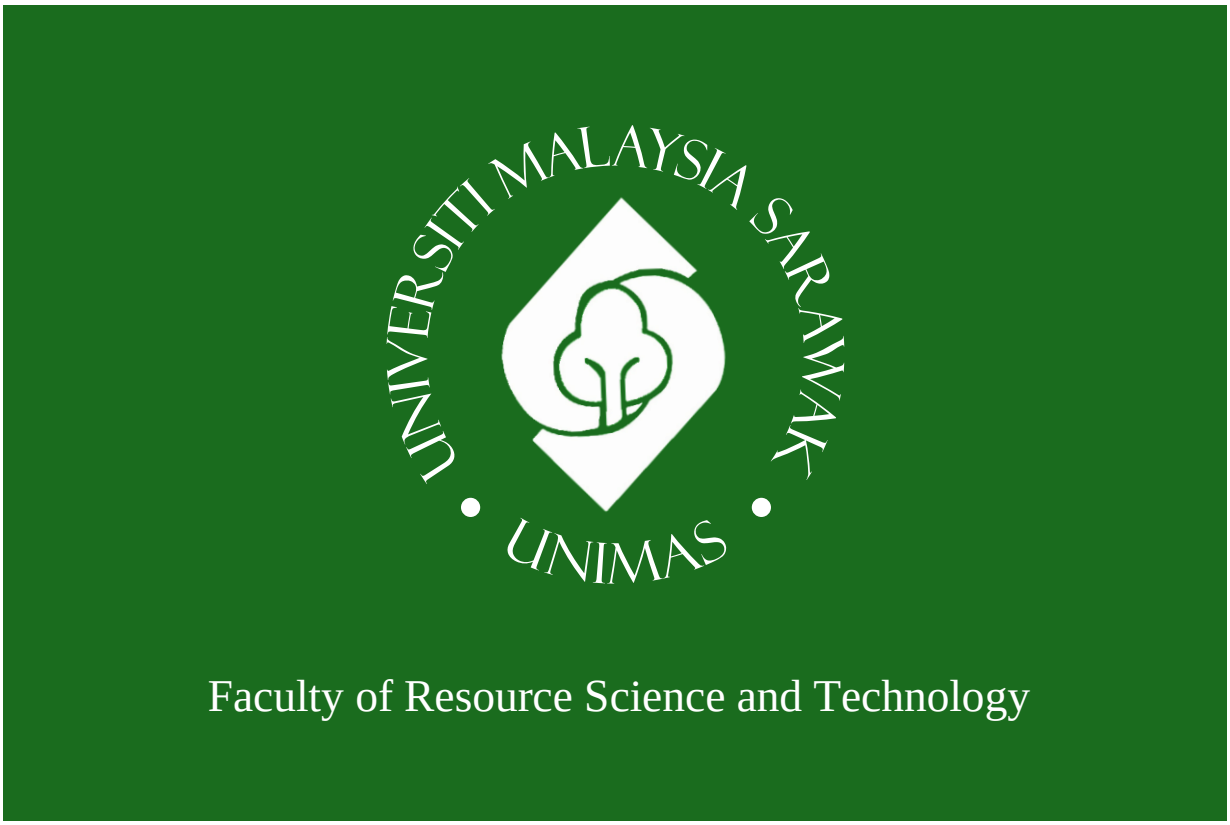

MORPHOLOGY, TOXICITY AND GENETIC STUDY ON FRESHWATER CYANOBACTERIA, *Anabaena* spp.

Lim Hong Chang

Bachelor of Science with Honours
(Aquatic Resource Science and Management)
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MORPHOLOGY, TOXICITY AND GENETIC STUDY ON FRESHWATER CYANOBACTERIA, *Anabaena* spp.

LIM HONG CHANG

This project is submitted in partial fulfillment of the requirement for the degree for
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DECLARATION

No portion of this work referred to in this dissertation has been submitted in support of an application for another degree of qualification of this or any other universities or institution of higher learning.

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LIM HONG CHANG
Aquatic Resource Science & Management
Faculty of Resource Science and Technology
University Malaysia Sarawak

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Cell Isolation, Establishment of Clonal Cultures and Preliminary Molecular Work on Freshwater Cyanobacteria, *Anabaena* spp.

Lim Hong Chang

Department of Aquatic Science
Faculty of Resource Science and Technology
Universiti Malaysia Sarawak

ABSTRACT

Three species of *Anabaena* sp. was isolated from freshwater aquaculture pond, P12 at Indigenous Fisheries Research and Production Centre, Tarat Inland Fisheries Division, Serian used for clonal culture establishment. In this study, modified ASN III found suitable for the clonal culture of *Anabaena* spp. in incubator with cool white fluorescent light intensity with 12:12 light and dark condition at 23^{o}C. Identification and cell isolation of *Anabaena* spp. was carried out using Olympus Inverted microscope. The target gene PC-IGS (Phycocyanin intergenic spacer) found in phycocyanin was amplified using PCβF, the forward primer, and PCαR, the reverse primer with a total PC-IGS PCR amplification fragment of 700bp. Toxin analysis using mouse bioassay showed that the extraction of *Anabaena* spp. (ANA10 and ANA11) killed mice within 24 hours. However, further toxin analysis using HPLC is necessary.

Keywords: *Anabaena* spp., modified ASN III, clonal culture, PC-IGS.

ABSTRAK

Tiga spesies Anabaena sp. *telah dipencilkan dari kolam ikan air tawar, P12 di Pusat Penyelidikan dan Pengeluaran Perikanan Sungai, Tarat, Serian digunakan untuk menghasilkan kultur klonal. Dalam kajian ini, pengubahsuaian ASN III didapati sesuai untuk pengkulturan klonal* Anabaena spp. *dalam pengeram dengan keamatan cahaya berpendarfluor sejuk dengan keadaan 12:12 terang dan gelap pada suhu 23^{o}C. Identifikasi dan pemencilan sel* Anabaena spp. *telah dijalankan dengan menggunakan Olympus mikroskop telangkup. Gen sasaran PC-IGS terdapat dalam fikosianin telah diamplifikasi dengan menggunakan primer PCβF dan PCαR menghasilkan jumlah fragmen amplifikasi PC-IGS PCR sebanyak 700bp. Toksin analisis dengan menggunakan ujian tikus menunjukkan bahawa pengekstrakan* Anabaena spp. *(ANA10 dan ANA11) membunuh tikus yang diuji dalam masa 24 jam. Walau bagaimanapun, toksin analisis dengan menggunakan HPLC perlu dilaksanakan.*

Kata kunci: Anabaena spp., *pengubahsuaian ASN III, kultur klonal, PC-IGS.*

1.0 INTRODUCTION

Cyanobacteria also known as cyanoprokaryotes constitute the most diverse group of plant kingdom. According to Fritsch (1945), cyanobacteria can be divided into five orders namely Chrooccocales, Chaemosiphonales, Pleurocapsales, Nostocales and Stigonematales. Order Nostocales can be divided into three families: Oscillatoriaceae, Nostocaceae and Scytonemataceae. Genera *Anabaena* is classified under Family Nostocaceae (Fig. 1). At present, Cyanophyceae records 150 genera and 1500 species from all over the world. The classification taxonomy of cyanobacteria is based on the morphological features and their nomenclature is ruled by the Botanical Code (BC).

They are the most archaic organisms on the earth that grow during the first three billion years of the earth's history that is during the Precambrian period. In this period, these algae absorbed CO_2 from the earth's atmosphere and released O_2, thus helped this green earth planet hospitable to other organisms. The tiny members of this group dominated the biota in the Proteorozoic Era, an Era between 2.35 and 0.5 billion years ago. This Era is known as the "Age of Cyanophyceae" (Hoek *et al.*, 1995).

Cyanobacteria are polymorphic, prokaryotic microorganisms with single cell, which are visible under the microscope as colonies and trichomes organization. They resemble with gram-negative bacteria in cellular organization and green plants in oxygenic photosynthesis (Stanier and Cohen, 1977) and named variously that is Cyanophytes, Cyanophyceae and most recently Cyanoprokaryotes (Fabbro and Mc Gregor, 2003). They are a group of chlorophyll containing thalloid plants of the simplest type having no true root, stem, leaves or leaf like organs.

Cyanobacteria are aerobic phototrophs. They require only water, carbon dioxide inorganic substances and light for the growth. Cyanobacteria have tremendous potential in environment management; as soil conditioners; biofertilizers; biomonitors of soil fertility; water quality; ameliorator agents; feed for animals; protein supplements and rehabilitation of degraded ecosystems through bioabsorption of metals (Whitton and Potts, 2000). Cyanobacteria which may be heterocystous (Wolk *et al.*, 1999) or non-heterocystous (Stal, 2000) are known as the largest contributors to the second most important biological process (biological nitrogen fixation) on this planet to fix the atmospheric nitrogen.

Besides their vast beneficial aspects there are some harmful aspects of cyanobacteria. Most common harmful aspect of cyanobacteria is formation of algal blooms, which cause several harms to the human and animal. A bloom of cyanobacteria is a common term used to describe an increase in the number of algal cells to a point where they can seriously reduce the water quality. They can discolour water, form surface scum, produce unpleasant tastes and odors, and create problems for aquatic life. Blooms of cyanobacteria can produce health and environmental hazards in water, including water used for drinking or recreation purposes. The blooms are linked to eutrophication of water. As the bloom ages and begins to die, concentration of toxins may increase. Some toxin may persist for more than three months before they are degraded by sunlight and microbial activity (Khare and Kumar, 2006).

Anabaena sp. can produced anatoxins (neurotoxins) and some may produce microcystins (hepatotoxins). The water that contaminated with cyanobacteria bloom may cause health effects through drinking water that containing the toxin or by recreational water contact through skin. Water contaminated by cyanobacteria can cause disease such as nausea,

headache, vomiting, abdominal pain, diarrhea, gastroenteritis, muscle weakness, pneumonia, and paralysis through drinking water. After the drinking water had boiled, the algae will died and release the toxin into the water (Gupta *et al.*, 2006). Nearly all animals including sheep, horses, ducks, fish and wild animals can be poisoned by cyanobacteria. Animals affected with the toxins may show weakness, pale colour mucous membranes, mental derangement, bloody diarrhea and death.

The toxicity in clonal culture of cyanobacteria is needed to be evaluated. The toxins that produce from the clonal culture cyanobacteria can be then isolate and study their effects upon living organisms. The toxic strain can be analyzed by using High Performance Liquid Chromatography (HPLC) to identify the toxin content in terms of cell level. Generally it is difficult in taxonomy study if just based on the visible morphology of the cyanobacteria for identification as some of the morphology of the cell might change due to different culture conditions. The complementary molecular data is needed because each type of species has different genetic sequence.

1.1 Objectives

1. To isolate *Anabaena* spp. from selected aquaculture pond and establish clonal culture of the species in laboratory conditions.

2. To obtain the genetic data on the *Anabaena* spp. from Malaysia water.

3. To detect the toxicity of the selected *Anabaena* spp.

KINGDOM: Monera

DIVISION: Cyanophyta

CLASS: Cyanophyceae

ORDER: Chroococcales

ORDER: Chaemosiphonales

ORDER: Pleurocapsales

ORDER: Stigonematales

ORDER: Nostocales

FAMILY: Nostocaceae

GENERA: *Anabaena*

Fig. 1: Systematic of cyanobacteria (*Anabaena* sp.) taxon according to Fritsch (1945).

2.0 LITERATURE REVIEW

This section will includes three subsections namely morphology of cyanobacteria, clonal culture of cyanobacteria, *Anabaena* spp. and genetic study.

2.1 Morphology of cyanobacteria

The blue-green algae, class Cyanophyceae contain chlorophyll α which differs from the chlorophyll of those bacteria which are photosynthetic, and also by the fact that free oxygen is liberated in blue green algal photosynthesis but not in that of the bacteria. In light of these considerations, while acknowledging that they have close affinities with the bacteria, the blue-green algae have been retained among the algae. Beside chlorophyll α, the other pigments and plastid organization that can found in cyanobacteria are c-phycocyanin, allophycocyanin, c-phycoerythrin, β-carotene and several xanthophylls. Among these pigments, phycocyanin can only be found in cyanobacteria. The storage product is cyanophycin granules (arginine and aspartic acid) and polyglucose (Harold & Michael, 1985).

Traditional methods of identification and the taxonomy of prokaryotic microalgae, cyanobacteria are based on morphological characteristics according to the Rules of International Code of Botanical Nomenclature. Morphology is a key factor and allows the use of manuals with tentative identification keys and the basic information about cyanobacteria for phycologist, bacteriologist, and researchers in ecological, experimental or applied biology (Hiroki *et al.*, 1998). According to Li *et al.* (1997), *Anabaena*, has been

identified based on morphological characteristics in many ecological or environmental studies. However, the identification of this genus is complex as some morphological and physiological characteristics change or may not be expressed in the culture for example the absence of akinete in trichome of *Anabaena* sp. Therefore, it should be study by combine morphological and genetic approaches.

The trichomes of the Family Nostocaceae are un-branched and form heterocysts and akinetes at maturity. Filamentous morphology is the result of repeated cell divisions occurring in a single plane at right angles to the main axis of the filament. The multicellular structure consisting of a chain of cells is called a trichome. The trichome may be straight or coiled. Cell size and shape show great variability among the filamentous cyanobacteria. Vegetative cells may be differentiated into heterocysts (having a thick wall and hyaline protoplast, capable of nitrogen fixation) and akinetes (large thick-walled cells, containing reserve materials, enabling survival under unfavourable conditions) (Mur *et al.*, 1999). Due to the presence of gas vacuoles, *Anabaena* spp. has buoyancy allows them to float at water surface, where sufficient light is available.

Some of the morphological attributes useful for the identification of *Anabaena* spp. (Fig. 2) includes life form (planktonic, benthic, endophytic), trichome form (bundle, solitary), trichome shape (straight form, regularly coiled, irregularly coiled), trichome diameter (µm), mucilaginous sheath (presence, absence), coil diameter and distance (µm), cell shape (spherical, barrel shaped, short barrel shape , cylindrical, ellipsoidal, short ellipsoidal, quadrate), apical cell shape (rounded, conical, obtuse conical), gas vacuole (presence, absence), heterocyst shape (spherical, subspherical, cylindrical, barrel shaped, ellipsoidal,

oval, oblong), heterocyst sheath (presence, absence), heterocyst width and length (µm), akinete (solitary, in pair, in series more than three), akinete location (adjacent to one side of heterocyst, adjacent to both sides of heterocyst, far from heterocyst, rarely far from heterocyst, rarely adjacent to heterocyst, irregularly located), akinete morphology (lemon shaped, barrel shaped, ellipsoidal, subspherical, oval, spherical, oblong, bent shaped), akinete apices (rounded, flattened, acute, protracted, constricted), akinete membrane (thick, thin, smooth, with fine spine, with coarse spine), color of akinete (colorless, yellowish brown, pale brownish, pale greenish, yellowish), akinete diameter and length (µm) (Hiroki *et al.*, 1998).

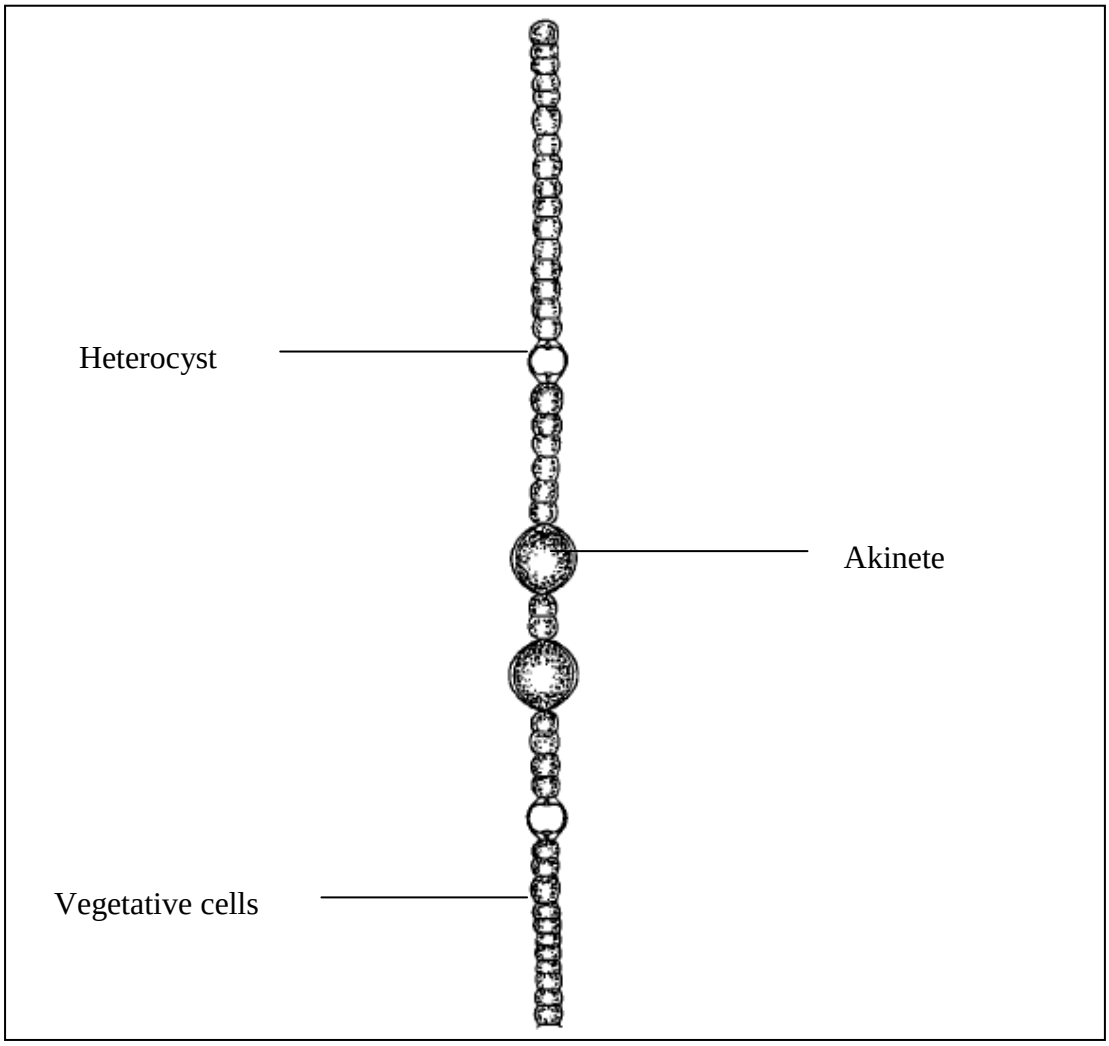

Fig. 2: Morphological attributes of *Anabaena* sp. (Mur *et al.*, 1999).

2.2 Clonal culture of cyanobacteria, *Anabaena* spp.

Freshwater display a wealth of environments and algal flora. The distribution of cyanobacteria in freshwater depends not only on the selective action of the chemo-physical environment but also on the algae's ability to colonize a particular environment. Freshwater media are divided broadly into three categories: synthetic, enriched and soil water. The media that were applied for the culture were synthetic. Synthetic (artificial) media are designed primarily to provide simplified, defined media, for both careful experimental studies and routine maintenance of cultures (Watanabe, 2004).

Various culturing media have been developed and used for isolation and cultivation of freshwater algae. Some of them are modifications of previous recipes to meet a particular purpose. According to Rippka (1988), for the isolation and maintenance of heterocystous cyanobacteria, it is advisable to omit the source of combined nitrogen ($NaNO_3$ or KNO_3) from the media and replace their nitrate content eventually by an appropriate amount of a non-nitrogenous anion, chlorides.

With the presence of heterocysts, heterocystous cyanobacteria are able to fix elemental (gaseous) nitrogen, independent of other combined nitrogen source. The nitrogen-fixing enzyme complex nitrogenase is quickly inactivated upon exposure to oxygen. In heterocystous species, protection of nitrogenase from oxygen is accomplished in part by loss during differentiation of the oxygen evolving part of photosynthesis. Freeing the heterocysts from the vegetative cells of *Anabaena* demonstrated that heterocysts alone were

able to fix nitrogen (Harold & Michael, 1985). Heterocysts increase in number when nitrogen in the environment has been depleted.

Heterocysts may be terminal or intercalary in the trichome and may be evenly distributed among the vegetative cells. Akinete develops from a vegetative cell that becomes enlarged and filled with food reserves (cyanophycin granules). After a period of dormancy, the akinete may germinate giving rise to a vegetative trichome. Potassium nitrate and ammonium chloride inhibit akinete formation (Tyagi, 1974).

2.3 Genetic study

Wilmotte and Golubic (1991) stated that the current status of cyanobacterial systematic whereby the identification of particular species based on morphology is not easy. Variable environmental conditions may affect the recording of phenotypes based on expressed proteins, such as photosynthetic pigments and isozymes, or plasmid content. Similarly, the toxicity of a strain may alter as a result of varying culture conditions (Doers and Parker, 1988). The photosynthetic apparatus of a cyanobacterium contains chlorophyll α and specific accessory pigments, including allophycocyanin, phycocyanin (PC) and phycoerythrin (Glazer, 1984). Phycocyanin and the other biliprotein pigments of the phycobilisomes are the major light-harvesting antennae in photosystem II of Cyanobacteria, Rhodophytes, Cryptophytes (Glazer, 1989).

The distribution of phycocyanin in aquatic microorganisms makes the study of phycocyanin gene sequence heterogeneity ideal for the classification of freshwater

Cyanobacteria. The entire phycocyanin operon contains genes coding for two bilin subunits and three linker polypeptides (Belknap and Hazelkorn, 1987). The intergenic spacer (IGS) between the two bilin subunit genes designated β (*cpcB*) and α (*cpcA*), of the phycocyanin operon was chosen as a potentially highly variable region of DNA sequence useful for the identification of Cyanobacteria to the strain level. Amplification of the phycocyanin-intergenic spacer (PC-IGS) sequence, via PCR (Mullis and Faloona, 1987), from extracted DNA and crude lysates of non-axenic environmental isolates of cyanobacteria was possible.

Neilan *et al.* (1995) study stated that alignments of the available phycocyanin peptide and gene sequences were the basis for the design of two PCR primers which were non-degenerate for the current database sequences. This lack of primer redundancy was possible because of the presence of completely conserved regions within both the α and β subunits of the phycocyanin operon. The primers situated within these functional subunits and flanking the variables intergenic spacer were designated PCβF, the forward primer, and PCαR, the reverse primer will yield approximately a total fragment of 685bp PCR products on *Anabaena* sp. that possesses PC-IGS sequence. The restriction fragment length polymorphisms (RFLPs) detected by digestion of the PC-IGS PCR products with a range of 4-bp and 6-bp recognizing restriction endonucleases provided signature profiles specific to the genus, species and population classifications of *Anabaena* spp.

3.0 MATERIALS AND METHODS

This section will cover five parts namely field sampling, cell culture, genetic study, mouse bioassay and flow chart, respectively.

3.1 Field sampling

The freshwater water samples were collected from pond P12 at Indigenous Fisheries Research and Production Centre, Tarat Inland Fisheries Division, Serian (Fig. 3) on 5 September 2007. The water was sampled from the surface and the water quality was recorded which includes pH, turbidity, DO, temperature and TDS using HORIBA W-22D. The collected water samples were stored inside cooler box before sending back to laboratory for clonal culture.

The permission to collect sample for this research was applied and approved by Controller of Wild Life/ Controller of National Parks and Nature Reserves, Forests Department Sarawak with the permit number NPW.907.4.2(II)-90.

3.1.1 Sampling site

Fig. 3: Map shown the sampling site i.e. Indigenous Fisheries Research and Production Centre, Tarat Inland Fisheries Division, Serian.

3.2 Cell culture

This part consisted of few subsections i.e. modified ASN III medium preparation, pre-sterilization and cleaning procedures, single-cell isolation technique and clonal culture establishment.

3.2.1 Modified ASN III medium preparation

The clonal culture was carried out using modified ASN III medium (Rippka *et al.*, 1979) with omission of some components inside the medium includes $NaNO_3$, NaCl, KCl and $MgCl_2.6H_20$ (Table 1). 1L of trace metal (Table 2) was prepared as mixed stock solution stored in 1L Schott bottle and kept in refrigerator at 4^oC. Separate stock solution of each macronutrient were prepared at a concentration of 10-fold of the final concentration. Each macronutrient was weighted accordingly to the gram unit needed and diluted with 100mL distilled water.

Approximately 10ml of each prepared macronutrient and 1ml of trace metal were added in 1L of distilled water. The mixture is then transferred into 1L Erlenmeyer flasks (capped with cotton bung) or 1L Schott bottle to be sterilized at 120^oC for 20 minutes at 1.0atm (autoclave) . The media were left cold at room temperature before use for clonal culture. The requirements in *Anabaena* spp. culture establishment includes filtered freshwater, nutrient contents (modified ASN III medium), incubator (illumination of 12:12 light-dark condition at 23^oC).

Table 1: Composition of modified ASN III

Component (s)	Concentration (g/L)
K_2HPO_4	0.02
$MgSO_4.7H_2O$	0.035
$CaCl_2.2H_2O$	0.0134
Citric acid	0.003
Ferric Ammonium Citrate	0.003
EDTA (Disodium salt)	0.0005
Na_2CO_3	0.01
Trace metal[#]	1.0ml/L

Table 2: Trace metal[#]

Component(s)	Concentration (g/L)
H_3BO_3	2.86
$MnCl_2.4H_2O$	1.81
$ZnSO_4.7H_2O$	0.222
$Na_2MoO_4.2H_2O$	0.39
$CuSO_4.5H_2O$	0.079
$CoCl_2.6H_2O$	0.0494

Source: Hughes *et al.*, (1985) and Rippka *et al.*, (1979).

3.2.2 Pre-sterilization and cleaning procedures

The standard cleaning method consists of immersing the vessels overnight in a neutral detergent bath (commercial detergent for laboratory use), followed by scrubbing with a brush and sponge for certain apparatus (Masanobu and Mary, 2004). The apparatus were rinsed several times with running water to remove the detergent. For Pasteur pipettes and tips, ELMA ultrasonic sonicator was used to completely remove the detergent, remained chemicals or cells for 2-3 hours. The final rinsed was with distilled water and the cleaned apparatus were dried in drying cabinet protected from dust.

The Pasteur pipettes used for transferring cells and samples that required in sterile condition were plugged with cotton wool at the wide end, wrapped with aluminium foil, kept in stainless steel canister and autoclaved. The Erlenmeyer flasks used for culturing (250mL or 500mL) can be capped with silicon rubber plugs or silicon rubber plugs with extended shield over the lip of the flask. Since it is not available in laboratory, traditional cotton plugs were made. The Erlenmeyer flasks prepared for culturing were filled with approximately 10% of distilled water accordingly to the total volume of the flask. The mouth of Erlenmeyer flasks were capped with cotton bung, wrapped with aluminium foil and autoclaved.

Besides culturing in Erlenmeyer flasks, it can be pre-cultured in test tube. The preparations include the cleaning of test tubes (washed with tap water and rinsed with distilled water) and filled with approximately 30-40mL of culture medium. The test tubes were capped with plastic caps and autoclaved, prepared for culturing.