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# An Improved Method for Purification of Marine Cyanobacteria Isolated from the Gulf of Thailand

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**Thirty five marine cyanobacteria isolates obtained from water samples collected from the Gulf of Thailand of the Chumpon and Chonburi provinces. They were purified by homogenizing, washing, lysozyme, antibiotics and dilution. Successful purifications were checked by morphological characterization (plate culture and scanning electron microscope) and molecular methods (Multiplex randomly amplified polymorphic DNA and 16S rRNA gene). This study presents the results of axenic cyanobacteria were obtained on ASNIII agar plates and scanning electron microscopy. The DNA fingerprints of all axenic marine cyanobacteria were divided into eight clusters and identified using 16S rRNA gene sequence correlatively close to *Geitlerinema* sp., *Leptolyngbya valdenosa*, *Leptolyngbya* sp., *Synechococcus* sp., *Pseudanabaena limnetica* (*Oscillatoria limnetica*) and Oscillatoriales cyanobacterium.**

**Key words:** Cyanobacterial diversity, Molecular identification, Phylogenetic trees, Systematic purification.

Cyanobacteria are interesting for biotechnologists because they produced widely important secondary metabolites[1] which widely diverse activities such as herbicide, algaecide, bacteriocide, fungicide, insecticide, antiviral, anticancer, etc.[2, 3]. They live in diverse wide range of terrestrial and marine conditions[4]. However, study on genetic diversity of cyanobacterial strains are difficult to achieve because, cyanobacteria have the variable morphological appearance depending on the prevailing environmental conditions[5]. They have morphological change when grown under different culture in artificial media. Therefore, they have been estimated that as many as 50% of cyanobacterial strains have been misidentified[6]. Moreover, they have various heterotrophic bacteria that adhere to cell surface, inside bundles of filaments and the amorphous mucilaginous of cells in large numbers[7, 8]. In order to avoid interference by these bacteria in physiological and molecular studies on the target cyanobacteria, an axenic culture of cyanobacteria is needed[9, 10]. Several established procedures have been suggested for the purification of axenic cyanobacterial culture. However, some of the procedures involving many laborious steps and consistent results were difficult to achieve. In addition, the procedure may be specific to certain cyanobacteria species[4, 9]. Although a single technique may be used to remove the associated heterotrophic bacteria or contaminants from the cyanobacteria. Since cyanobacteria are widely diverse groups, exhibiting enormous variations in growth, morphology and metabolic capabilities, any particular approach cannot guarantee success of purification[10]. Therefore, the main objective of this study were to obtain axenic culture of marine cyanobacteria isolated from the Gulf of Thailand

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by subjecting the culture to sequential treatment, including homogenizing, washing, treated with lysozyme, addition of antibiotics and serial dilution.

## MATERIALS AND METHODS

### Sample collection and cyanobacterial isolation

Sea water was sampled from the Gulf of Thailand, Chumpon and Chonburi provinces (11.61905ºN 10.8476812ºE to 10.70144ºN 99.3665500ºE and 12.93047ºN 100.87787ºE to 13.28771ºN 100.91078ºE, respectively), between Jan and Feb 2010. The water samples of four sample sites were collected at 0-1 m depth from marine surface using 20 µM mesh of plankton net, placed in 1 L sterile Schotte bottles and enriched at 25 °C, light intensity of 20 µmol photons $m^{-2}s^{-1}$ provided by cool white fluorescent tubes with a light/dark cycles of 12h/12h for 7 days.

One thousand mL of water samples were vacuum filtered through a sterile 0.2 µm pore size filters nylon membrane (Whatman), which were then resuspended in 5 mL ASN III medium tubes and dispersed by vortex mixer. The 1 mL cell suspension was diluted in sterile ASN III medium and mixed with 1.4% (w/v) ASN III agar at 40 °C then poured into the Petri dishes and solidified at room temperature. Plates were sealed with parafilm and incubated in an inverted position under light condition as mentioned above for 1 to 2 months. The plates were incubated until colonies appeared. Single colonies were then re-isolated and transferred to 50 mL of liquid medium.

### Purification of cyanobacteria with modified methods

Each marine cyanobacterial isolate was homogenized in 500 µL of ASN III medium using an homogenizer bar and washed by centrifugation. 500 µL of culture were washed with 900 µL of sterile ASN III medium mixed with buffer (100 mM HEPES, pH 6.8, 10 mM EDTA), then mixed well by vortex and centrifuged at 6000 g for 5 min. Pellets of cyanobacterial isolates were resuspended in liquid ASN III medium, and the procedure was repeated three to six times. The pellets were suspended and treated by lysozyme followed the methods described by Kim *et al*.[11] and Sarchizian and Ardelean[12]. The reaction of samples were resuspended in sterile ASN III medium supplement with ampicillin (100 µg $mL^{-1}$), penicillin (100 µg $mL^{-1}$), imipenem (100 µg $mL^{-1}$) and kanamycin (100 µg $mL^{-1}$)[13, 14] separately in 1 mL medium. The inoculum was obtained from a liquid ASN III culture, previously ''starved'' (kept in dark) for 72 h to deplete the cells of their endogenous carbon reserves. After incubation, cells were washed with 900 µL of sterile ASN III medium by centrifugation at 6000 x g for 5 min and the procedure was repeated six times. About 100 µL of the rinsed cyanobacterial solution were serially diluted (1:10, 1:100, 1:1000, 1:10,000 and 1:100,000) and poured into plates with ASN III agar medium. The plates were incubated under conditions previously described. Finally, axenic marine cyanobacteria from each dilution plates were picked and transferred to 20 mL vial tubes containing 5 mL of ASN III medium mixed with 2.5% NaCl in distilled water and incubated at previous conditions without shaking.

### Checking purity by cultural plates and SEM

Aliquots (20 µL) from vials without visible contamination were mixed Luria-Bertani (LB) suplemented 2.5% NaCl medium plates[15] and incubated at the same condition for 14 days. The plates with no contamination were inoculated on ASN III medium and incubated for 14 to 18 days, until cyanobacterial growth was apparent. Further contamination tests were carried out with these samples, and axenic cultures were transferred to 20 mL vials with ASN III medium. The axenic marine cyanobacterial isolates from culturing on ASN III agar were treated by ethanol and acetone series in a routine manner for SEM specimens preparation. The samples were subjected to fix and dehydrate, and were observed under a SEM (JSM-5910LV; JEOL, USA).

### Extraction and quantification of total genomic DNA for RAPD analysis and 16S rRNA gene sequence.

The method of DNA extraction and quantification followed the methods described by Bolch *et al*[16] and Saha *et al*[17]. Approximately, 30-40 µL packed cell volume was re-suspended in 200 µL of TE buffer (50 mM Tris : 10 mM EDTA, pH 8.0) with 50 µL of lysozyme (25 mg $mL^{-1}$ SDDW, store at -20 ºC up to a maximum of week) and incubated (60-90 min, 37 ºC). Immediately after lysozyme treatment, 50 µL of 10% sodium dodecyl sulphate (SDS) was added and mildly vortexed. About 10 min, the tubes were incubated (30-60 min, 60 ºC) with 3 µL of proteinase-K (20 mg $mL^{-1}$ SDDW, stored as aliquots at -20 ºC). Thereafter , 300 µL of

ice-cold neutralization solution (3 M potassium acetate, pH 5.5) was added and gently mixed by inverting 4-6 times prior to placing in an ice bath and added with 300 µL of hot saturate buffer phenol (pH 7.9): chloroform : isoamyl alcohol (25: 24 : 1). The lysate was then centrifuged (10 min. 15000 x g) to remove major amount of polysaccharides, proteins and total DNA was transferred to a new microcentrifuge tube. The supernatants were re-extracted with 300 µL of chloroform: isoamyl alcohol (24:1) till clear supernatant was obtained. DNA was precipitated with 1 mL of chill absoluted ethanol (-20 ºC), gently mixed by inversion and was incubated overnight (60 min, -20 ºC). The precipitate DNA was obtained by centrifugation (15 min, 15000 x g) and washed with 70% cold ethanol before centrifuged again at 15000 x g for 15 min. The pellets were dried (30 min) and re-suspended in 50 µL of SDDW. Thereafter, 3 µL of DNA was incubated for 10 min at 37 ºC to digest away all contaminating RNA. DNA quality and quantity were checked on 1% (w/v) agarose gel dissolved in TAE buffer. DNA on agarose gel electrophoresis was run at 100 volt for 1 h compared with standard DNA (Biolab England Inc.).

**Checking purity of axenic marine cyanobacterial cultures by randomly amplified polymorphic DNA (RAPD)**

DNA fingerprinting of multiplex RAPD were amplified by two random 10-mer of CRA 22 and CRA 23 primers[5] obtained from OPERON Techniques Inc., USA and was performed in a C-100 TM Programmable Thermocycler (Biorad). The PCR reaction mixture was performed in a total volume of 25 µL including 2 µL of 25 ng $\mu L^{-1}$ DNA, 1 µL of 10 mM dNTPs, 1.2 µL CRA 22 or CRA 23 primer, 2.5 µL of 10X PCR buffer, 0.2 µL Taq polymerase (Biolab England Inc.,) and 12 µL sterile HPLC water. The programmable cycle was predenaturation step at 94°C for 4 min followed by 35 cycles of denaturing (95°C for 1 min), annealing (45°C for 30 sec), extension (72 °C for 1 min) and post extension (72 °C for 5 min). The 12 µL of amplified PCR products were loaded and separated on 1.8% agarose gels in 0.5X TAE buffer, electrophored for 2.5 h at 100 volts and then stained with ethidium bromide for 1 h. The banding pattern was viewed and photographed using gel imager TM 1200 documentation (Biorad) and the dendrogram was constructed using method decribed by Prabina *et al*. [18] and Premanandh *et al*[19].

Amplifying 16S rRNA gene was amplified by CYA 359F and CYA 781R primers [20] obtained from OPERON Techniques Inc., USA and was performed in a C-100 TM Programmable Thermocycler (Biorad). The PCR reaction mixture was performed in a total volume of 25 µL including 2 µL of 25 ng $\mu L^{-1}$ of eight axenic marine cyanobacterial DNA templates, 0.5 µL of 10 mM dNTPs, 0.25 µL of 20 µM CYA 359F and CYA781R primers, 2.5 µL of 10X PCR buffer, 0.8 µL Taq polymerase (Biolab England Inc.), and 18.59 µL sterile HPLC water. The programmable cycle was predenaturation step at 94°C for 5 min followed by 35 cycles of denaturation (94 °C for 1 min), annealing (60 °C for 30 sec), and extension (72°C for 60 sec). Finally, post extension was done at 72 °C for 7 min. Then, PCR product was checked using 12 µL of amplified PCR products mixed with loading dye were loaded on 1.8% agarose gels dissolved in 0.5X TAE buffer, DNA from PCR products separated by agarose gel electrophoresis for 1 h at 100 volts was stained with ethidium bromide for 1 h**.** Then, the PCR products were purified by PCR purification kit (Biobasic) and directly sequenced at Biobasic Inc., Canada. The nucleotide sequences (partial 16S ribosomal RNA sequences) were analyzed by using BLAST program at http://www.ncbi.nlm.nih.gov and aligned using SeaView program version 4[21]**.** The cyanobacterial 16S rRNA gene sequences were aligned using the multiple sequence alignment tools in MUSCLE. This was followed by conversion to a distance matrix. The distance matrix was used to reconstruct a phylogenetic tree by the neighbor-joining (NJ), with multiple substitutions corrected and positions with gaps excluded. The number of bootstrap trials were set to 100 and 10,000, respectively.

## RESULTS

**Purification and selection of marine cyanobacterial strains**

Using the lysozyme method combined with an antibiotics treatment (ampicillin, penicillin, imipenem and kanamycin) and physical separation as washing, centrifugation and serial dilution with ASN III and LB agar plates after incubation for 14

days and found that contamination was reduced. Bacterial colonies formed on LB agar about 2 - 6 $x10^5$ c.f.u. $mL^{-1}$ [Fig. 1(A)] and colonies formed on ASN III agar about $7x10^3$ c.f.u. $mL^{-1}$ [Fig. 1(B)]. Purified marine cyanobacteria were isolated using four antibiotics, washing and dilution to $10^{-3}$ found the number of cyanobacterial colonies containing between 10 to 35 colonies [Fig. 1(B)] and also found cyanobacterial colonies grew separately from resistant bacteria.

Bacterial contaminants were not detected on both LB agar and ASN III agar culture plates (Fig. 2) and on the surface of cyanobacteria when observed by SEM (Fig. 3). The results from RAPD indicated the potential to use the RAPD marker method for detecting purity and selection of cyanobacterial isolates from genomic DNA extracted of thirty five cyanobacterial isolates. The primer CRA22 and CRA23 were optimized for identifying cyanobacterial strains that produced the similarity of DNA fingerprint of some isolates and produced totally 10-30 amplified DNA fragments of size ranging from 1,900 bp to 100 bp (Fig. 4). DNA fingerprinting from present study also analysed the genetic relatedness of different cyanobacterial isolates with two primers for revealed cyanobacteria as closely related to each isolate and were classified to eight clusters (Fig. 5). For example, the cluster I was closely related group composed of cyanobacterial strains BM34, BM38, BM44, BM46, LT010, LT06, LT07, LT03, LT02, LT22, LT10 and LT5 with DNA fragments of size ranging from 100 bp to 1,900 bp. The cluster II was closely related group composed of cyanobacterial strains LT19, LT37, LT41, LT42, LT017, BM3, BM3N, BM23, BM37 and BM43 with DNA fragments of size ranging from 150 bp to 1200 bp. The cluster III was closely related group composed of cyanobacterial strains BM1, BM20, BM26, BM28 and LT37 with DNA fragments of size ranging from 500 bp to 1400 bp. The cluster IV was closely related group composed of cyanobacterial strains LT23,

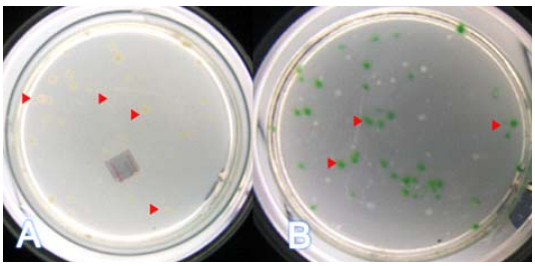


**Fig. 1.** Characteristic of heterotrophic bacterial colonies on Luria-Bertani agar with 2.5% NaCl (A) and axenic marine cyanobacterial colonies (red arrows) on ASN III agar (B)

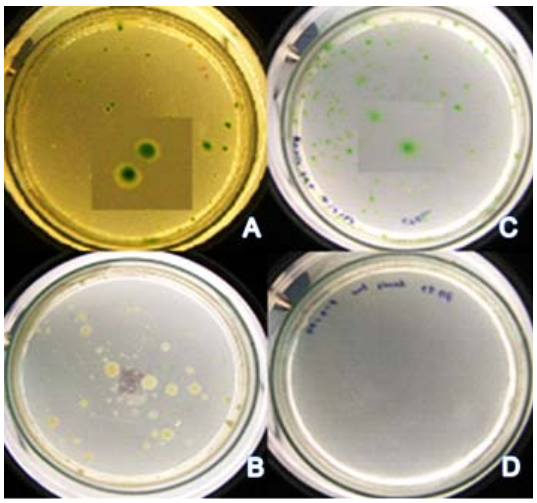


**Fig. 2.** Characterization of non-axenic cyanobacteria strain BM 46 and heterotrophic bacteria on ASN III agar (A) and Luria-Bertani agar (B). Axenic cyanobacteria strain BM 46 obtained on ASN III agar (C) and non-colonial bacteria on LB plates (D) after purification

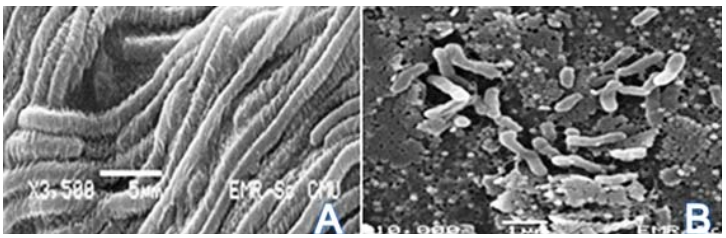


**Fig. 3.** Scanning electron micrograph of axenic strains after purified. (A) *Oscillatoria limnettica*. Scale bar 5 µm; (B) *Synechococcus* sp. Scale bar 1 µm

and LT34 with DNA fragments of size ranging from 100 bp to 1400 bp. The cluster V was closely related group composed of cyanobacterial strains BS, BS1, BS4, and BS6 DNA with DNA fragments of size ranging from 550 bp to 1,600 bp. The cluster VI was closely related group composed of cyanobacterial strains BS11 and BS12 with DNA fragments of size ranging from 150 bp to 1,600 bp. Cyanobacterial cluster VII and VIII found only one isolate in each cluster, they were cyanobacterial strains BS22 and PY4 with DNA fragments of size ranging from 800 bp to 1,350 bp and 500 bp to 1,700 bp respectively. The similarity of each isolate from RAPD showed purity that can be distributed to cluster group in order to ensure the supply of quality cyanobacterial isolates to be identified by 16S rRNA.

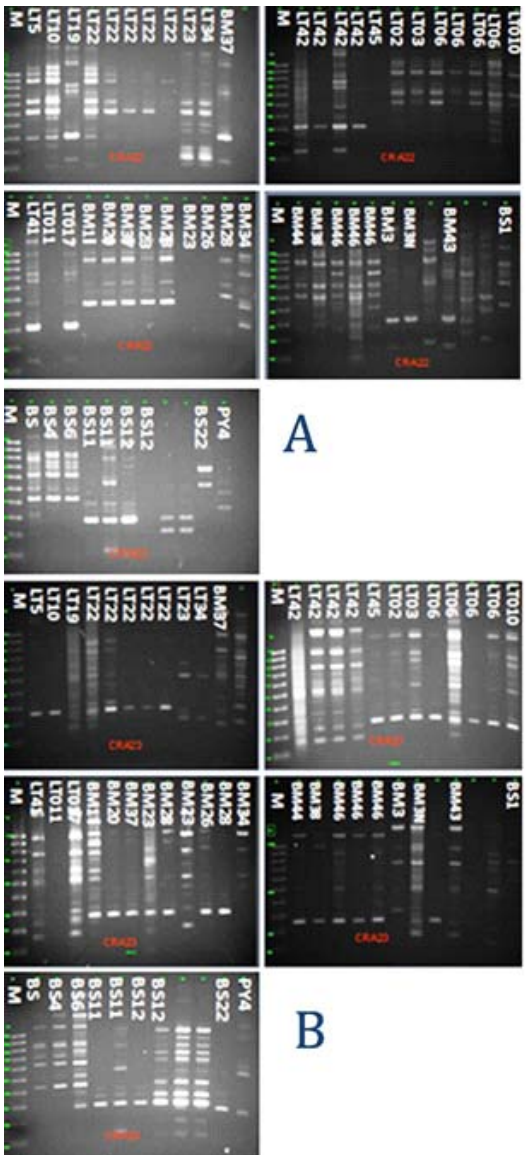


**Fig. 4.** RAPD profile of axenic marine cyanobacterial isolates amplified with primer CRA22(A) and CRA23 (B) from four sampling sites of Chumpon and Chonburi provinces which sampling sites: BM (Bore Mou), LT (Lamthan), BS (Bang San) and PY (Pathaya). The marker lanes (M) contain 100 to 1000 bp in the 1 kb ladder

**Identification of the axenic marine cyanobacteria**

DNA sequence analysis was performed using the partial 16S rRNA sequences. A total of 395 nuclecleotides was determined in the partial 16S rRNA gene sequences of eight cyanobacterial strains chosen by RAPD. The sequence comparisons with validly published cyanobacteria indicated the cyanobacterial strains LT10 and BS22 strains were most closely related to *Oscillatoria limnetica* (Accession no. AF410934.1) with a 99% similarity value. The cyanobacterial strains LT37 and BS1 were most closely related to *Leptolyngbya valderiana* BDU41001 (Accession no. GU186898.1) with a 98% similarity value. The cyanobacterial strains LT19 strain was most closely related to *Leptolyngbya* sp. AECC1324 (Accession no. EU729062.1) with a 98% similarity value. The cyanobacterial strain LT34 was most closely related to Oscillatoriales cyanobacterium (Accession no. AB491863) with a 98% similarity value. The cyanobacterial strain PY4 strain was most closely related to *Geitlerinema* sp. (Accession no. FJ396032.1) with a 98% similarity value. The cyanobacterial strain BS11 were most closely related to *Synechococcus* sp. (Accession no. EF473719) with 98% similarity value. The phylogenetic tree indicated that the eight axenic marine cyanobacteria could be recognized by six clusters. For example, The cyanobacterial strains BS22, LT10, BS1 and LT37 were recognized to be close to two different clusters while cyanobacterial strains BS11, PY4, LT34 and LT19 were recognized to be different clusters (Fig. 6).

## DISCUSSION

Lysozyme is very effective in eliminating cyanobacteria associated heterotrophic bacteria by digesting peptidoglycan of bacterial cell wall[22].

It can eliminate Gram negative bacteria[23] while cyanobacteria and Gram positive bacteria are more resistant to lysozymatic digestion at concentrations up to 1.2 g $L^{-111}$ such as *Streptococcus suis*[22]. Therefore, antibiotics can still be used after lysozyme treatment. The ampicillin, penicillin, imipenem and kanamycin were applied separately since, using a cocktail made marine cyanobacteria produced severe discoloration and death of some isolates. Some method of purification had been reported a cocktail of antibiotics containing penicillin, streptomycin and tetacyclin which were more effective than using a single antibiotic[9]. This method used imipenem, a ß-lactum antibiotic derivated from *Streptomyces cattleya* with a broad spectrum against aerobic and anaerobic Gram

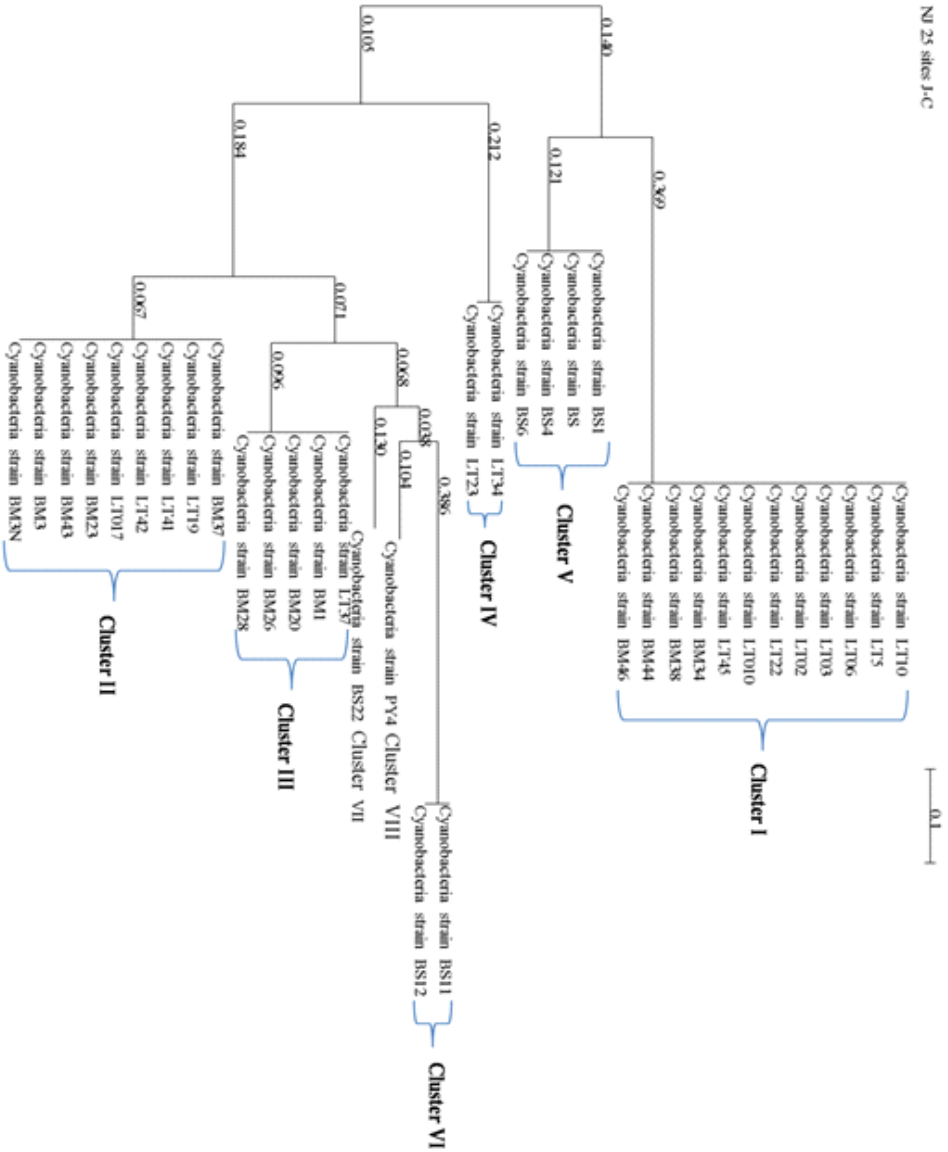


**Fig. 5.** Dendogram representing the relationships between axenic marine cyanobacterial isolates based on neighbor-joining cluster analysis of the RAPD profiles derived using two primers and Jacquard similarity coefficient

positive bacteria as well as Gram negative ones through inhibiting peptidoglycan biosynthesis[13]. Moreover, imipenem does not affect cyanobacterial growth[14] and has been used as the most effective antibiotic for obtaining an axenic culture[4, 13, 16]. However, it was reported that it can not kill some heterotrophic bacteria such as *Nodularia spumigem* KNUA005's *Rhizobium* sp. CB1 and *Brevundimonas* sp. CB2[14]. Therefore, kanamycin was finally used after imipenem treatment to eliminate resistance bacteria because it is an aminoglycoside antibiotic which prevents mRNA translation by affecting the 30S ribosomal subunit[13, 14]. The results showed that it still could

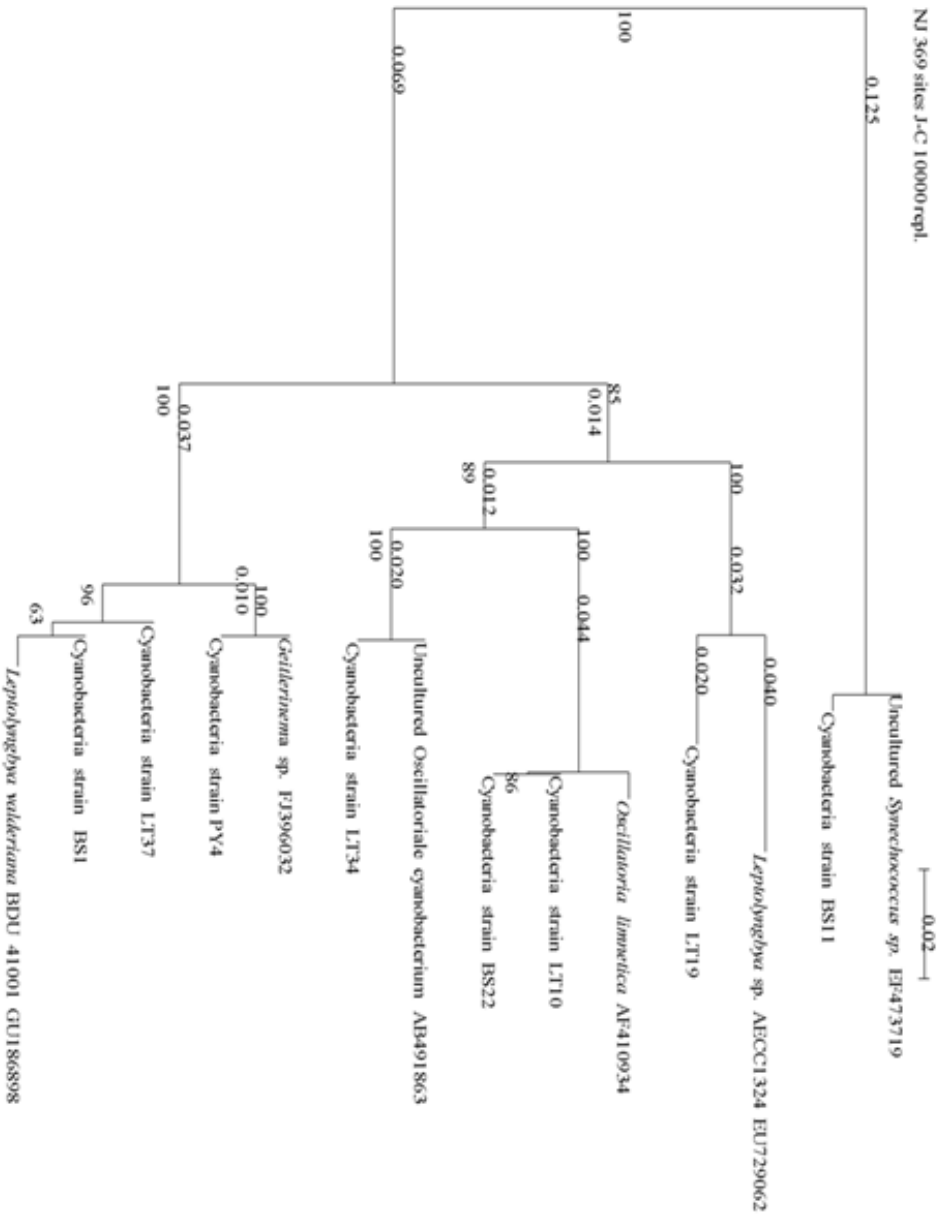


**Fig. 6.** Phylogenetic estimate on relationships of eight axenic marine cyanobacterial strains based on neighbour-joining analysis of partial 16S rRNA gene sequences. Values above the lines represent bootstrap values (10,000 replicates). Bar, 0.02 nuclotide substitutions per position

not remove completely bacterial contaminants that correlated with result of Han *et al*.[13]. Hence, washing and the final step as dilution are essential for the establishment of axenic culture. Washing cyanobacterial cells by centrifugation in ASN III medium was done during homogenization, lysozyme and antibiotics because it effectively reduced the number of bacterial contaminants by removing the free cell contaminants from the cyanobacterial cells. Azma *et al*.[9] claimed the bulk of the smallest contaminants could be removed by using centrifugation technique. Finally, the dilution to $10^{-3}$ was appropriate to make a single cyanobacterial cells disperse to form colonies and dispense heterotrophic bacterial colonies separated from cyanobacterial colonies. Moreover, this dilution is more appropriate than other dilutions since, axenic cyanobacterial colony was obtained (10 to 35 colonies) and resistance heterotrophic bacteria forming colonies was separated from cyanobacterial colonies.

Normally, axenic cyanobacterial isolates were obtained after proving no contamination in culture on both ASN III agar plate and LB agar plates while several report used SEM[11, 13] and RAPD fingerprinting[18]. Moreover, RAPD fingerprinting could be classified cyanobacterial strain[5]. Although, the result of classification by RAPD correlated with the 16S rRNA gene but RAPD showed a good fit revealing discriminatory power of taxonomic cyanobacteria more than 16S rRNA gene. As a result of the multiplex RAPD marker allows the detection of multilocus gene of whole cyanobacterial genome where regions with higher variability used short primer of arbitrary sequence[24] while Drancourt and Raoult[25] claimed 16S rRNA gene allows identifying using single locus of conserve gene. In addition, 16S RNA genes of marine cyanobacteria in this study is not 100% homology with any sequence so the difference between closest and next closest match to the unknown strain is <0.5% divergence (>99.5% similarity)[26]. In conclusion, this is the first successfully established axenic cultures from thirty five marine cyanobacterial isolates from the Gulf of Thailand. It is very effective in obtaining axenic strains not only unicellular cyanobacteria but also filamentous cyanobacteria.

## ACKNOWLEDGEMENTS

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