phases. The little different significance obtained by error of taking cell culture. Statistic analysis result also showed that there was no significant between the variances. The result indicated that *Oscillatoria limosa* was tolerated to these salt concentrations

4.3.1.2 *Phormidium laminosum.*

Salt concentration from 0.5 to 1.5 g/L showed had no effect on growth rate of *Phormidium laminosum*, including the time of growth phase and rate of growth to compare with the control. The dropped curve of the 1.5 g/L variance was caused by the error of culture volume taken for measurement.

4.3.1.3 *Phormidium* sp.

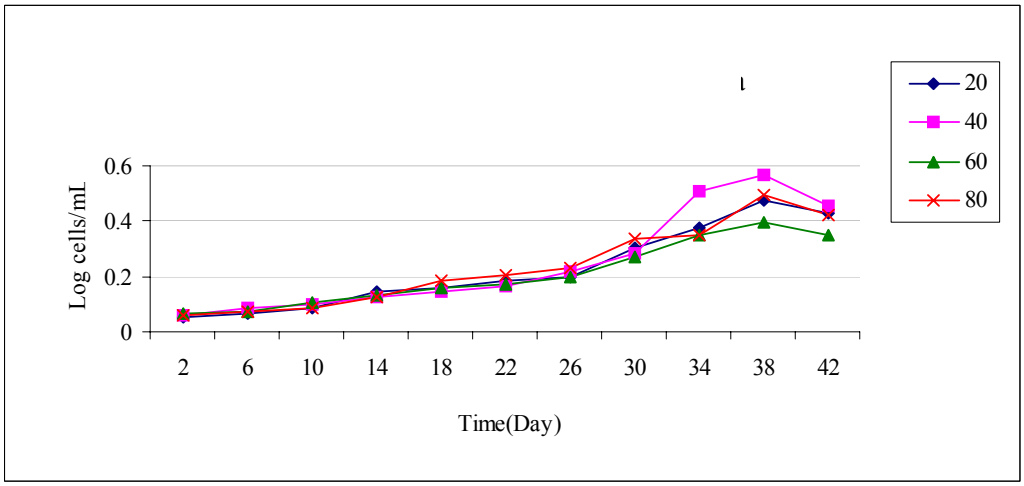


Figure 4.32 Effect of salinity on growth of *Oscillatoria limosa*

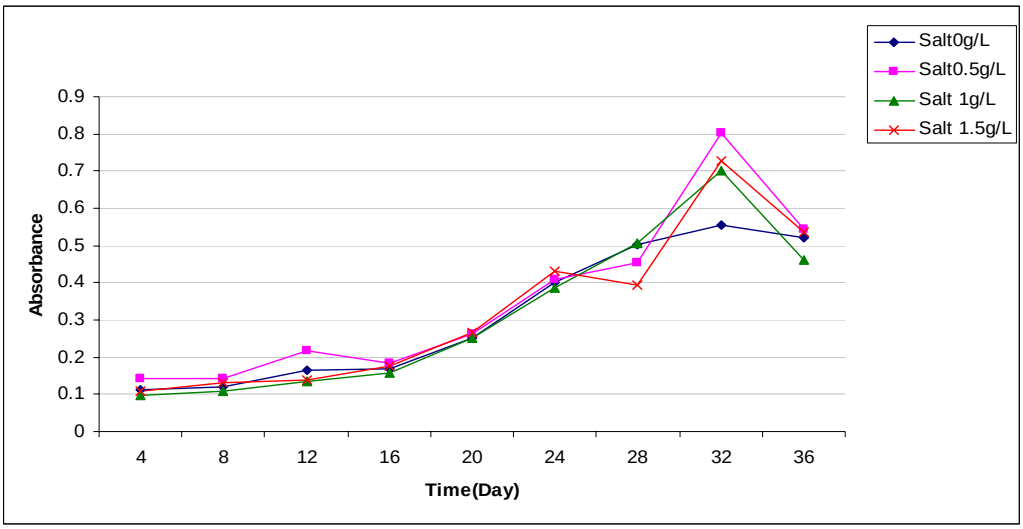


Figure 4.33 Effect of salinity on growth of *Phormidium laminosum*

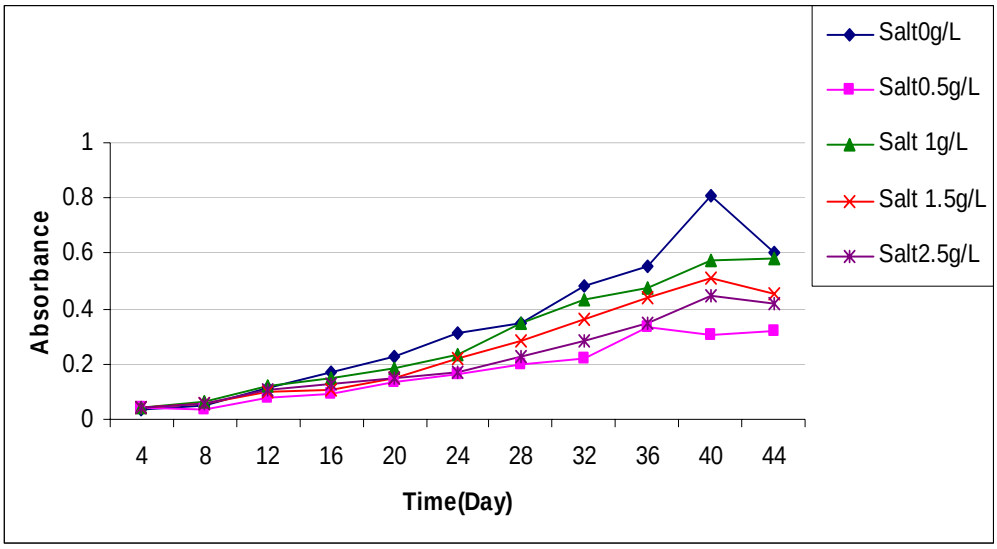


Figure 4.34 Effect of salinity on growth of *Phormidium* sp

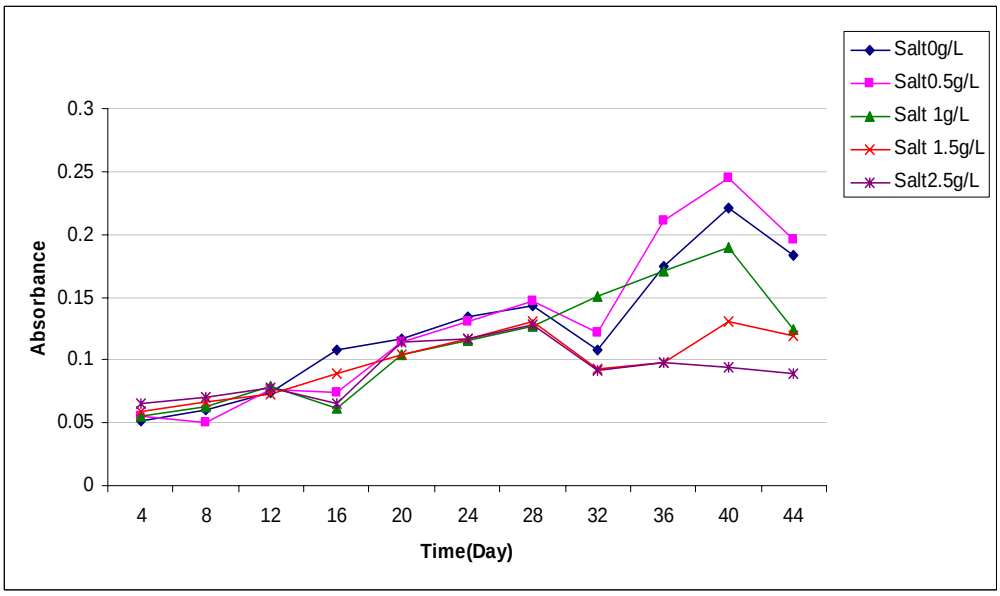


Figure 4.35 Effect of salinity on growth of *Scytonema javanicum*

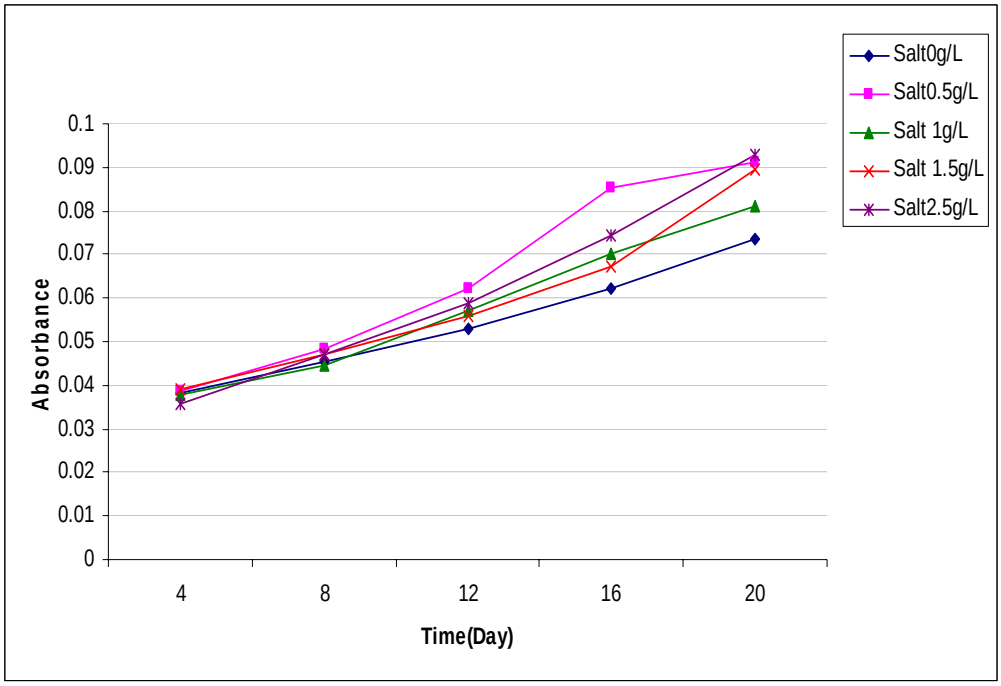


Figure 4.36 Effect of salinity on growth of *Nostoc punctiforme*

4.3.2. Effect of salinity on chlorophyll *a*

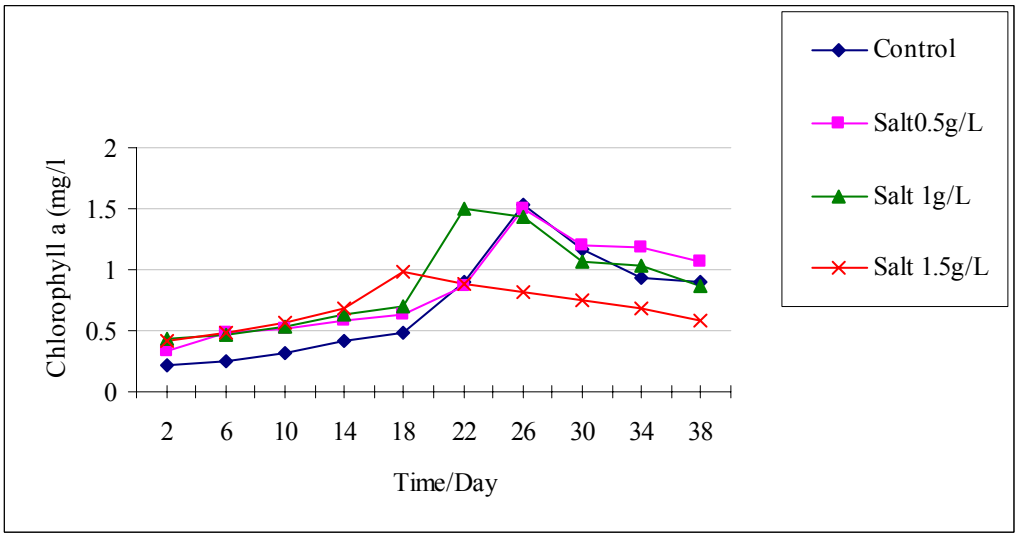


Figure 4.37 Effect of salinity on chlorophyll *a* content of *Oscillatoria limosa*

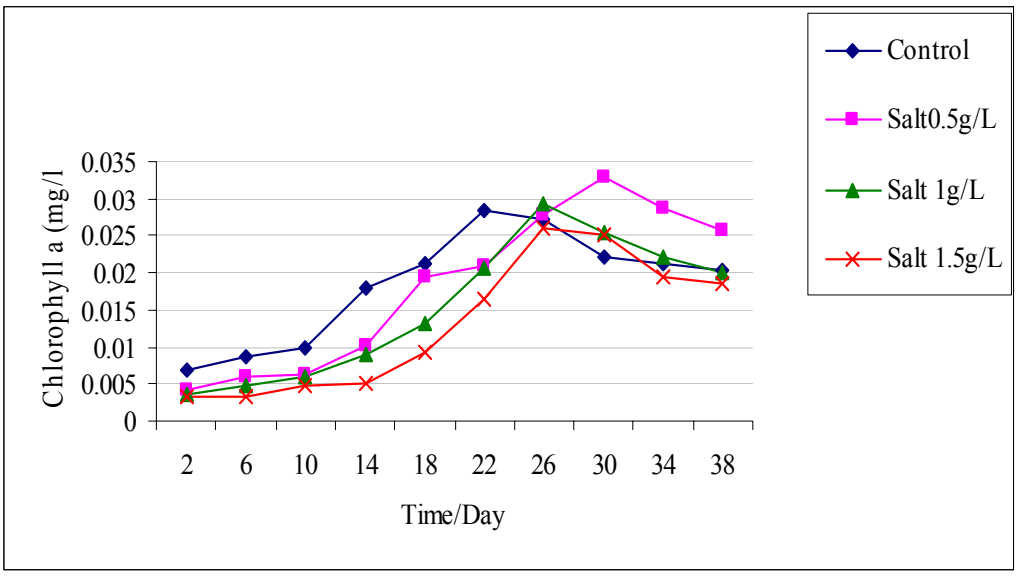


Figure 4.38 Effect of salinity on chlorophyll *a* of *Phormidium laminosum*

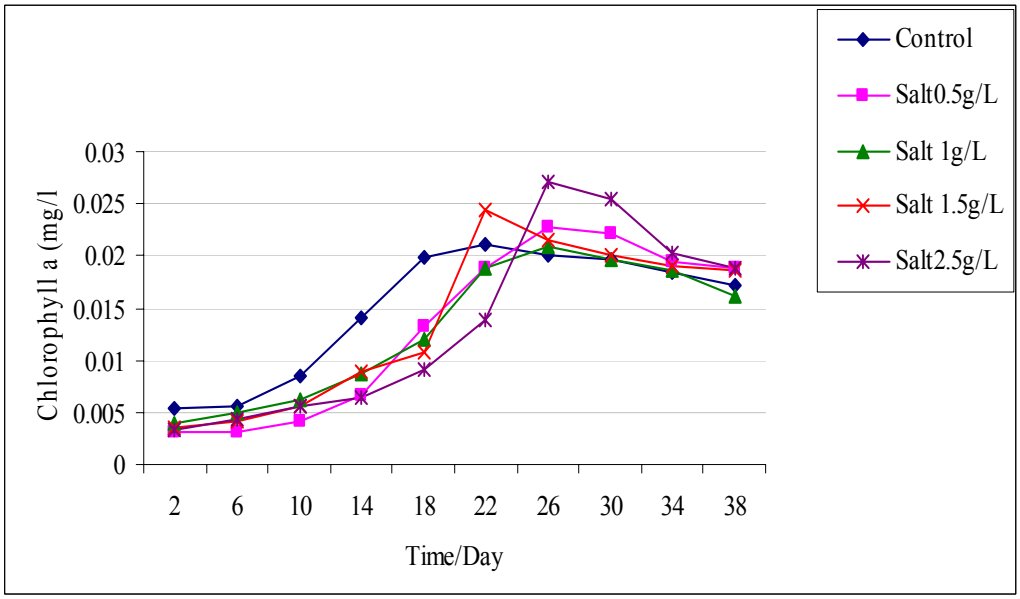


Figure 4.39 Effect of salinity on chlorophyll *a* content of *Phormidium* sp

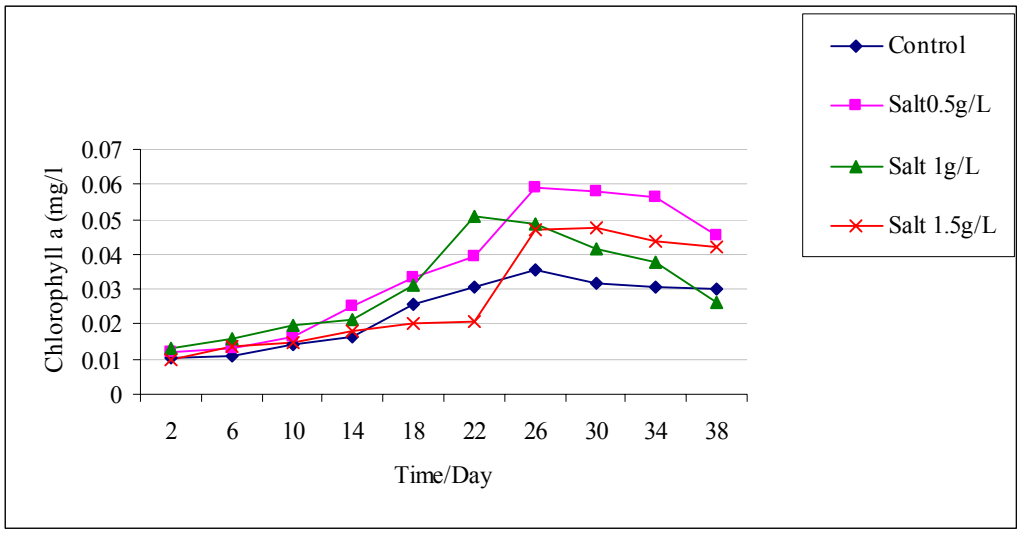


Figure 4.40 Effect of salinity on chlorophyll content of *Scytonema javanicum*

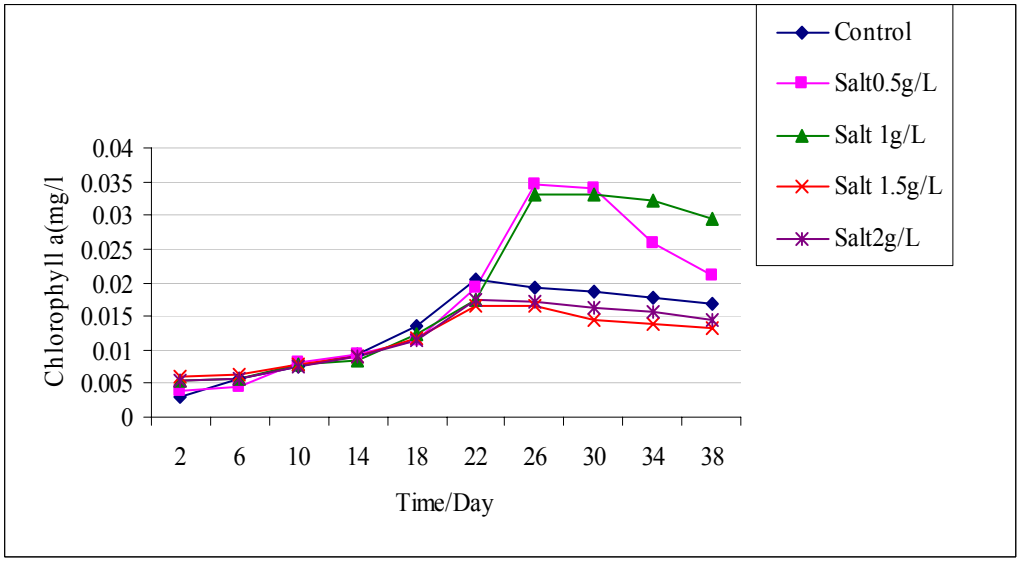


Figure 4.41. Effect of salinity on chlorophyll *a* content of *Nostoc punctiforme*

4.3.3 Effect of salinity on beta-carotene content.

Five filamentous cyanobacteria were grown in five salt concentration: control, no salt; 0.5 g/L, 1g/L, 1.5g/Land 2.5 g/L, but as for the *Oscillatoria limosa* and

Phormidium laminosum were failed at the early of experiment. Therefore results of the last variance obtained only from three strains namely: *Nostoc punctiforme*, *Scytonema javanicum*, and *Phormidium* sp. The culture of fives filamentous cyanobacteria were taken at their mid- exponential growth phase for β-carotene measurement. Results obtained as showed in the figure 4.42

According to the results showed that *Nostoc punctiforme* contained β-carotene higher than other strains, with 84 mg/L, this measured at salt concentration 1 g/L. Nostoc also content high amount of β-carotene in the salt concentration 0.5 to 1.5 g/L, with 64-60 mg/L of β-carotene, respectively. β-carotene was found lowest at control with 21.34 mg/L and also found that β-carotene in *Nostoc* decreased when salt concentration high at 2.5 g/L, and decreased 43% to compare with the maximum amount. *Phormidium laminosum* was the next strain content high amount of β-carotene but highest was at 0.5 g/L salt concentration, 63.25 mg/L. As for the *Phormidium laminosum* found high amount of β-carotene at low salt concentration, especially at zero to 0.5 g/L was effective for β-carotene accumulation, while when salt concentration was increased the amount of β-carotene was decreased down to 32% at 1.5 g/L. *Phormidium* sp. had a moderate amount of β-carotene to compare with other strain and content a similar quality at 0.5 to 1.5 g/L, 31-35 mg/L. The amount of β-carotene found slightly decreased when salt concentration reached 2.5 g/L (22.6 mg/L). Cyanobacterial strain that contained lowest amount of β-carotene was *Scytonema javanicum.* The amount of 6.3 mg/L was measured at salt concentration from 0 to 0.5 g/L. Then β-carotene found decreased sharply from 1 g/L to 2.5 g/L which left only 6.6% to compare with the maximum amount measured in this experiment. The result of this study suggested that salt concentration in the

medium had a significant effect in the production of β-carotene, mostly in *Nostoc punctiforme* and *Scytonema javanicum*.

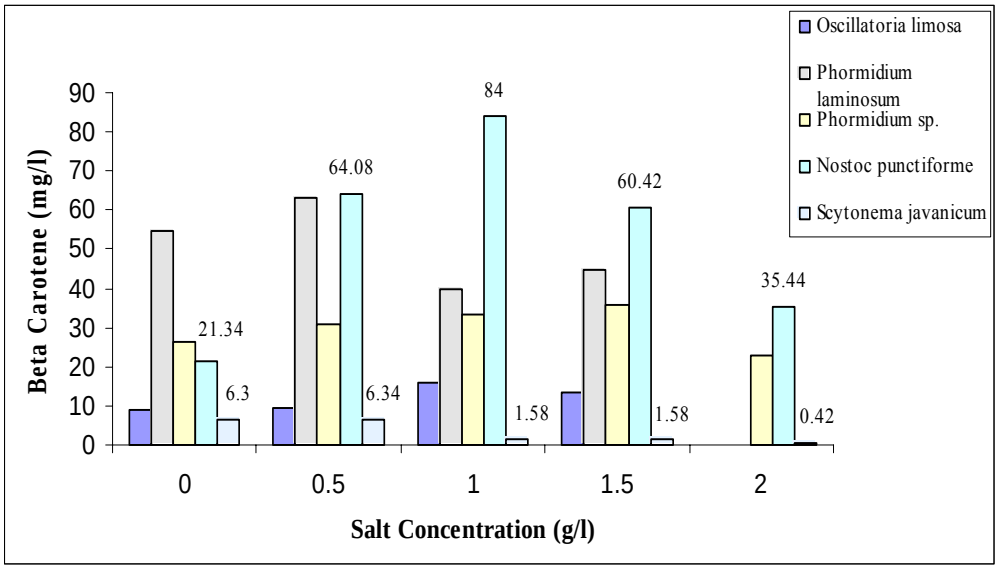


Figure 4.42 Effect of salinity on β-carotene

4.4 Spectroscopic Analysis

A spectral data set was obtained by using infrared spectroscopy which measures bond vibrations of functional groups within organic compounds such as lipid, protein, carbohydrate and nucleic acid contained in the cyanobacterial cells. Fingerprint- like infrared spectra are acquired and used for strain discrimination. The results obtained were compared with a previous published study (Kansiz *et al.*, 1999) which used FTIR spectroscopy to analyse and discriminate freshwater cyanobacteria.

The spectra of 7 cyanobacterial strains were collected at early, mid and late exponential growth phase with 30 spectra acquired in each growth phase. The recorded spectra display the typical bands associated with biological samples (see Figure 4.43).

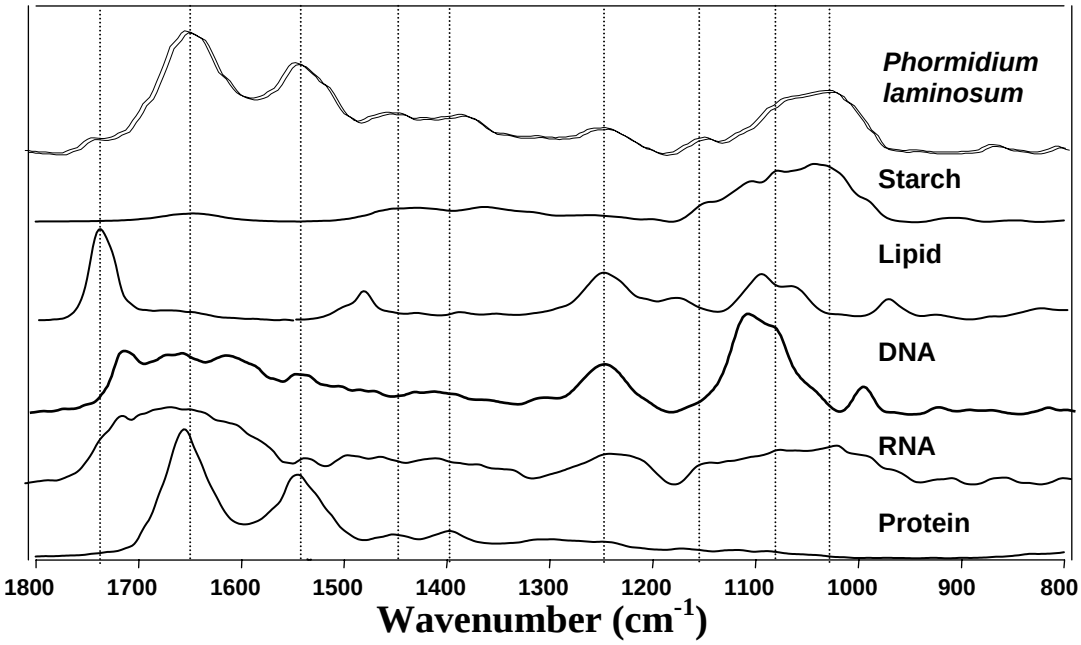


Figure 4.43 Showing an FTIR spectrum of *Phormidium laminosum* versus spectra from the major macromolecular components found in the cells.

4.4.1 Averaged spectra

30 spectra IR spectra collected from each of the 7 strains at each of the three growth phases were averaged (Figure 4.46). Band assignments attributable to the major macromolecular classes of lipid, carbohydrates, nucleic acids and proteins were observed. These are summarized below.

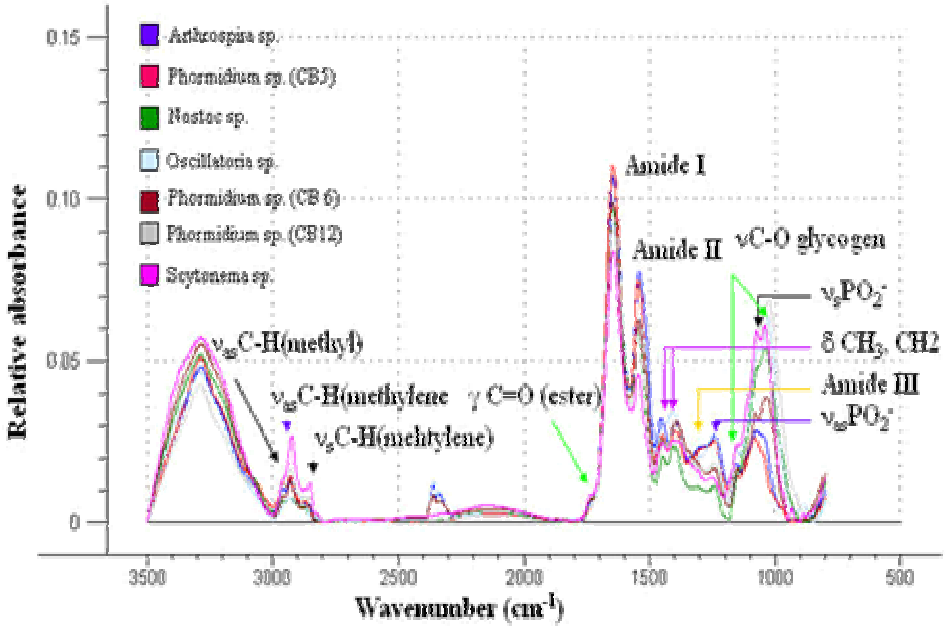


Figure 4.44 Averaged spectra at early phase

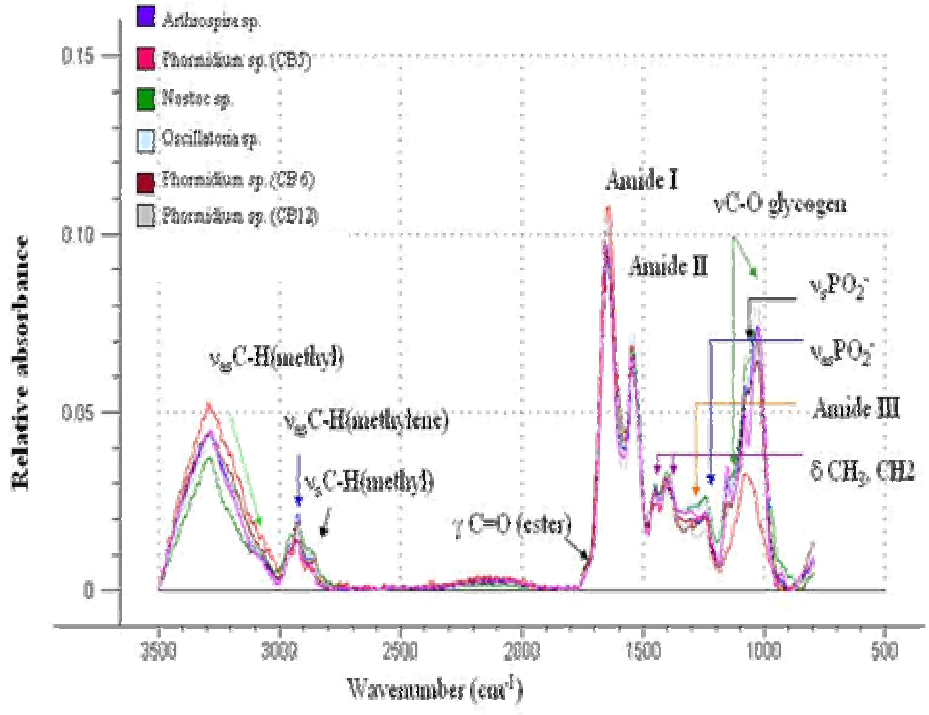


Figure 4.45 Averaged spectra at late-phase

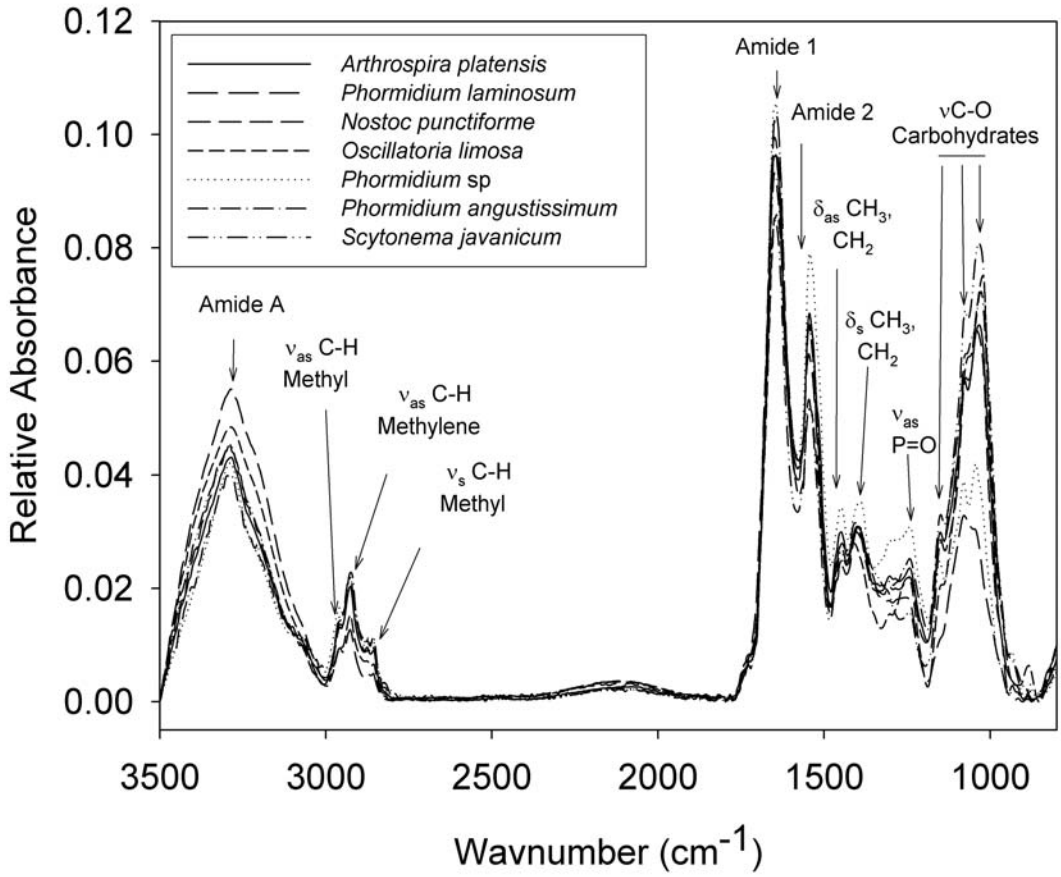


Figure 4.46 Showing average spectra from 30 spectra acquired from each of the 7 cyanobacterial strains at the mid exponential growth phase. The major bands in the spectrum are identified.

4.4.2 Band Assignments

Lipid group vibration

Bands between 2970 and 2850 cm^{-1} acquired due to the carbon-hydrogen (C-H) stretch absorption of the hydrocarbon chain. These are attributed to the asymmetric

C-H stretching of methyl (2970 cm^{-1}), and methylene (2920 cm^{-1}) and symmetric stretching vibration of methyl (2850 cm^{-1}) group of lipids. These vibrations also result from hydrocarbon chains within carbohydrate molecules as well. The ester group exhibited the very weak symmetric C=O stretching vibration at 1747 cm^{-1} is uniquely attributed to lipids.

Protein group vibration

Amide vibration found at 1700-1540 cm^{-1}. The peptide group, the structural repeating unit of proteins, gives up to 9 characteristics bands named amide A,B, I,II,…VII. The amide I and II which are the most intense bands of the protein infrared spectrum. The amide I band between 1600-1700 cm^{-1} is mainly associated with the C=O stretching vibration and is directly related to the backbone conformation of proteins. Amide II results mainly from the N-H bending vibration.

Phosphate group vibration

Phosphate group vibration result in bands found at 1240 and 1080 cm^{-1} which is the vibration of the P=O group in the phosphodiester backbone of nucleic acids (DNA, RNA) and nucleotides such as ATP. The symmetric vibration of phosphate group found at 1080 cm^{-1} and asymmetric is found at 1240 cm^{-1}.

Carbohydrate stretching vibrations

The -O-C stretching vibrations from carbohydrates are found at 1150 cm^{-1}, 1058 cm^{-1} and 1030 cm^{-1}. In cyanobacteria these IR bands result mainly from starch.

4.4.3 Spectral variability

Figure 4.47 shows the averaged spectra of *Nostoc punctiforme* cells harvested at the mid exponential growth phase. There are 26 spectra which were collected from three replicates of cultures, from these spectra all the bands from the major macromolecular classes described above were observed, namely lipid, protein, phosphate group from DNA and RNA and bands from carbohydrates. The small spectrum at the bottom of the figure is obtained by calculating the standard deviation for each wavenumber value across the data set. This gives a measure of variation between samples at different regions of the spectrum. The variability from spectrum to spectrum was quite small compared with the over all absorbance of the spectra (less then 10%), which is typical for all species measured in the study.

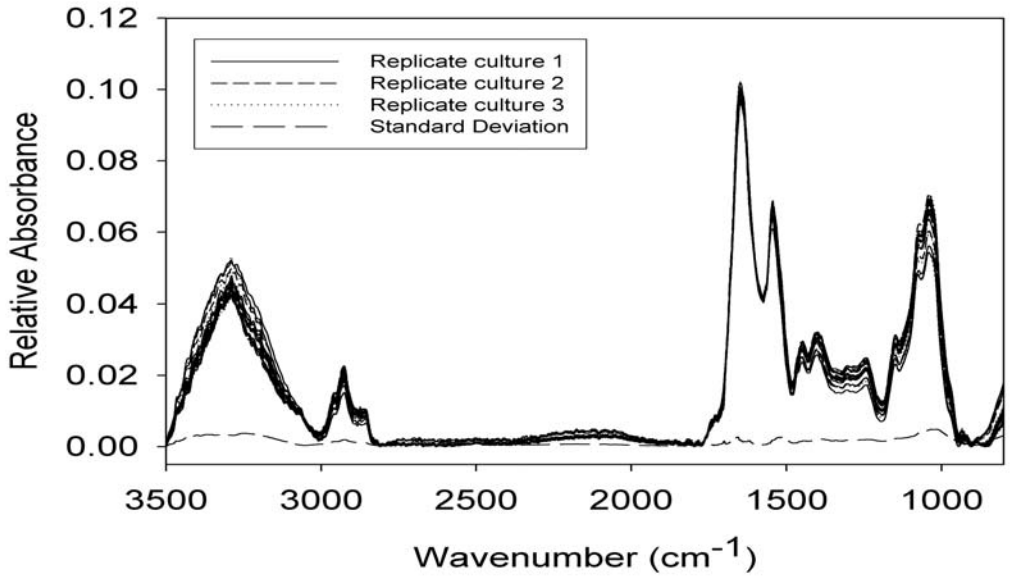


Figure 4.47 26 mid-exponential *Nostoc punctiforme* spectra drawn from 3 replicate cultures. The standard deviation curve represents the standard deviation of absorbance for each sample at each wavenumber value.

4.4.4 Changes in macromolecular composition of cyanobacteria over the growth phases

The relative absorbance of bands in the FTIR spectrum attributable to macromolecular classes such as proteins or carbohydrates in microalgal cells have been shown to correlate very well with the relative concentration of these macromolecules measured by conventional chemical extraction methods (Giordano *et al.*, 2001; Beardall *et al.*,2001). In this study we used the ratio of the absorbance of the most intense band in the spectrum attributable to carbohydrates (the band at 1030 cm^{-1} for the C-O stretching vibrations from carbohydrates) to the absorbance of the major protein band (the amide I band at 1650 cm^{-1}) as an indicator of changes in the relative concentrations in protein and carbohydrate in cells over the exponential growth phase. We measured the ratio of the absorbance of the bands at 1030cm^{-1} and 1650 cm^{-1} obtained from FTIR spectra for each cyanobacterial strain tested at the early, mid and late exponential growth phase (Figure 4.48). The results show that in most strains there is a steady increase in this ratio from the early to late exponential growth phase. This is most marked in *Arthrospira platensis* where there is more than a threefold increase, whereas in *Phormidium laminosum* and *Phormidium angustissimum* the ratio is quite invariant from the early to late exponential phase. Decreasing protein concentration and increasing concentrations of storage molecules such as lipids or carbohydrate are consistently seen in cyanobacterial and eukaryotic microalgal cultures that become limited of a major nutrient such as nitrogen or phosphorus (e.g. Beardall *et al*., 2001; Giordano *et al.,* 2001). The spectral changes seen in some species may indicate limitation of N or P as cultures reach the late exponential phase. This is consistent with reports that batch cultures of cyanobacteria

enter the stationary phase of growth because of major nutrient limitation (Henriksen *et al*., 2002)

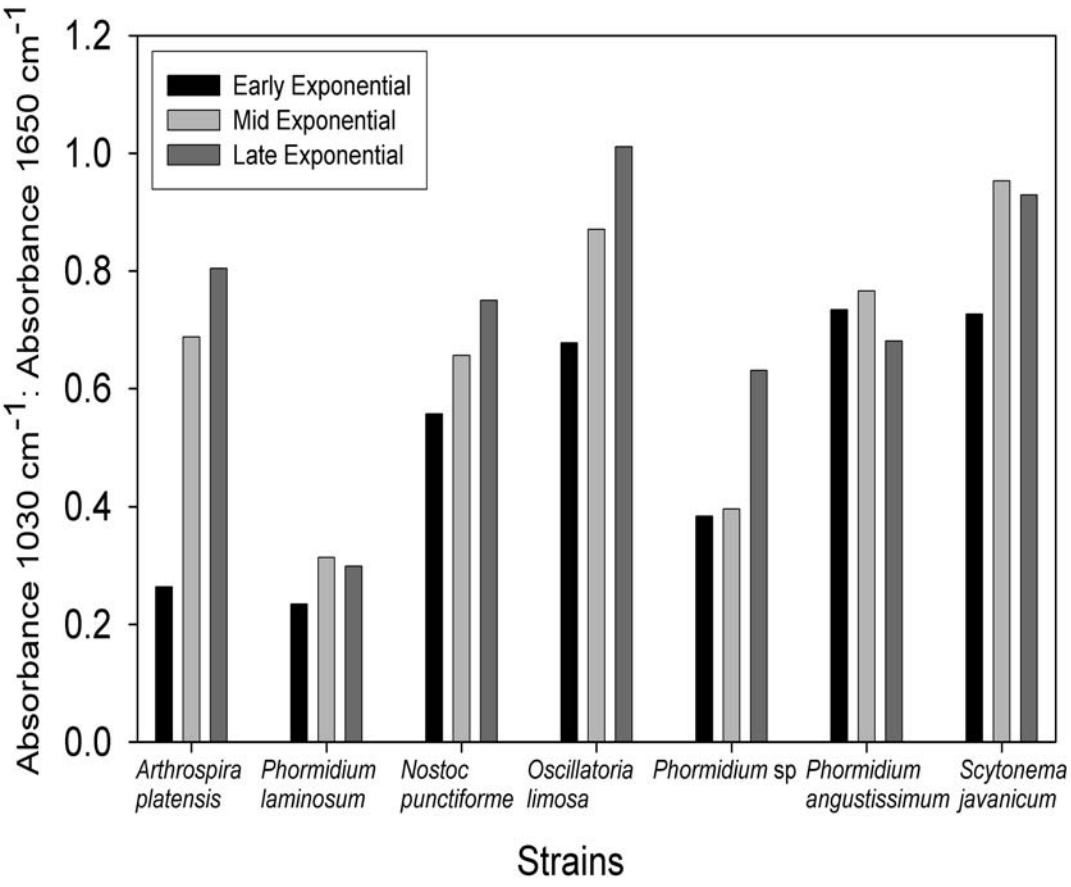


Figure 4.48 Showing the ratio of the band from carbohydrates at 1030 cm^{-1} to the amide I band at 1650 cm^{-1} from baseline corrected, vector normalised FTIR spectra acquired from the 7 cyanobacterial strains at early, mid and late exponential growth phases.

4.4.5. Examination of changes in macromolecular composition during the exponential growth phase using principal component analysis

The multivariate statistic method Principal component analysis (PCA) can be used to reduce the complexity of spectral data. This is done by defining vectors called principal components (PCs). Principal component 1 (PC1) is first defined in a direction that describes the largest variation in the data set. The next principal component (PC2) is the drawn orthogonally to PC1 in a direction that defines the next largest portion of variance in the dataset, which will be independent from variance described by PC1. Any number of PCs can be drawn each orthogonal to each other and hence describing independent sources of variation. In practice, the first few PCs define the structural variance (in this case chemical differences) and the lower PCs define noise (in this case noise in the spectrometer and the like).

PCA scores plots can be drawn where samples (in this case individual spectra) are projected onto plots drawn using various numbers of PCs (e.g. a PC1 vs PC2, 2-dimensional scores plot). Samples that are at the same position on a scores plot have identical characteristics in reference to variation defined by the PC axes, whereas samples that are furthest away from each other vary the most in the defined characteristics. Loadings plots are used to determine which variables are responsible for differences shown in the scores plots. In the analysis of spectroscopic data a 1-dimensional loadings plot is usually used to analyse which parts of the spectrum (wavenumber variables) are responsible for the patterns that may be observed in the scores plot.

PCA models were made using 6 PCs from spectra from early, mid and late exponential phases for each strain in the spectra range 1800 to 950 cm^{-1}. Scores plots

showed that PC1 defined the major spectral variation between samples. For example, Figure B shows the PC1 vs PC2 scores plot for all *Arthrospira platensis* spectra acquired in all three exponential growth phases. The scores plot clearly shows clustering of the spectra from the three different growth phases. The most distinct is the early exponential spectra which all have a PC1 score of about -0.3. Mid and late exponential spectra are more similar to one another in terms of variation along PC1 with mid exponential growth phase spectra with PC1 scores between 0 and +0.2 and late exponential spectra with PC1 scores between +0.05 and 0.3. The PC loadings plot shows that the amide I and amide II bands at 1650 and 1540 cm^{-1} respectively, are negatively loaded, whereas the spectral region from 1150 to 950 cm^{-1} (attributed to C-O stretching from carbohydrates) is positively loaded.

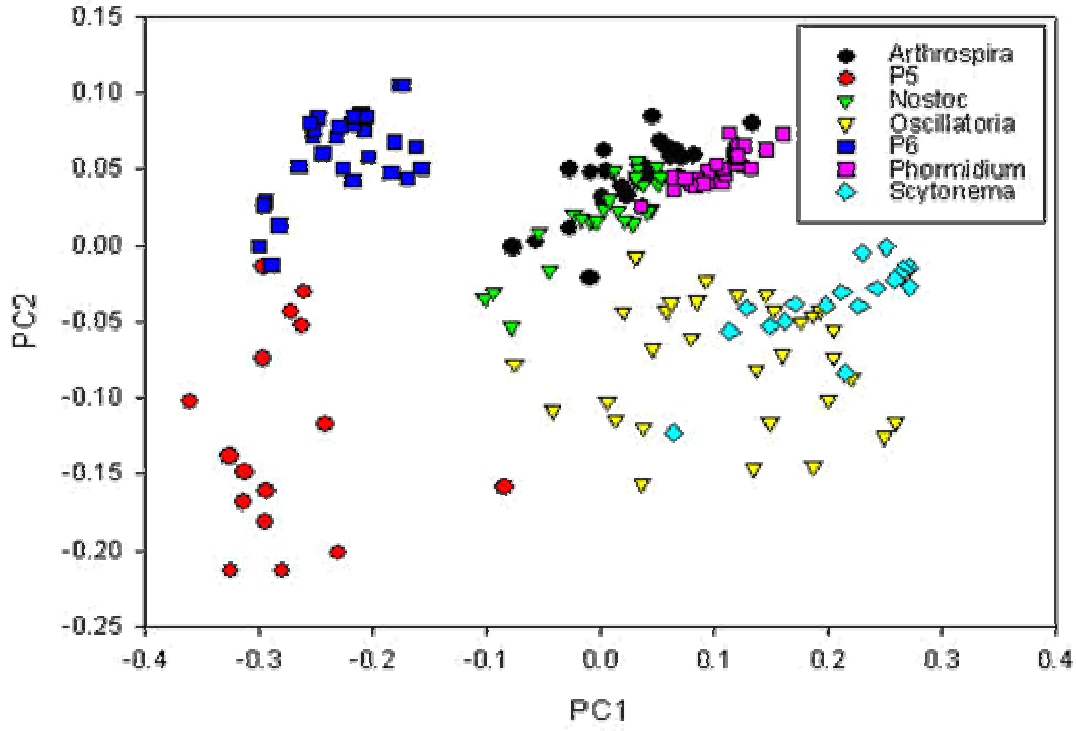


Figure 4.49 PCA scores and loading plot of 7 cyanobacterial strains

Loadings plots are used to interpret scores plots by considering that positively scored samples will have high values for variables that are positively loaded and low values for variables that are negatively loaded, and vice versa for negatively scored samples. In Figure B the early exponential phase spectra are all negatively scored and hence will have high absorbance values for the negatively loaded amide bands and low absorbances for the positively loaded C-O stretching bands from carbohydrates. Conversely, the late exponential phase spectra that are all positively scores will have lower amide band and higher C-O stretching absorbances compared with early exponential phase spectra. This confirms what was assumed from the analysis of the band ratios presented in Figure 4.48 that early exponential phase *Arthrospira platensis*

spectra have lower absorbance from bands attributed to carbohydrate and higher absorbance from protein bands compared to late exponential phase spectra.

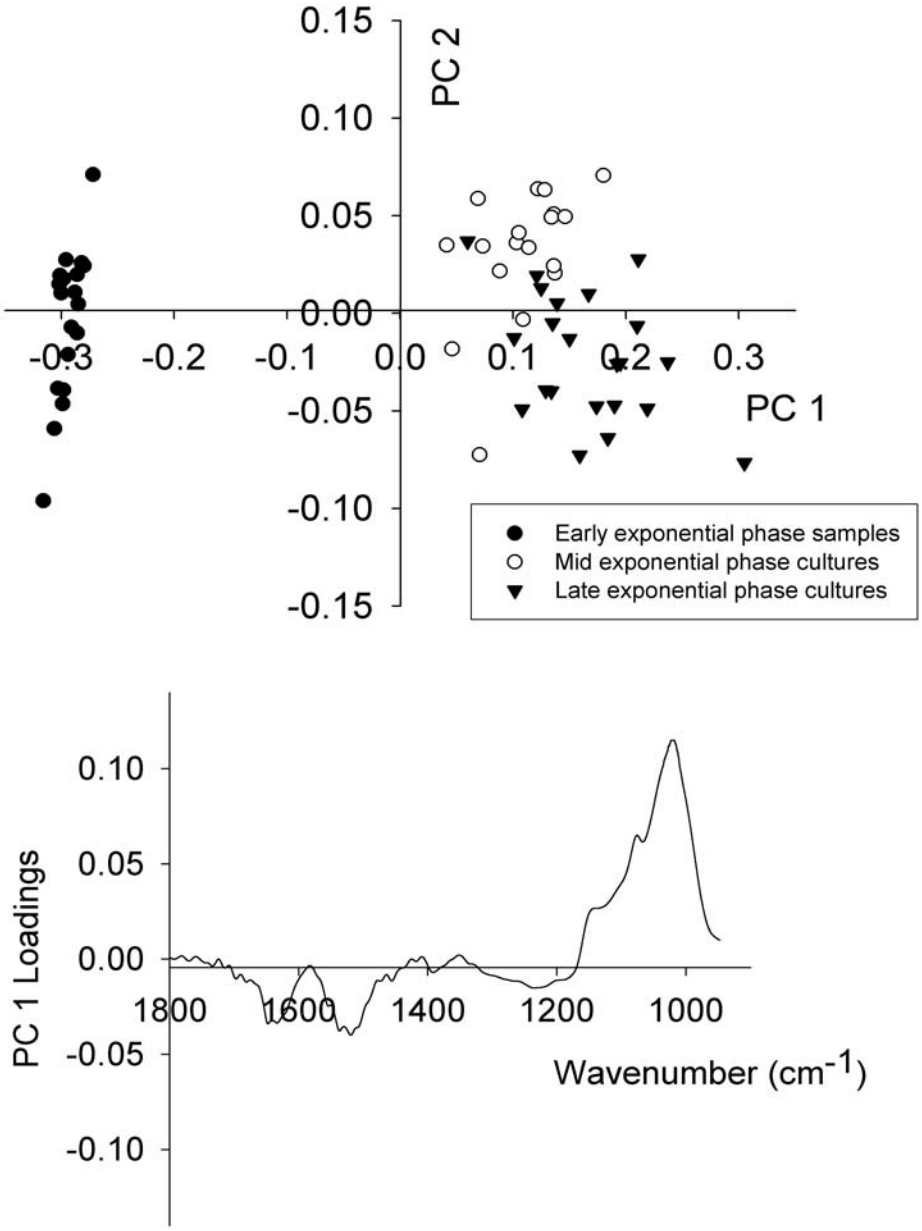


Figure 4.50 Showing PC1 vs PC2 scores plot for all *Arthrospira platenesis* FTIR spectra acquired during the early, mid and late exponential phases of growth, and PC1 loadings for the dataset.

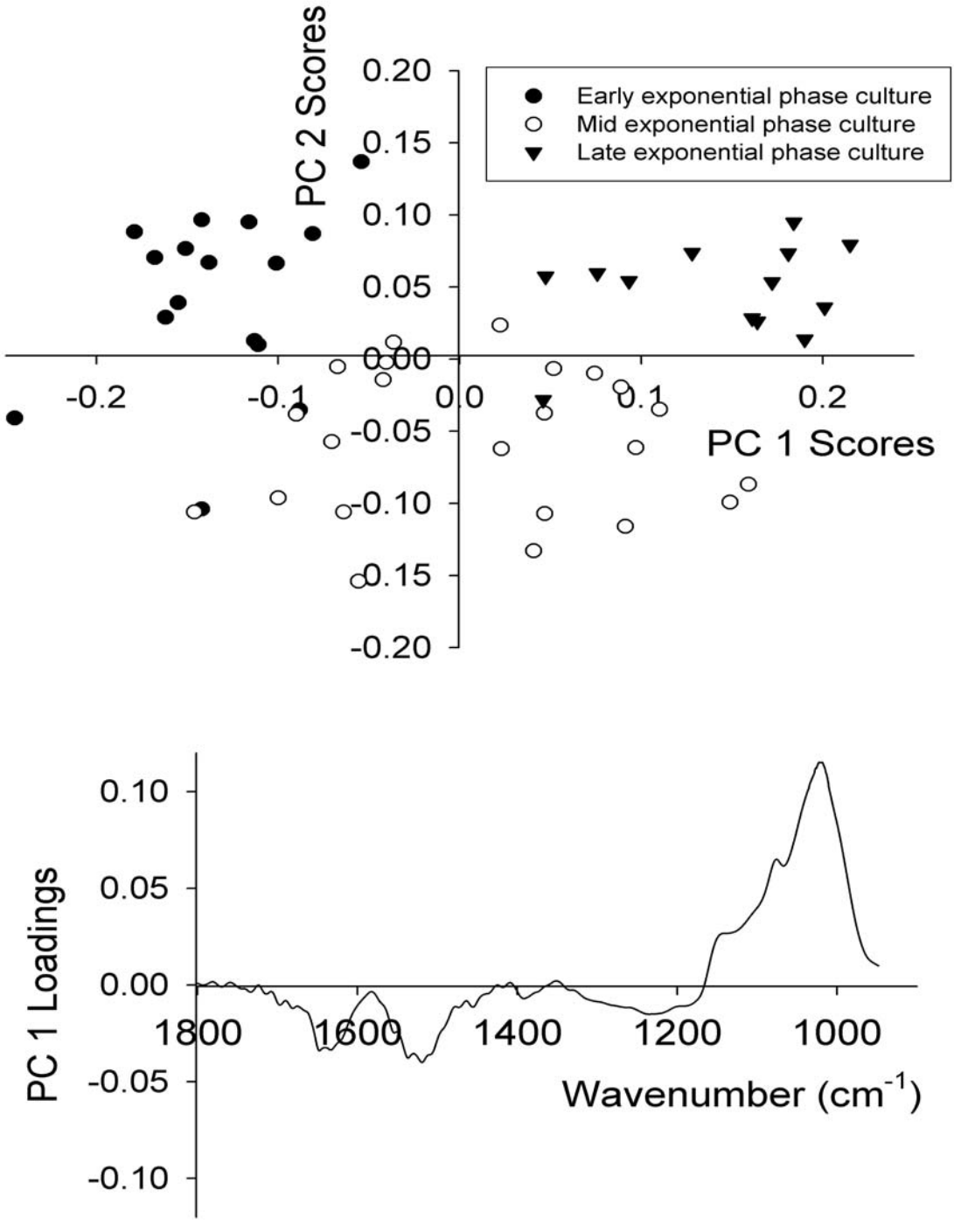


Figure 4.51 Showing PC1 vs PC2 scores plot for all *Oscillatoria limosa* FTIR spectra acquired during the early, mid and late exponential phases of growth, and PC1 loadings for the dataset.

Figure 4.51 shows PC1 vs PC2 scores and loadings plots for *Oscillatoria limosa*. These show the same trends as shown in Figure 4.52. Again early, mid and late exponential spectra form distinct clusters and these are separated in terms of amide and C-O stretching absorbance, with early exponential spectra being high in amide and low in C-O stretching absorbance and vice versa for late exponential phase spectra. These trends were exhibited to greater or lesser degree in all the species tested (data not shown).

4.4.6 Differences between strains using PCA

PC1 vs PC2 scores plots for the all the combined mid exponential spectra shows clustering of all the strains (Figure 4.52). Most differentiation seems to occur along PC1. The loadings plots for PC1 shows that differences between the clusters is due to differing amide and C-O stretch bands, with these bands being oppositely correlated (Figure G). *Phormidium laminosum* and *Phormidium* sp. are shown to have similar characteristics due to the close proximity of the clusters of spectra for the two strains on the 2-D scores plot. Both strains have negative PC1 scores indicating that these two strains have the lowest levels of C-O stretching absorbance and the highest levels of amide absorbance amongst the 7 strains tested.

The spectra regions that differentiate the different strains are the same regions that were shown to undergo change during exponential growth (Figures 4.51, 4.52, and 4.53), which suggests that changes in growth may affect the accuracy of classification based on PCA modeling. This is explored in the next section where classification of mid-exponential spectra alone is compared with classification of the entire dataset composed of all early, mid and late exponential phase spectra.

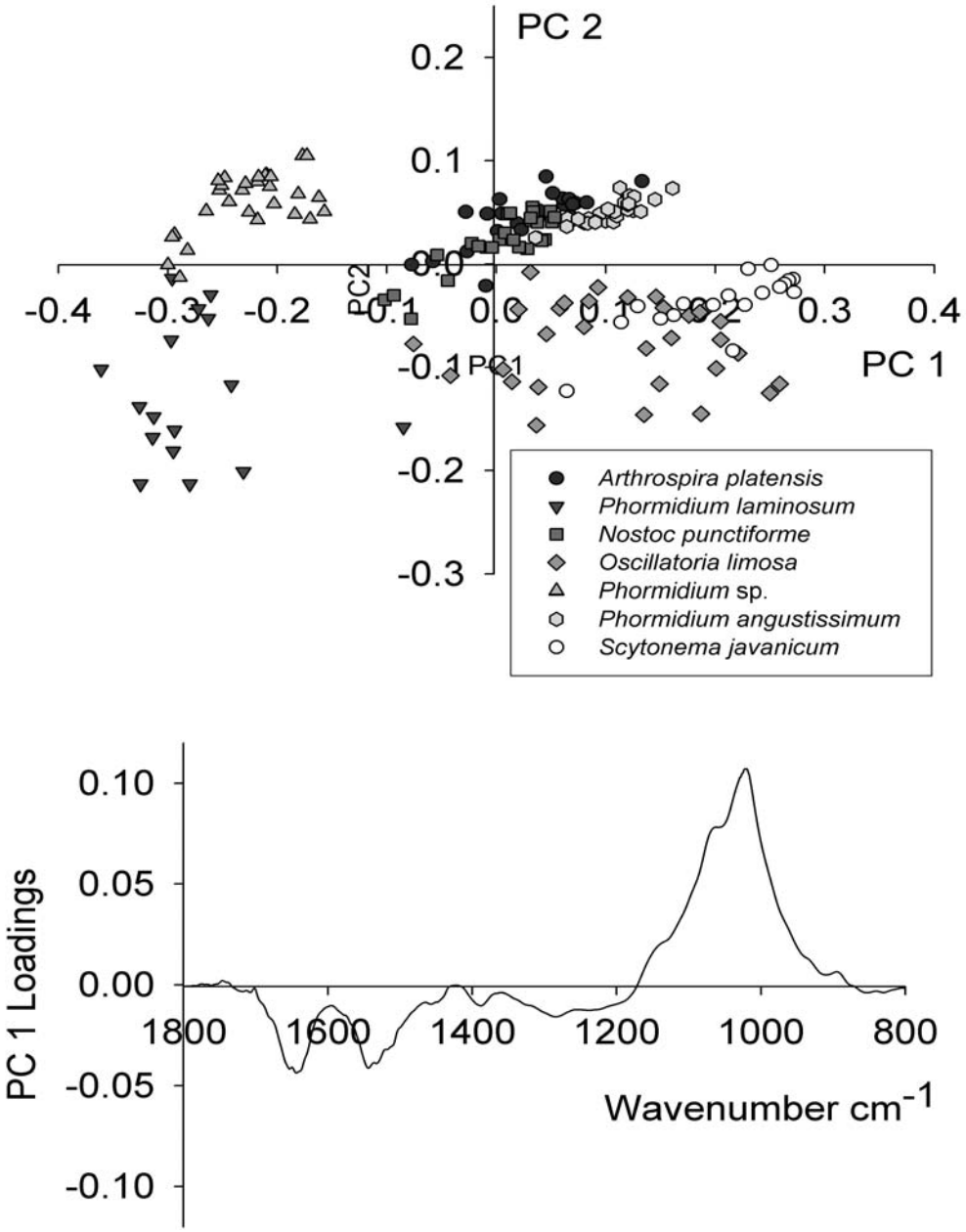


Figure 4.52 Showing PC1 vs PC2 scores plot for all mid exponential FTIR spectra, and PC1 loadings for the dataset.

4.4.7 Classification of cyanobacterial strains using Soft Independent Modeling by Class Analogy (SIMCA)

Soft Independent Modeling by Class Analogy is a PCA classification method. SIMCA is based on the idea that members of a class exhibit a particular class pattern and all members of the class are more similar to members within their class than to members of another class. SIMCA uses PCA models to define the individual classes and compares new objects for classification with these models. Because the data classes are known in advance SIMCA is called a supervised classification method. SIMCA is regarded as being more powerful than traditional classification methods such as Linear Discriminant Analysis (LDA), particularly when used to analyse data where the number of variables is large and the number of subjects is small, such as with spectroscopy data.

The first step in SIMCA classification is called the training stage. This involves making Principal Component models for each class of objects from a selected training set. The second stage is called the classification stage where new objects from a test set are compared with the established PC models. Figure H illustrates the concept behind SIMCA classification showing a new object being compared two PC classes. There are a number of possible outcomes to the classification process: an object is correctly classified; it is incorrectly classified; it is classified to two or more classes; or it is not classified to any of the defined classes.

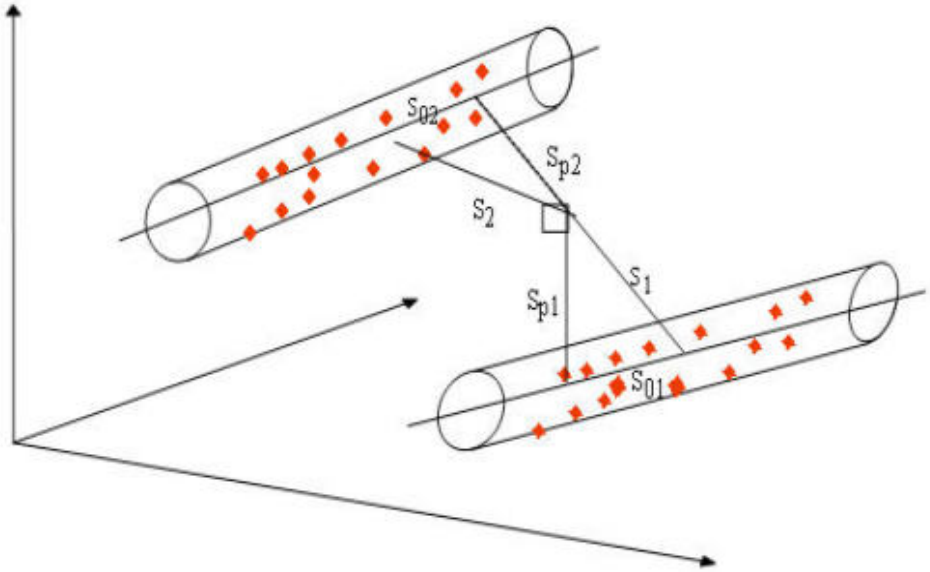


Figure 4.53 SIMCA classification of a new object (represented by the square) to either of two PC models (represented by the cylinders).

In this study spectra from each strain, which defined each class, were divided randomly into two-thirds comprising a training set and one third to a test set. PC models were derived using typically 6 PCs. A number of different models were defined using different region of the spectrum (shown in Figure 4.53) in attempt to determine whether different spectral windows resulted in more accurate classification. In addition, SIMCA classification was applied to the combined dataset of early, mid and late exponential spectra as well as the mid exponential spectra alone, in an attempt to discover whether the chemical changes observed over exponential growth would have an effect on the accuracy of classification.

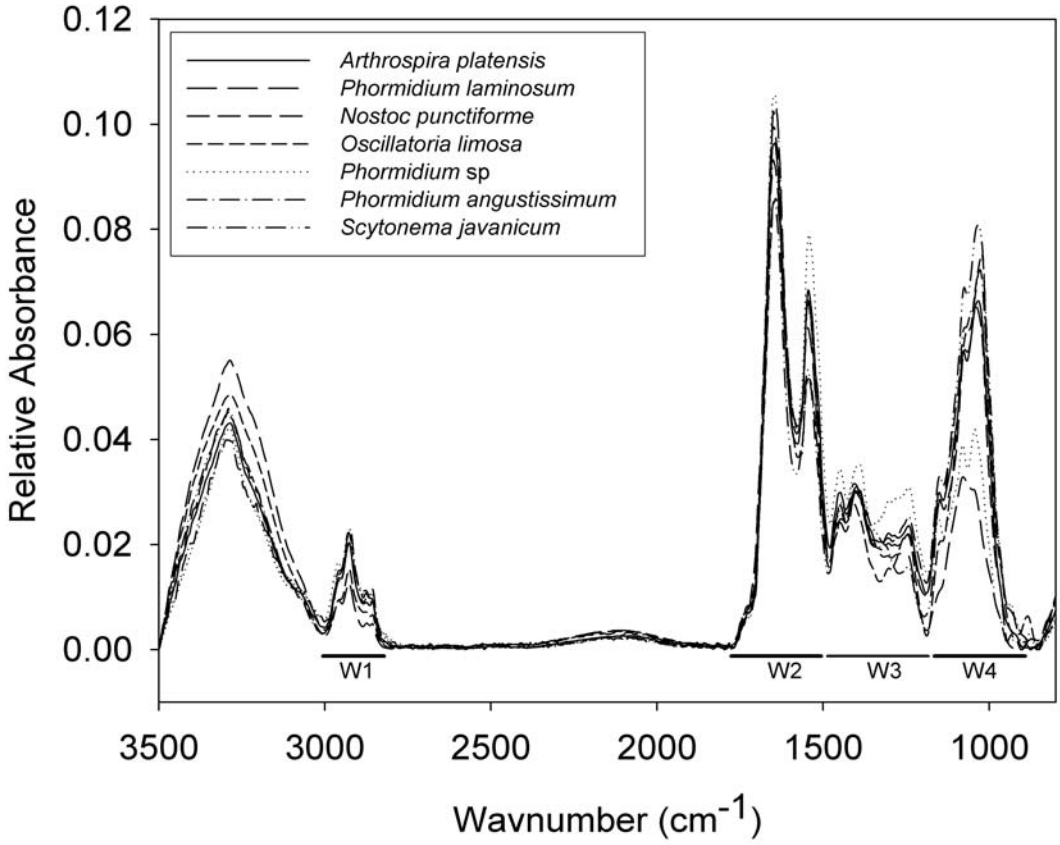


Figure 4.54 Showing the different spectral windows (W1, W2, W3, and W4) used in the SIMCA classification. The whole range from 3500-800 cm^{-1} was also used as well as a combination of W2, 3 and 4.

The results of the SIMCA classification are shown in Table A. The best classification result of 93% classification of 58 test spectra was achieved using the mid exponential growth phases data and the C-O stretching region of the spectrum (W4). The next best classification spectrum was the amide region (W2) with 85% correct classification. These two spectral region were also shown to be the most accurate for the discrimination of cyanobacteria in the study by Kansiz *et al*. (2001)

SIMCA classification was still quite accurate when using the combined data from all exponential growth phases with 81% classification out of 149 test spectra using the combined spectral region from 1800-950 cm^{-1} (W2+W3+W4). These results indicate classification results for these organisms would probably be enhanced by harvesting cells for spectral acquisition under consistent culture conditions, however the method is still quite accurate even when cells are harvested throughout exponential growth even with the macromolecular changes that do occur over this period.

Table 4.55 Percentage correct classification for SIMCA classification.

	Entire spectrum	**W1**	**W2**	**W3**	**W4**	**W2+W3+W4**
Mid exponential spectra	76	81	85	45	93	81
All exponential phase spectra	61	46	60	59	60	81

The number of mid exponential phase spectra in the test set was 59; the number of spectra in the test set combining all exponential phases was 134. The entire spectral range was 3500-800 cm^{-1}. The spectral windows W1, 2, 3, and 4 are defined in figure 4.55.

CHAPTER V

CONCLUSIONS

This dissertation investigated the distribution of soil-borne cyanobacteria in the Nakhon Ratchasima Province and related this to measurements of physico-chemical factors such as salinity, pH, soil type and texture. The effect of differing photon fluxes and salt concentrations on growth and pigment accumulation in a number of filamentous cyanobacterial strains isolated from saline soils was then investigated. In addition, the study pioneered the development of a method, based on Attenuated Total Reflectance Fourier Transform Infrared (ATR/FTIR) spectroscopy, for the rapid, accurate and non-destructive discrimination of soil-borne cyanobacteria, based on their FTIR spectra.

Seven filamentous cyanobacterial strains, namely: *Oscillatoria limosa*, , *Phormidium laminosum*, *Phormidium* sp., *Scytonema javanicum*, *Nostoc punctiforme*, *Phormidium angusitissimum* and *Arthrospira platensis*, were isolated and purified by microbiological techniques. Of these, five strains were used for the study of the effects of light and salinity on pigment content and growth rates. Cells were grown in BG 11 liquid, cyanobacterial medium and treated by exposure to photon fluxes of 20, 40, 60, and 80 μmol $m^{-2}s^{-1}$ and then grown in salt concentration 0, 0.5, 1.0, 1.5 and 2.5 g NaCl L^{-1}. Sub-samples were harvested from lag phase to late exponential growth phase for chlorophyll measurement and harvested at mid-exponential growth phase for β-carotene extraction and

measurement. This part of the work was aimed at exploring the potential for exploiting cyanobacteria found in saline soils for biotechnology processes such as β carotene harvesting.

The work involving ATR/FTIR spectroscopy was aimed at developing a rapid and inexpensive spectroscopic method to discriminate soil-borne cyanobacteria. Previous work has demonstrated that FTIR spectroscopy could be used to discriminate freshwater cyanobacteria, but this technique used a very expensive FTIR microscope. The new FTIR/ATR method developed here employs a relatively inexpensive ATR attachment that can be connected to any existing FTIR spectrometer, and costs a fraction of what is needed to purchase an FTIR microscope.

Cell cultures of all 7 strain were grown and sub-samples were harvested at early, mid and late exponential growth phase to acquire spectra by ATR/FTIR spectroscopy. More than 700 IR spectra were acquired. The multivariate statistical technique Principal Component Analysis (PCA) was used for extraction and interpretation of the systematic variance in the spectral data set. PCA was used to analyse changes in cellular composition which occurred over the exponential growth phase. A technique known as Soft Independent Modeling by Class Analogy (SIMCA) which uses PCA models was then used to discriminate strains. The effect on the accuracy of the SIMCA discrimination of changes in macromolecular composition of the cells, which was observed throughout the exponential phase of growth, was assessed.

5.1 Effects of soil properties and cyanobacteria distribution

6 soil properties such as pH, Electro-conductivity, total nitrogen, available phosphorous and potassium, and type of soil were analysed. pH ranged from very acidic 5,7 at Ban Tatalkhae to moderately alkaline 8,0 at Ban Tanot. The other sites were neutral to slightly alkaline. Electro-conductivity measured in a range from 0.12ms/cm at Ban KhokPhet to the highest in Ban Donri at 1.5 ms/cm. Total nitrogen was high in Ban Rai and Ban Talatkhae at around 0.15%, and lowest in Ban Tanot and Ban Chat at around 0.051%. Available Phosphorus was high in Ban Takotung at 82 ppm and lowest was in Ban Takhro at 8.8 ppm. Potassium was at its highest in Ban TaKoTung at 225 ppm and the lowest value was measured in Ban Donri The soil types found were sandy loam, silt loam and clay loam.

The high abundance of filamentous cyanobacterial strains was found in Ban NonthongLang with 60% of all species named in the study found in this region and the lowest abundance was in Ban Tanot 10% of the total species isolated. pH, N,P and K content appeared to have little effect on abundance of cyanobacteria whereas soil type and salinity appeared to have more of an influence. Cyanobacteria appeared to be least abundant in dry, sandy soils which had high salinity. The effect of soil water content on species abundance was corroborated by the small number of strains isolated during the dry season (2 strains) compared to the wet season (25 strains).

5.2 Effect of photon flux on growth, chlorophyll *a* and β-carotene

Photon fluxes at 20 and 40 μmol $m^{-2}s^{-1}$ had no effect on growth of all strains from the lag phase to late exponential growth phase. The treatments with 60-80μmol $m^{-2}s^{-1}$ slowed the growth of all species except *Oscillatoria limosa* during the early exponential phase of growth. Photon fluxes of 80μmol $m^{-2}s^{-1}$ inhibited growth of all species, except *Oscillatoria limosa*, which shown tolerance to all four selected levels of photon fluxes. *Scytonema javanicum Nostoc punctiforme* were the most sensitive to photon fluxes. Light affected chlorophyll *a* content in *Nostoc punctiforme* and *Scytonema javanicum,* especially with prolonged exposure to 80μmol $m^{-2}s^{-1}$ which appeared to result in photo-bleaching of chlorophyll in these strains. In *Scytonema javanicum* chlorophyll *a* was elevated at the lowest photon flux and β-carotene content also high in the first two photon flux levels. β-carotene content was not affected by photon fluxes of 20 to 60 μmol $m^{-2}s^{-1}$ in the other strains The results indicate all but one of the five strains tested had a preference for low light, reflecting their normal soil-borne environment, and will produce the most biomass under these conditions. These lower photon fluxes were also optimal for production of chlorophyll and in some strains, β carotene. These results have important implications for the selection of optimal growth conditions for biotechnology processes that might be established that employ these organisms for the production of pigments or biomass.

5.3 Effect of salinity on growth, chlorophyll a and β-carotene content

Salinity from 0-1.5 g NaCl L^{-1} did not effect growth in any of the strains tested. Growth in *Nostoc punctiforme* and *Scytonema javanicum* was slightly affected by the highest salinity level (2.5 gL^{-1}) which became evident at the late exponential growth phase. *Scytonema javanicum* contained the lowest amount of β-carotene in all salt concentrations. β-carotene levels were elevated in *Nostoc punctiforme* at a maximum at salt concentrations of 1g NaCl L^{-1}, and was optimal in the range of 0.5–1.5 g NaCl L^{-1}. β-carotene *Phormidium laminosum* was highest when grown in the salinity range of 0-0.5 g NaCl L^{-1}.was at a maximum at a salt concentration of 0.5 g NaCl L^{-1}. Salinity at 2.5 g NaCl L^{-1}. reduced β-carotene content in all strains. The results of the study suggested that there are optimal salinity ranges for the production of β-carotene in the soil-borne cyanobacteria that were isolated from the saline soils, which appears to be species dependent. This has a bearing on the selection of strains and growth conditions for commercial production of β carotene. For instance, a highly saline tolerant strain such as *Nostoc punctiform* may be well suited to commercial harvesting of β carotene because it produces most β carotene at salinity levels which would inhibit or slow the growth of many potential contaminant organisms such as eukaryotic algae that have air-borne spores such as *Chlorella* sp..

5.4 ATR/FTIR spectroscopic data.

A method was developed whereby FTIR spectra with an excellent signal to noise ratio could be acquired from cyanobacterial films air-dried onto an ATR crystal substrate. The method was rapid and very reproducible. The FTIR spectra of all strains displayed bands typical of those of microalgae and cyanobacteria. The major bands were those from: lipids with bands from C-H stretching vibrations found between 2970-2850 cm^{-1} and from ester carbonyl stretching at around 1730 cm^{-1}; bands attributed to proteins, particularly the amide I at 1650 cm^{-1} and the amide II at 1540 cm^{-1}; bands from phosphate group stretching vibrations found at 1240 and 1080 cm^{-1} resulting mainly from the phosphodiester groups of nucleic acids; and bands from C-O stretching vibrations from carbohydrates found between 1150-1000 cm^{-1}. Each strain had a unique spectral profile, with most variation between strains occurring in the C-O stretching region and the amide region. Both these regions changed during growth phase and this varied between the strains. In most strains there appeared to be a decrease in amide absorbance and an increase in the absorbance of C-O stretching bands as the batch cultures progressed through the early, mid to late exponential growth phases. This was related to other spectroscopic studies of algae where these spectral changes similar to these were caused by limitation in either nitrogen or phosphorus which resulted in protein levels in the cells declining and carbohydrate increasing. These findings were corroborated by Principal Component Analysis which showed that in most strains that early mid and late exponential phase cells were distinct in terms of macromolecular composition and the separation was the result of high protein and low carbohydrate absorbance in

the early exponential phase and low protein and high carbohydrate absorbance in late exponential phase cells, with mid exponential cells somewhere in between. These results showed that the ATR/FTIR spectroscopic method in combination with PCA was a powerful approach for monitoring macromolecular change in cyanobacteria during growth and has the potential to be used as an on-line monitoring tool for algal biotechnology processes.

PCA was also explored as a method to classify and discriminate the cyanobacterial strains. PCA scores plots revealed that the cyanobacterial strains were spectrally distinct. Loadings plots showed that the clustering of strains on PCA scores plots was mainly due to differences in absorbance of amide and C-O bands. Since these were the bands that also altered during the exponential growth phase, we wanted to test what affect growth phase had on classification based on the FTIR spectroscopy data. PCA models were made using only the mid exponential spectra, as well as defining PCA models using spectra from all the growth phases combined. Soft Independent Modeling by Class Analogy (SIMCA) was used as the classification method, with two thirds of spectra being used for training and one third for testing. Different spectral regions were employed in the PCA models to see which part of the FTIR spectrum was most useful for discriminating the strains.

SIMCA was found to classify the strains with an accuracy of better than 90% when the mid exponential spectra alone were tested. Accuracy was reduced to about 80% when all the growth phases were combined. Greatest discrimination was achieved when the spectral region from 1800-950 cm^{-1} was used, which included the amide bands and the C-O stretching bands. These results indicate that

ATR/FTIR spectroscopy combined with chemometric classification methods such as PCA and SIMCA constitute a powerful, rapid, non-destructive and potentially automated way of classifying soil-borne cyanobacteria.

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APPENDICES

APPENDIX A

SAMPLES SAMPLING SITES

Figure A1 A. Ban NonThongLang, B. Ban Rai, C. Ban TaKoTung (dry season, D. Ban Chat (dry season)

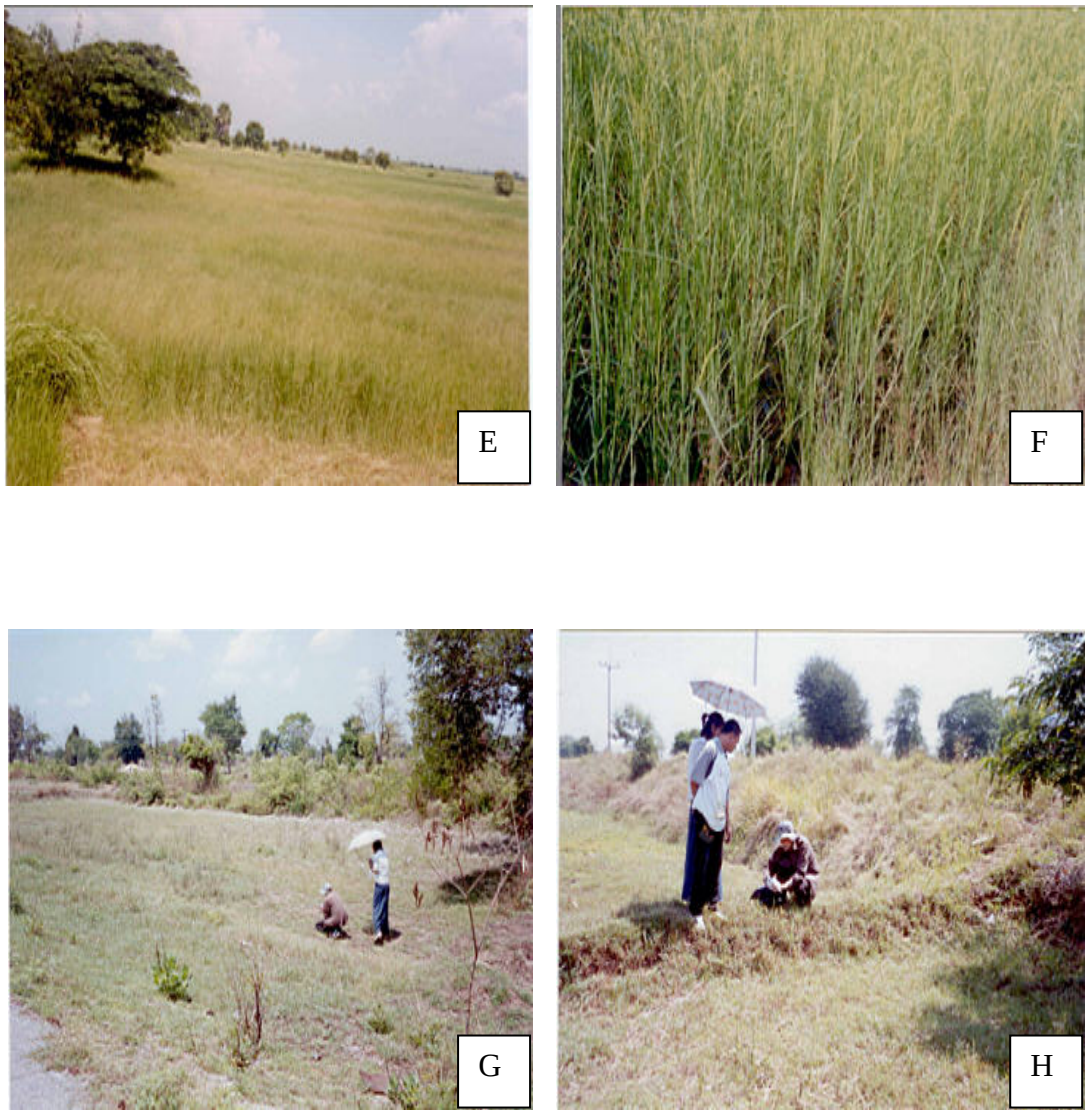

Figure A1 (Continued). E. Ban TakoTung in wet season, F. Ban Khok Phet, G. Ban Donri, H. Ban NonThongLang (second site)

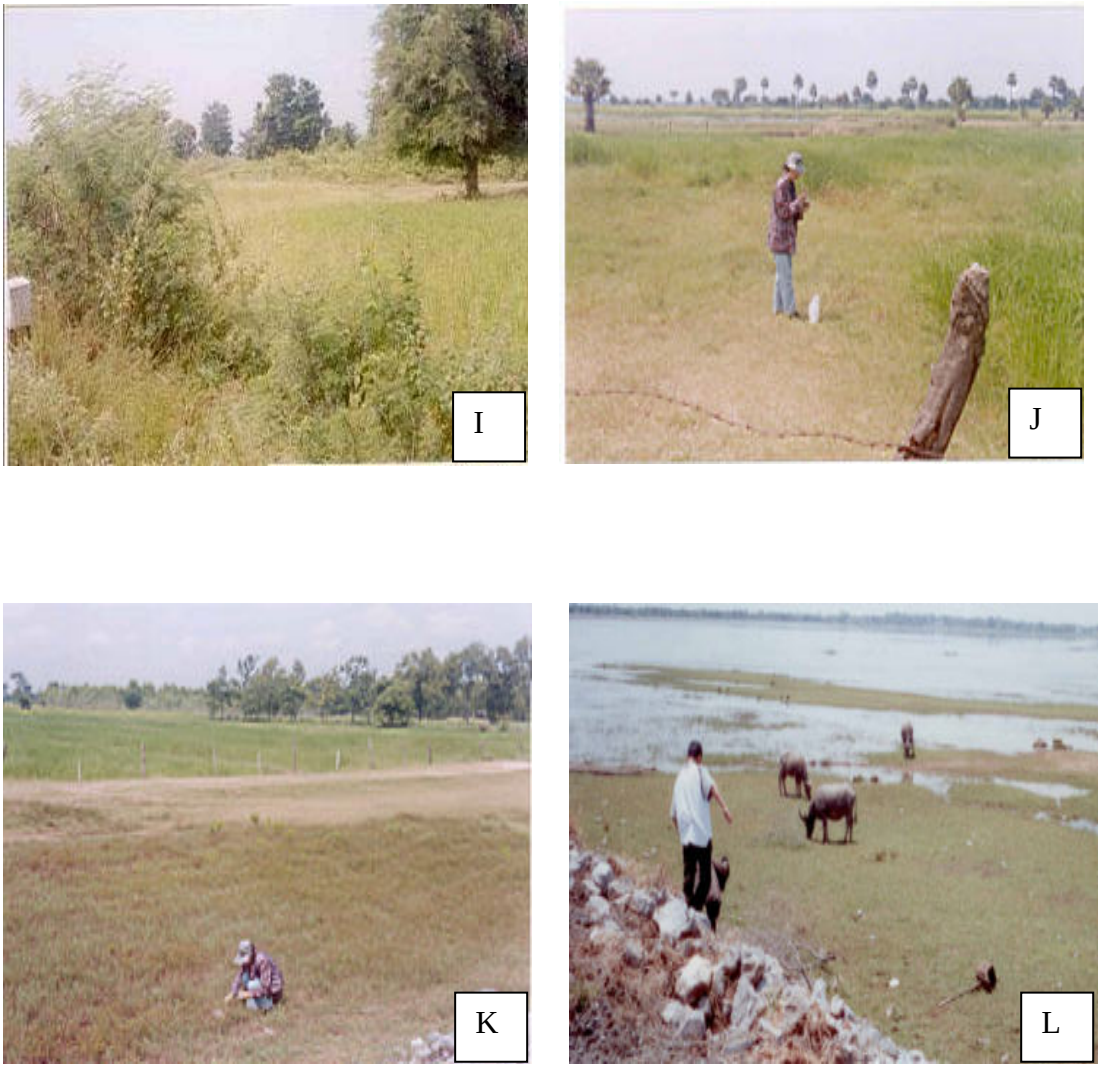

Figure A1 (Continued). I. Ban NongBuaTung, J. Ban TakoTung, K. Reservoir Ban Takhro (first site), L. Reservoir BanTakhro (second site)

Figure A1 (Continued) M. Ban TalatKhae, N. Ban Tanot

APPENDIX B

CULTURE MEDIUM AND REAGENTS PREPARATION

BG-11 MEDIUM FOR BLUE GREEN ALGAE

Solution A

1.	$NaNO_3$	1.500	g
2.	K_2HPO_4	0.040	g
3.	$MgSO_4$	0.070	g
4.	$CaCl_2.7H_2O$	0.036	g
5.	Citric acid	0.006	g
6.	Ferric ammonium citrate	0.006	g
7.	EDTA (Disodium salt)	0.001	g
8.	Na_2CO_3	0.020	g
9.	Trace metal mix A5	1.000	mL
10	Agar (if needed)	10.000	g
11.	Distilled water	1.000	L

The pH should be 7.1 after sterilization

Solution B. Trace metal mix A5

1.	H_3BO_3	2.860	g
2.	$MnCl_2.4H_2O$	1.810	g
3.	$ZnSO_4.7H_2O$	0.220	g
4.	$NaMoO_4.2H_2O$	0.390	g
5.	$CuSO_4.5H_3O$	0.079	g

6.	$Co(NO_3)_2.6H_2O$	49.400	g
7.	Distilled water	1.000	g

Preparation of medium

A. Solid media

All the components of solution A were dissolved in the distilled water, 500mL. All the component of solution B also was dissolved in 1 liter distilled water. The mixture were mixed thoroughly and took 1 ml of solution B added to solution A and brought volume up to1.0L. The medium was mixed and added 10g of agar then gently heated until dissolved. The medium was sterilized and used for isolation cultivation and maintenance of cyanobacteria.

Liquid medium

Liquid medium was prepared by the same procedure of solid medium but no agar. After sterilization medium was neutralized by HCL 10%.

APPENDIX C

INSTRUMENTATIONS

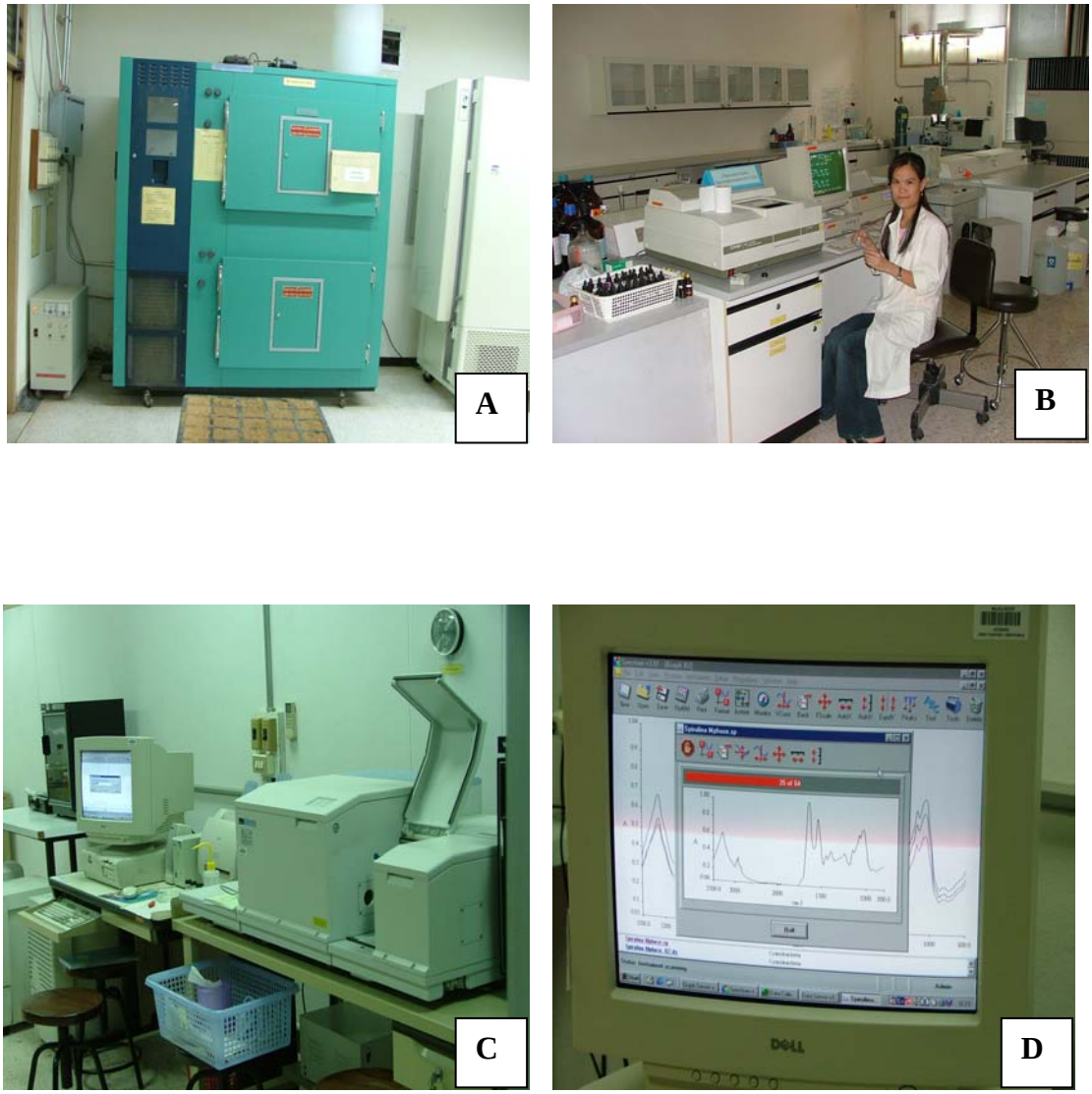

Figure 1C A. Growth cabinet used to culture cyanobacteria, B. UV-Visible spectrophotometer, C. Fourier Transform Infrared (FTIR) microspectroscopy with Attenuated Total Reflectance (ATR). D. IBM – compatible PC running spectrum FTIR version 3.1

.

Figure 1C (Continued). E. Total Reflectance (ATR) accessory, F. Vacuum filtration with glass fiber filter

APPENDIX D

CHEMICAL PROPERTIES AND PATTERNT OF SOIL SAMPLES

Table 1 D Soil properties analysis.

Villages	Sample Sites	Parameters					Particle size			
		pH (1:1H2O)	EC (ms/cm)	N (%)	P (ppm)	K (ppm)	Sand	Silt	Clay	
1. Ban Khokphet	1	7.8	0.17	0.043	13.8	150	70.94	17.51	11.55	SL
	2	6.6	0.16	0.104	31.09	55	61.81	23.16	15.04	SL
	3	6.7	0.03	0.031	12.21	73	73.81	18.49	7.7	SL
2. Ban Rai	4	7.5	0.2	0.14	33.08	164.5	53.74	28.04	18.23	SL
	5	7.7	0.23	0.144	12.62	163	20.77	55.14	24.09	SIL
	6	7.3	0.28	0.185	1.99	278.5	37.31	49.23	13.47	SIL
3. Ban NonThongLang	7	7.3	2.1	0.08	42.59	281	63.95	28.3	7.75	SL
	8	5.3	0.07	0.095	14.36	99.5	36.14	47.07	15.89	SL
	9	6.9	2.4	0.049	24.07	131	56.86	33.2	9.95	SL
4. Ban Takhro	10	7.5	0.12	0.22	9.76	80	57.34	37.39	28.62	SL
	11	7.4	0.06	0.041	15.98	67.5	83.04	30.1	12.56	LS
	12	7.5	0.23	0.039	0.85	173	40.22	12.02	4.95	L
5. Ban Nong BuaTung	13	6.4	0.26	0.085	11.46	179.5	42	33.91	24.09	L
	14	6.3	0.33	0.077	15.68	133.5	45.53	35.72	18.75	L
	15	6.3	0.33	0.051	12.81	127.5	52.8	28.99	18.21	SL

Table 1 D (Continued)

Villages	Sample Sites	Parameters					Particle size			
		pH (1:1H2O)	EC (ms/cm)	N (%)	P (ppm)	K (ppm)	Sand	Silt	Clay	
6. Ban TaKoTung	16	7.5	0.19	0.069	88.34	128.5	72.06	17.8	10.14	SL
	17	6.9	0.36	0.135	44.37	300	25.07	36.04	38.89	CL
	18	7.2	0.58	0.11	115.8	249	23.74	44.21	32.05	CL
7. Ban TaLatKhae	19	6.5	0.5	0.228	24.15	93	56.34	32.1	11.55	SL
	20	5.5	0.28	0.216	21.77	158.5	22.11	34.82	43.07	C
	21	5	0.39	0.03	7	130.5	33.99	37.39	28.62	CL
8. Ban Chat	22	7.5	0.383	0.016	27.32	87.5	75.84	19.28	4.88	LS
	23	7.7	0.395	0.025	33.08	139	73.42	18.95	7.63	SL
	24	7.7	0.379	0.117	12.62	261	51.53	28.24	20.23	L
9. Ban Done Ri	25	7.2	0.371	0.137	71.31	120.5	72.24	20.59	7.17	SL
	26	7.5	0.454	0.024	16.57	59	78.63	18.45	2.92	LS
	27	7	0.382	0.196	89.57	74.5	58.61	25.22	16.18	SL
10. Ban TaNot	28	8	0.22	0.102	20.04	136.5	53.53	33.05	13.41	SL
	29	7.9	0.81	0.014	17.15	106	79.06	16.56	4.38	LS
	30	8	0.04	0.038	4.04	63	77.66	17.91	4.43	LS

Note: SL- Sandy Loam; LS- Loamy sand; L- Loam; SIL- Silt loam; C- Clay; CL- Clay loam

APPENDIX E

EFFECT OF LIGHT AND SALINITY ON GROWTH, CHLOROPHYLL AND β - CAROTENE

Table 1 E .Effect of light on cell density of *Phormidium* sp.

No./Date	Time (Days)									
	2	6	10	14	18	22	26	30	34	38
1	0.055	0.093	0.106	0.173	0.183	0.198	0.227	0.238	0.375	0.371
2	0.043	0.099	0.142	0.178	0.211	0.215	0.393	0.644		
3	0.045	0.102	0.115	0.186	0.228	0.259	0.402			
4	0.064	0.149	0.253	0.241	0.288	0.326				
5	0.062	0.133	0.15	0.227	0.246	0.315	0.318	0.664	0.63	0.427
6	0.072	0.148	0.165	0.254	0.275	0.354	0.463	0.46	0.492	0.469
7	0.077	0.126	0.164	0.205	0.327	0.612	0.527	0.524		
8	0.056	0.107	0.132	0.216	0.259	0.308	0.489	0.481	0.64	0.462
9	0.061	0.11	0.175	0.218	0.249	0.272	0.536	0.556	0.565	0.446
10	0.055	0.104	0.203	0.221	0.247	0.267	0.435	0.601	0.442	0.437
11	0.054	0.106	0.114	0.186	0.192	0.248	0.475	0.458	0.431	0.39
12	0.049	0.108	0.153	0.195	0.204	0.237	0.442	0.433		

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Table 2E Effect of salinity on cell density of *Phormidium sp*

No./Date	Salt	Time (Days)									
	conc	2	6	10	14	18	22	26	30	34	38
1	0	0.055	0.087	0.137	0.178	0.244	0.257	0.322	0.467	0.518	0.555
2	0	0.046	0.129	0.174	0.216	0.335	0.403	0.564	0.549	1.26	0.576
3	0	0.049	0.13	0.193	0.286	0.358	0.384	0.558	0.641	0.643	0.684
4	0.5	0									
5	0.5	0.051	0.117	0.136	0.214	0.258	0.307	0.334	0.486	0.415	0.453
6	0.5	0.057	0.124	0.143	0.181	0.226	0.285	0.315	0.505	0.502	0.506
7	1	0.064	0.13	0.142	0.174	0.218	0.35	0.366	0.422	0.543	0.547
8	1	0.068	0.117	0.145	0.182	0.234	0.337	0.442	0.502	0.514	0.524
9	1	0.06	0.121	0.16	0.187	0.257	0.363	0.495	0.505	0.674	0.675
10	1.5	0.056	0.11	0.114	0.144	0.243	0.358	0.444	0.503	0.643	0.536
11	1.5	0.065	0.087	0.099	0.16	0.229	0.272	0.381	0.439	0.498	0.459
12	1.5	0.059	0.092	0.115	0.147	0.179	0.227	0.26	0.376	0.394	0.364
13	2.5	0.065	0.085	0.102	0.153	0.176	0.222	0.29	0.335	0.542	0.468
14	2.5	0.058	0.128	0.144	0.137	0.187	0.229	0.294	0.356	0.359	0.356
15	2.5	0.052	0.111	0.136	0.147	0.147	0.237	0.277	0.362	0.436	0.436

Table 3 E Effect of salinity on cell density of *Scytonema javanicum*

No./Date	Salt	Time (Days)									
	conc	2	6	10	14	18	22	26	30	34	38
1	0	0.05	0.054	0.069	0.103	0.123	0.133	0.148		0.163	0.239
2	0	0.056	0.069	0.074	0.102	0.105	0.127	0.138	0.158	0.185	0.216
3	0	0.049	0.059	0.078	0.117	0.121	0.142	0.145	0.165	0.174	0.208
4	0.5	0.054	0.0064	0.077	0.108	0.132	0.142	0.15	0.186	0.228	0.239
5	0.5	0.057	0.066	0.072	0.102	0.103	0.124	0.129		0.217	0.225
6	0.5	0.055	0.08	0.079	0.0104	0.107	0.127	0.162	0.178	0.187	0.271
7	1	0.058	0.053	0.08	0.087	0.098	0.113	0.122	0.152	0.178	0.201
8	1	0.051	0.064	0.069	0.088	0.099	0.112	0.127	0.165	0.178	0.173
9	1	0.057	0.072	0.089	0.0109	0.114	0.12	0.131	0.135	0.157	0.194
10	1.5	0.058	0.07	0.078	0.089	0.104	0.12	0.138	0.143	0.145	0.205
11	1.5	0.059	0.067	0.07	0.088	0.107	0.117	0.128	0.136	0.148	0.187
12	1.5	0.059	0.063	0.07	0.092	0.103	0.114	0.125			
13	2.5	0.066	0.07	0.076	0.091	0.107	0.117	0.124	0.138	0.148	0.147
14	2.5	0.065	0.073	0.082	0.01	0.117	0.117	0.126	0.137	0.146	0.134
15	2.5	0.063	0.069	0.075	0.096	0.118	0.118	0.135			

Table 4 E Effect of light on cell density of *Phormidium laminosum*

No./Date	Time (Days)										
	2	6	10	14	18	22	26	26	30	34	38
1	0.061	0.078	0.079	0.086	0.108	0.117	0.148	0.184	0.234	0.268	0.416
2	0.06	0.072	0.074	0.088	0.126	0.125	0.128	0.192	0.24	0.287	0.356
3	0.062	0.071	0.077	0.094	0.105	0.129	0.136	0.214	0.288	0.328	0.35
4	0.059	0.062	0.075	0.092	0.11	0.117	0.128	0.234	0.234	0.274	0.282
5	0.05	0.06	0.068	0.086	0.118	0.123	0.13	0.187	0.245	0.252	0.307
6	0.061	0.075	0.08	0.085	0.103	0.126	0.128	0.18	0.275	0.259	0.259
7	0.055	0.062	0.089	0.108	0.11	0.123	0.134	0.174	0.245	0.252	0.401
8	0.059	0.072	0.08	0.092	0.114	0.135	0.146	0.172	0.345	0.363	0.422
9	0.066	0.08	0.088	0.097	0.124	0.142	0.159	0.166	0.305	0.355	0.422
10	0.055	0.073	0.078	0.086	0.102	0.115	0.117	0.204	0.254	0.259	0.215
11	0.06	0.076	0.086	0.091	0.101	0.106	0.128	0.164	0.227	0.233	0.218
12	0.055	0.074	0.079	0.086	0.112	0.112	0.129	0.193	0.253	0.274	0.257

Table 5 E Effect of light on cell density of *Oscillatoria limosa*

No./Date	Time (Days)									
	2	6	10	14	18	22	26	30	34	38
1	0.065	0.087	0.14	0.153	0.169	0.174	0.341	0.348	0.44	0.43
2	0.057	0.077	0.158	0.159	0.197	0.207	0.298	0.388	0.584	0.496
3	0.076	0.092	0.143	0.155	0.181	0.208	0.272	0.389	0.395	0.36
4	0.089	0.098	0.123	0.159	0.167	0.266	0.37	0.381	0.568	0.44
5	0.095	0.103	0.125	0.143	0.174	0.189	0.266	0.568	0.575	0.473
6	0.073	0.095	0.119	0.134	0.146	0.201	0.223	0.579	0.567	0.456
7	0.075	0.125	0.136	0.146	0.15	0.197	0.237	0.271	0.401	0.439
8	0.069	0.098	0.147	0.163	0.197	0.216	0.27	0.282	0.382	0.22
9	0.077	0.085	0.116	0.156	0.16	0.179	0.295	0.501	0.412	0.398
10	0.068	0.087	0.125	0.236	0.253	0.256	0.355	0.359	0.567	0.481
11	0.071	0.081	0.115	0.129	0.148	0.185	0.298	0.306	0.409	0.354
12	0.072	0.098	0.134	0.181	0.203	0.261	0.352	0.39	0.515	0.434

Table 6 E Effect of salinity on cell density of *Oscillatoria limosa*

No./Date	Time (Days)									
	2	6	10	14	18	22	26	30	34	38
1	0.044	0.058	0.099	0.106	0.129	0.132	0.227	0.274	0.279	0.468	0.414
2	0.043	0.076	0.09	0.104	0.126	0.179	0.233	0.245	0.32	0.363	0.346
3	0.037	0.052	0.075	0.097	0.193	0.219	0.238	0.312	0.371	0.395	0.382
4	0.044	0.061	0.068	0.088	0.129	0.134	0.218	0.355	0.385	0.411	0.356
5	0.052	0.063	0.064	0.095	0.125	0.129	0.148	0.272	0.342	0.462	0.438
6	0.053	0.078	0.086	0.103	0.133	0.154	0.226	0.297	0.344	0.462	0.315
7	0.035	0.065	0.089	0.126	0.152	0.164	0.223	0.254	0.362	0.402	0.367
8	0.047	0.085	0.098	0.11	0.146	0.151	0.181	0.194	0.268	0.276	0.268
9	0.048	0.055	0.086	0.097	0.124	0.158	0.179	0.195	0.272	0.399	0.234
10	0.049	0.067	0.088	0.097	0.152	0.182	0.189	0.326	0.234	0.396	
11	0.044	0.063	0.079	0.09	0.142	0.16	0.192	0.327	0.252	0.416	
12	0.046	0.095	0.106	0.116	0.14	0.174	0.365	0.379	0.288	0.442	

Table 7 E Effect of salinity on cell density of *Nostoc punctiforme*

No./Date	Time (Days)					
	2	6	10	14	18	22
1	0	0.043	0.047	0.049	0.058	0.071
2	0	0.034	0.046	0.05	0.064	0.075
3	0	0.038	0.043	0.06	0.064	0.075
4	0.5	0.038	0.046	0.066	0.083	0.087
5	0.5	0.04	0.052	0.07	0.087	0.099
6	0.5	0.038	0.047	0.051	0.086	0.088
7	1	0.039	0.042	0.057	0.079	0.089
8	1	0.036	0.049	0.059	0.062	0.071
9	1	0.039	0.042	0.056	0.07	0.083
10	1.5	0.041	0.045	0.057	0.07	0.09
11	1.5	0.037	0.047	0.055	0.067	0.085
12	1.5	0.039	0.049	0.056	0.065	0.093
13	2.5	0.034	0.044	0.059	0.074	0.089
14	2.5	0.036	0.045	0.061	0.077	0.096
15	2.5	0.037	0.052	0.056	0.072	0.094

Table 8 E Effect of light on cell density of *Nostoc punctiforme*

No./Date	Time (Days)				
	2	6	10	14	18
1	0.040	0.042	0.055	0.122	0.117
2	0.038	0.045	0.066	0.127	0.114
3	0.041	0.048	0.065	0.120	0.095
4	0.049	0.055	0.065	0.085	0.081
5	0.040	0.047	0.067	0.078	0.078
6	0.039	0.041	0.056	0.064	0.058
7	0.043	0.049	0.058	0.072	0.078
8	0.046	0.066	0.067	0.083	0.084
9	0.038	0.045	0.059	0.072	0.084
10	0.035	0.045	0.060	0.069	0.082
11	0.045	0.050	0.057	0.074	0.087
12	0.049	0.055	0.065	0.086	0.083

Table 9 E Effect of salinity on β - carotene of *Oscillatoria limosa*

No	Photon flux		NaCl	Rettime		Area	Amt/Area	Amount		
	Lux	Mean	mg/l		(Mean)	(mAU*s)		(ppm)	mg/g	Mean
1	2850	40	0	12.646		62.68603	0.00485624	0.30442	9.16	
2	2850	40	0	12.667	12.661	63.64521	0.00485832	0.30921	9.17	9.16
3	2850	40	0	12.672						
4	2850	40	0.5	12.657		61.53576	0.00485366	0.29867	9.06	
5	2850	40	0.5	12.687	12.673					9.46
6	2850	40	0.5	12.675		71.01602	0.004872445	0.34602	9.86	
7	2850	40	1							
8	2850	40	1	12.67	12.676	99.89239	0.00490771	0.49024	16.12	16.1
9	2850	40	1	12.682						
10	2850	40	1.5	12.675		89.85459	0.00489802	0.44011	12.19	
11	2850	40	1.5	12.684	12.686	81.45473	0.00488808	0.39816	12.59	13.5
12	2850	40	1.5	12.7		109.5645	0.00491536	0.53855	15.64	

Table 10 E Effect of salinity on β - carotene of *Phormidium laminosum*

No.	Photon flux			Rettime		Area	Amt/Area	Amount		
	Lux	Mean			(mean)	(mAU*s		(ppm)	mg/g	mean
1	2850	40	0	12.572						
2	2850	40	0	12.556	12.567					
3	2850	40	0	12.573		131.0126	0.00492831	0.64567	54.66	54.7
4	2850	40	0.5	12.56		81.22768	0.00488778	0.39702	30.3	
5	2850	40	0.5	12.557	12.552	74.30522	0.00487785	0.36245	68.3	63.3
6	2850	40	0.5	12.539		175.1721	0.00494497	0.86622	91.16	
7	2850	40	1	12.525		82.83938	0.00488986	0.40507	48.79	
8	2850	40	1	12.54	12.534					39.8
9	2850	40	1	12.537		69.59881	0.00486996	0.33894	30.81	
10	2850	40	1.5	12.534		61.78322	0.00485422	0.29991	39.34	
11	2850	40	1.5	12.563	12.551	68.31752	0.00486763	0.33254	73.77	44.5
12	2850	40	1.5	12.558		124.9723	0.00492511	0.6155	20.5	

Table 11 E Effect of salinity on β - carotene of *Phormidium* sp.

No.	Photon flux			Rettime		Area	Amt/Area	Amount		
	Lux	Mean			(Mean)	(mAU*s)		(ppm)	mg/g	Mean
1	2850	40	0	12.82		164.613	0.0049418	0.8135	24.2	
2	2850	40	0	12.85	12.83	235.563	0.0049577	1.1678	31.7	26.5
3	2850	40	0	12.82		164.966	0.0049419	0.8152	23.6	
4	2850	40	0.5							
5	2850	40	0.5	12.82	12.82	192.534	0.0049494	0.9529	29.8	
6	2850	40	0.5	12.82		214.648	0.0049541	1.0634	32.3	31.1
7	2850	40	1	12.8		201.129	0.0049514	0.9959	29.7	
8	2850	40	1	12.81	12.8	231.774	0.0049571	1.1489	34.5	33.2
9	2850	40	1	12.81		244.942	0.0049591	1.2147	35.2	
10	2850	40	1.5	12.81		245.165	0.0049591	1.2158	34.2	
11	2850	40	1.5	12.81	12.8	27.1233	0.0046751	1.268	37.6	35.7
12	2850	40	1.5	12.78						
13	2850	40	2.5	12.8		134.005	0.0049298	0.6606	18.6	
14	2850	40	2.5	12.78	12.78	181.605	0.0049467	0.8983	24	22.6
15	2850	40	2.5	12.76		179.474	0.0049462	0.8877	25.3	

Table 12 E Effect of salinity on β - carotene of *.Scytonema javanicum*

No.	Photon flux			Rettime		Area	Amt/Area	Amount		
	Lux	Mean			(Mean)	(mAU*s)		(ppm)	mg/g	Mean
1	2850	40	0	12.58		5.95962	0.003541	0.0211	5.53	
2	2850	40	0	12.59	12.6	6.54705	0.0036714	0.024	7.06	6.3
3	2850	40	0	12.61						
4	2850	40	0.5	12.62						
5	2850	40	0.5	12.61	12.62	7.1395	0.0037812	0.027	5.27	6.34
6	2850	40	0.5	12.62		11.847	0.0042636	0.0505	8.86	
7	2850	40	1	12.62						
8	2850	40	1	12.62	12.61					1.58
9	2850	40	1	12.6		3.32252	0.0023873	0.0079	1.58	
10	2850	40	1.5	12.62		3.1241	0.0022218	0.0069	1.65	
11	2850	40	1.5	12.64	12.62					1.58
12	2850	40	1.5	12.59		3.24334	0.0023237	0.0076	1.51	
13	2850	40	2.5	12.62						
14	2850	40	2.5	12.61	12.61	6.94513	0.0037472	0.026	0.46	0.42
15	2850	40	2.5	12.59		5.29411	0.0033583	0.0178	0.38	

Table 13 E Effect of salinity on β - carotene of . *Nostoc punctiforme*

No.	Photon flux			Rettime		Area	Amt/Area	Amount		
	Lux	Mean			(Mean)	(mAU*s		(ppm)	mg/g	Mean
1	2850	40	0	12.62		16.9655	0.0044839	0.0761	21.7	
2	2850	40	0	12.65	12.63	41.6169	0.0047863	0.1992	21	21.3
3	2850	40	0							
4	2850	40	0.5	12.65						
5	2850	40	0.5	12.64	12.64	43.3791	0.0047947	0.208	57.8	64.1
6	2850	40	0.5	12.64		38.2869	0.0041682	0.1826	70.4	
7	2850	40	1	12.66						
8	2850	40	1	12.63	12.65	42.4152	0.0047902	0.2032	102	84
9	2850	40	1	12.67		28.3889	0.0046893	0.1331	66.5	
10	2850	40	1.5	12.64		38.2327	0.0047679	0.1823	97.8	
11	2850	40	1.5	12.64	12.65					60.4
12	2850	40	1.5	12.67		40.1847	0.0047789	0.192	49.2	
13	2850	40	2.5	12.64		36.4929	0.0047571	0.1736	42.4	
14	2850	40	2.5		12.66					35.4
15	2850	40	2.5	12.67		27.3452	0.0046777	0.1279	28.4	

Table 14 E Effect of salinity on chlorophyll *a* in average of *Oscillatoria limosa*

No.	Salt Conc.	Days									
		2	6	10	14	18	22	26	30	34	38
1	0	0.25	0.322	0.422	0.476	0.898	1.541	1.164	0.941	0.896	0.742
2	0.5	0.48	0.521	0.584	0.633	0.872	1.505	1.207	1.179	1.064	1.025
3	1	0.467	0.532	0.635	0.7	1.502	1.432	1.07	1.035	0.867	0.867
4	1.5	0.486	0.565	0.679	0.988	0.878	0.81	0.75	0.679	0.589	0.512

Table 15 E Effect of salinity on chlorophyll *a* of *Phormidium laminosum*

No.	Salt Conc.	Days									
		2	6	10	14	18	22	26	30	34	38
1	0	0.0088	0.0099	0.018	0.0212	0.0284	0.0272	0.0222	0.0211	0.0204	0.0198
2	0.5	0.00608	0.0062	0.0102	0.0195	0.021	0.0277	0.0328	0.0288	0.0258	0.0211
3	1	0.0048	0.00608	0.0089	0.0133	0.0205	0.0292	0.0253	0.0221	0.0199	0.0176
4	1.5	0.0033	0.00474	0.0051	0.0092	0.0164	0.0259	0.025	0.0193	0.0185	0.0169

Table 16 E Effect of salinity on chlorophyll *a* of *Phormidium* sp.

No.	Salt Conc.	Days									
		2	6	10	14	18	22	26	30	34	38
1	0	0.0055	0.0086	0.014	0.0199	0.0212	0.02	0.0197	0.0185	0.0172	0.0168
2	0.5	0.0032	0.0042	0.0066	0.0132	0.0189	0.0228	0.0221	0.0194	0.0188	0.0175
3	1	0.0049	0.0062	0.0087	0.012	0.0188	0.0209	0.0196	0.0187	0.0162	0.0147
4	1.5	0.0041	0.0055	0.0089	0.0108	0.0245	0.0215	0.02	0.0191	0.0186	0.0178
5	2.5	0.0034	0.0044	0.0055	0.0064	0.0092	0.0139	0.0272	0.0255	0.0203	0.0189

Table 17 E Effect of salinity on chlorophyll *a* of *Scytonema javanicum*

No.	Salt Conc.	Days									
		2	6	10	14	18	22	26	30	34	38
1	0	0.0108	0.0142	0.0163	0.0255	0.0304	0.0357	0.0317	0.0308	0.0299	0.0289
2	0.5	0.0132	0.0166	0.0249	0.0336	0.0392	0.0592	0.0579	0.0562	0.0452	0.0324
3	1	0.0159	0.0199	0.0215	0.0312	0.0511	0.0487	0.0415	0.0378	0.0261	0.0255
4	1.5	0.0137	0.015	0.0178	0.0202	0.0207	0.0472	0.0475	0.0435	0.0423	0.0337

Table 18 E Effect of salinity on chlorophyll *a* of *Nostoc punctiforme*

No.	Salt Conc.	Time (Days)									
		2	6	10	14	18	22	26	30	34	38
1	0	0.00562	0.0074	0.0092	0.0136	0.0204	0.0193	0.0185	0.0177	0.0168	0.0161
2	0.5	0.0046	0.0082	0.0094	0.0114	0.0193	0.0346	0.0341	0.0259	0.0211	0.0199
3	1	0.0058	0.0077	0.0085	0.0122	0.0174	0.0332	0.033	0.0321	0.0294	0.0258
4	1.5	0.0062	0.0077	0.0089	0.0118	0.0165	0.0164	0.0144	0.0139	0.0133	0.0128
5	2.5	0.0056	0.0076	0.0089	0.0115	0.0175	0.0171	0.0162	0.0155	0.0145	0.0137

Table 19 E Effect of light on β - carotene of *Phormidium* sp.

No.	Photon flux				Rettime	Area	Amt/Area	Amount		
	Lux	Mean	mcr.	Mean		(mAU*s)		(ppm)	mg/g	Mean
1	1200		19,8		12.781	60.99624	0.004853	0.295979	8.45	
2	1300	1233	21,4	20	12.787	74.93216	0.004879	0.365581	11.44	11.08
3	1200		19,8		12.804	93.43768	0.004902	0.458005	13.35	
4	2280		37,6		12.904	88.78996	0.004897	0.434792	12.08	
5	2850	2433	47,0	40	12.884	73.58553	0.004877	0.358855	11.15	13.95
6	2200		36,3		12.867	110.3421	0.004916	0.542433	18.63	
7	3380		55,7		12.83	96.67554	0.009048	0.474176	14.49	
8	4840	3970	79,8	60	12.887	107.9592	0.004914	0.530532	16.44	15.91
9	3690		60,8		12.897	104.4169	0.004911	0.51284	16.82	
10	4350		71,7		12.885	112.1439	0.004917	0.551432	16.69	
11	5730	4866	94,5	80	12.88	68.49725	0.004868	0.333442	11.36	13
12	4520		74,5		12.865	64.03799	0.004859	0.311171	10.95	

Table 20 E Effect of light on β carotene of *Oscillatoria limosa*

No.	Photon flux				Rettime	Area	Amt/Area	Amount		
	Lux	Mean	mcr.	Mean		(mAU*s)		(ppm)	mg/g	Mean
1	1200		19,8		12.526	87.22002	0.004895	0.426951	13.95	
2	1300	1233	21,4	20	12.519	80.31632	0.004887	0.392472	11.77	12.06
3	1200		19,8		12.516	67.13519	0.004865	0.326639	10.48	
4	2280		37,6		12.524	102.0164	0.00491	0.500851	16.29	
5	2850	2433	47,0	40	12.511	116.1174	0.00492	0.571277	16.5	14.66
6	2200		36,3		12.545	71.4285	0.004873	0.348082	11.19	
7	3380		55,7		12.495	181.3449	0.004947	0.897051	27.6	
8	4840	3970	79,8	60	12.508	97.56024	0.004906	0.478595	14.05	17.01
9	3690		60,8		12.492	62.46002	0.004856	0.30329	9.38	
10	4350		71,7		12.544	145.2075	0.004935	0.716566	19.53	
11	5730	4866	94,5	80	12.523	147.66	0.004936	0.728814	22.71	19.3
12	4520		74,5		12.551	64.03799	0.00491	0.505469	15.68	

Table 21 E Effect of light on β - carotene of *Phormidium laminosum*

No.	Photon flux				Rettime	Area	Amt/Area	Amount		
	Lux	Mean	mcr.	Mean		(mAU*s)		(ppm)	mg/g	Mean
1	1200		19,8		12.568	41.18257	0.004784	0.197021	8.99	
2	1300	1233	21,4	20,3	12.593	53.72871	0.004833	0.259682	17.04	14.03
3	1200		19,8		12.589	46.16162	0.004807	0.221889	16.08	
7	2280		37,6		12.531	38.07461	0.476692	0.181499	19.05	
8	2850	2433	47,0	40,3	12.571	61.39126	0.004853	0.297952	27.34	23.53
9	2200		36,3		12.599	51.13314	0.004825	0.246718	24.22	
4	3380		55,7		12.533	55.69512	0.004839	0.269503	20.37	
5	4840	3970	79,8	65,4	12.502	42.8841	0.004792	0.205519	19.25	18.17
6	3690		60,8		12.513	38.89609	0.004772	0.185601	14.31	
10	4350		71,7		12.592	66.46677	0.004864	0.323301	30.47	
11	5730	4866	94,5	80,2	12.599	27.75366	0.004682	0.129951	9.84	19.41
12	4520		74,5		12.607	42.88956	0.004792	0.205546	17.91	

Table 22 E Effect of light on β - carotene of *Scytonema javanicum*

No.	Photon flux				Rettime	Area	Amt/Area	Amount		
	Lux	Mean	mcr.	Mean		(mAU*s)		(ppm)	mg/g	Mean
1	1200		19,8							
2	1300	1233	21,4	20,3	12.552	9.46669	0.004079	0.038619	19.3	
3	1200		19,8		12.543	13.11195	0.004334	0.056825	28.4	23.85
4	2280		37,6		12.606	12.77286	0.004316	0.055131	27.55	
5	2850	2433	47,0	40,3	12.603	11.88727	0.004266	0.050708	25.35	28.01
6	2200		36,3		12.627	14.20208	0.004385	0.062269	31.15	
7	3380		55,7							
8	4840	3970	79,8	65,4	12.564	6.01068	0.003553	0.021358	10.7	17.71
9	3690		60,8		12.575	11.38488	0.004234	0.048199	24.1	
10	4350		71,7							
11	5730	4866	94,5	80,2	12.679	10.34969	0.004157	0.043029	21.5	18.5
12	4520		74,5		12.673	7.8052	0.003885	0.030321	15.5	

Table 23 E Effect of light on β carotene of *Nostoc punctiforme*

No.	Photon flux				Rettime	Area	Amt/Area	Amount		
	Lux	Mean	mcr.	Mean		(mAU*s)		(ppm)	mg/g	Mean
1	1200		19,8		12.657	10.67258	0.004183	0.044641	1.86	
2	1300	1233	21,4	20,3						3.34
3	1200		19,8		12.682	24.95914	0.004647	0.115994	4.83	
4	2280		37,6		12.643	19.86158	0.004558	0.090535	4.11	
5	2850	2433	47,0	40,3	12.661	26.58204	0.004669	0.124100	4.96	4.53
6	2200		36,3							
7	3380		55,7		12.665	21.96737	0.0046	0.101052	4.04	
8	4840	3970	79,8	65,4						4.1
9	3690		60,8		12.663	21.85652	0.004598	0.100499	4.16	
10	4350		71,7							
11	5730	4866	94,5	80,2	12.659	20.76357	0.004577	0.09504	4.32	4.41
12	4520		74,5		12.661	21.658	0.004594	0.099066	4.5	

APPENDIX F

THE CHANGE OF CHEMICAL COMPOSITIONAL VALUES DURING GROWTH PHASES

Table 1 F Bands values of *Arthrospira platensis*

CB early		CB middle		CB late	
Name	Value	Name	Value	Name	Value
2963	0.010258	2962	0.913384	2959	0.012848
2925	0.013631	2931	0.019837	2926	0.021428
2872	0,006197	2859	0.008918	2860	0.009079
1732	0.007327	1729	0.006867	1727	0.007140
1651	0.106870	1649	0.096202	1650	0.029244
1542	0.077397	1540	0.067089	1545	0.062776
1454	0.032175	1454	0.029721	1451	0.028622
1398	0.031247	1450	0.029917	1402	0.029097
1303	0.023475	1241	0.032522	1248	0.020519
1238	0.026672	1151	0.029925	1150	0.029539
1162	0.017748	1077	0.056803	1078	0.057625
1079	0.028379	1036	0.066292	1030	0.074102

Table 2 F Bands values of *Phormidium laminosum*

CB early		CB middle		CB late	
Name	Value	Name	Value	Name	Value
2962	0.011847	2961	0.009347	2963	0.011814
2931	0.014258	2927	0.012706	2879	0.007173
2871	0.006402	2859	0.004908	1934	0.014444
1724	0.007493	1739	0.005778	1737	0.00579
1646	0.110487	1642	0.103214	1650	0.107674
1546	0.074961	1540	0.059693	1545	0.068449
1453	0.026942	1451	0.024210	1450	0.024175
1396	0.028226	1399	0.030835	1392	0.031161
1287	0.023286	1255	0.018324	1252	0.020642
1248	0.025149	1146	0.011532	1153	0.011081
1152	0.014527	1080	0.032876	1080	0.032624
1082	0.026171	1037	0.028696	1039	0.027723

Table 3 F Bands values of *Nostoc punctiforme*

CB early		CB middle		CB late	
Name	Value	Name	Value	Name	Value
2964	0.006501	2960	0.013065	2959	0.015555
2930	0.007425	2927	0.020301	2929	0.020178
2882	0.004802	2857	0.0009240	2881	0.011484
1739	0.004714	1732	0.008083	1727	0.008765
1650	0.097289	1651	0.099538	1651	0.096049
1547	0.058375	1540	0.064460	1545	0.067234
1477	0.020192	1450	0.027492	1448	0.029597
1402	0.023504	1402	0.030200	1406	0.033144
1245	0.011268	1246	0.021983	1246	0.026216
1150	0.015778	1151	0.028488	1148	0.031793
1072	0.047868	1075	0.057105	1043	0.072079
1031	0.054222	1030	0.063574	1037	0.072136

Table 4 F Bands values of *Osillatoria limosa*

CB early		CB middle	
Name	Value	Name	Value
2963	0.005448	2962	0.009098
2934	0.007511	2927	0.015232
2879	0.007426	2872	0.007005
1727	0.006849	1740	0.006658
1645	0.092676	1644	0.085851
1538	0.063051	1539	0.051294
1446	0.031029	1409	0.027930
1406	0.034210	1303	0.015486
1245	0.026664	1252	0.021799
1153	0.022170	1149	0.020555
1070	0.053588	1068	0.060657
1031	0.063589	1023	0.075132

Table 5 F Bands values of *Phormidium* sp

CB early		CB middle		CB late	
Name	Value	Name	Value	Name	Value
2960	0.008179	2963	0.016317	2962	0.012019
2929	0.012770	2927	0.021904	2930	0,016191
2858	0.005096	2875	0.011240	1727	0.007095
1730	0.007014	1727	0.007628	1650	0.103268
1650	0.098933	1646	0.010556	1545	0.072175
1543	0.062596	1540	0.078044	1450	0.030371
1457	0.025783	1450	0.034180	1399	0.031497
1396	0.031445	1392	0.035185	1309	0.024434
1241	0.016867	1244	0.030826	1243	0.025464
1153	0.016752	1154	0.024475	1152	0.031829
1077	0.034032	1081	0.038893	1080	0.048241
1036	0.038708	1042	0.041727	1025	0.065797

Table 6 F Bands values of *Phormedium angustissimum*

CB early		CB middle		CB late	
Name	Value	Name	Value	Name	Value
2961	0.011512	2959	0.015000	2959	0.011497
2927	0.016924	2927	0.022787	2927	0.018969
2872	0,007820	2859	0.011309	2855	0.009098
1726	0.006711	1743	0.005571	1744	0.005929
1651	0.093754	1650	0.093221	1649	0.093831
1544	0.059260	1546	0.065974	1545	0.062473
1447	0.024538	1540	0.064737	1450	0.038164
1046	0.030551	1448	0.027917	1409	0.031612
1241	0.018071	1406	0.031578	1241	0.021739
1150	0.023021	1245	0.025156	1146	0.024460
1074	0.056479	1147	0.032842	1073	0.054963
1029	0.069056	1030	0.072188	1029	0.064279

Table 7 F Bands values of *Scytonema javanicum*

CB early		CB middle		CB late	
Name	Value	Name	Value	Name	Value
2964	0.012501	2959	0.014279	2955	0.012765
2926	0.026770	2927	0.022226	2929	0.019950
2858	0.011943	2875	0.011214	2872	0.019950
1738	0.008147	1649	0.085128	1729	0.006752
1646	0.083659	1640	0.085128	1648	0.085242
1546	0.045734	1540	0.051258	1545	0.053539
1454	0.022590	1450	0.026469	1452	0.026431
1406	0.025423	1407	0.029833	1404	0.027652
1252	0.013979	1247	0.015716	1245	0.016933
1150	0.023970	1147	0.028249	1151	0.027716
1075	0.059011	1076	0.068936	1074	0.065813
1043	0.060715	1031	0.080579	1032	0.079833

CURRICULUM VITAE

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