CHAPTER 3

Investigation of the diversity of actinomycetes from *Nostoc commune* Voucher ex Bornet&Flahault and *Nostochopsis* spp.

3.1 Introduction

Actinomycetes are Gram-positive bacteria, which are classified in the subclass *Actinobacteridae,* order *Actinomycetales.* All members of this order are characterized by high G+C content (>55 mol %) in their DNA [Stackbrandt *et al.*, 1997]. Actinomycetes are saprophytic soil inhabitants and are also the most widely distributed group of microorganisms in nature [Takizawa *et al.*, 1993].

Actinomycetes are found in freshwater and marine; aquatic environments habitats [Fenical and Jensen, 2006; Singh *et al*., 2006; Pathom-aree *et al*., 2006]. In aquatic habitats, taxonomically diverse actinomycetes exhibit unique physiological and structural characteristics. These help them to survive under extreme conditions of pressure, salinity and temperature, with the potential production of novel secondary metabolites. These bioactive compounds were not observed in actinomycetes, but were isolated from terrestrial habitats [Radajewski *et al*., 2002].

The dominant actinomycetes that were isolated from several samples collected from streams, rivers, lake mud, river sediments, beach sands, sponges and marine sediments were *Micromonospora* [Rifaat, 2003; Jensen *et al*., 2005a,b, Eccleston *et al*., 2008]. Besides, other actinomycetes genera; *Amycolatopsis*, *Marinophilus, Rhodococcus, Salinispora, Streptomyces* and *Williamsi*a were found in the aquatic habitats [Mincer *et al*., 2005; Williams *et al*., 2005; Kim *et al*., 2006; Kwon *et al*., 2006; Pathom-aree *et al*., 2006].

Not only did freshwater ecosystems prove to be good sources for isolation, but macroalgae were also representative of good habitats for actinomycetes, as well.

Descriptive studies of bacteria isolated from the surface of macroalgae were reported as early as 1875 [Johansen *et al*., 1999]. The interest in bacterial populations living in association with macroalgae has increased during recent decades. There have been 107 studies on bacterial communities associated to a total of 148 macroalgae (36 Chlorophyta, 46 Phaeophyceae, 55 Rhodophyta, 12 undetermined algae) within the last 40 years [Goecke *et al*. 2010]. Wiese *et al*. [2009] determined that almost 50% of a total of 210 isolates of the epiphytic bacterial community consisted of *Saccharina latissima, Streptomyces, Leifsonia, Amycolatopsis, Arthrobacter* and *Micrococcus.* Actinomycetes were also found from edible macroalgae (*Palmaria palmata*). This alga has traditionally been consumed raw on the coastal shoreline around Northern Ireland. This study revealed that 3 isolates were actinomycetes [Moore *et al*., 2002]. From the study of Cláudia *et al*. [2009], it was shown that communities of associated actinomycetes collected from marine organisms included algae. 16S rRNA sequecing-based analysis revealed that actinobacteria represents 30.4% of these communities.

In terms of cyanobacteria, both colonial and filamentous forms usually have bacterial cells attached to their cell surfaces, such as those found inside tight bundles of the filaments and reaching into the amorphous mucilaginous zone surrounding the colonies [Hoppe, 1981; Paerl, 1992; Nausch, 1996]. With regard to the interactions between cyanobacteria and their associated bacterial flora, some cases are regarded as being symbiotic and as having a, mutually-beneficial association , wherein the later benefits from organic substances released by the former returning remineralised inorganic nutrients, and from which carbon dioxide can be directly utilized by the host algae [Paerl *et al*., 1989]. A positive bacterial chemotaxis in heterocysts result was described by Paerl and Gallucci [1985]. In 2003, Salomon *et al*. studied the bacterial communities in *Nodularia spumigena* and found that the actinomycete strain displayed a negative effect on *N. spumigena*.

The research described in this thesis involved the study of the diversity of actinomycetes isolated from *N. commume* and *Nostochopsis* spp, edible cyanobacteria with mucilage. A pretreatment method and six isolation media were used to recover the actinomycete isolates which were classified into genus level based on morphological observations, as well as chemotaxonomic and molecular analysis.

3.2 Meterials and Methods

3.2.1 Study sites

This study of *Nostochopsis* spp. was conducted during the cool-dry season. In the rainy season, high turbidity obstructed these algal growths. On the other hand, *Nostoc commune* was collected during the rainy season, because it grows on moist basic soil. Important rivers or streams that revealed sustaining edible freshwater macroalgae in abundant levels of growth, were located in various parts of Thailand, especially in the North and Northeastern areas. *Nostochopsis* spp. was collected from the Nan River in Nan Province. The substrate in this site consisted of plenty of gravel and cobblestones. The water body was very clear and the temperature was low even in the summer season. The water velocity was very high. The conditions in this site were suitable for macroalgal growth (Figure 3.1 and 3.2A). Samples of *Nostoc commune* Voucher ex Bornet & Flahault which is the terrestrial species were collected on land from Ubon Ratchathani Province. In this study site, the specimens were collected from the moist soil of the football field in Khemmaraj School, Khemmaraj District. In the rainy season, the macroalgae grew abundantly on basic soil (Figure 3.1 and 3.2B).

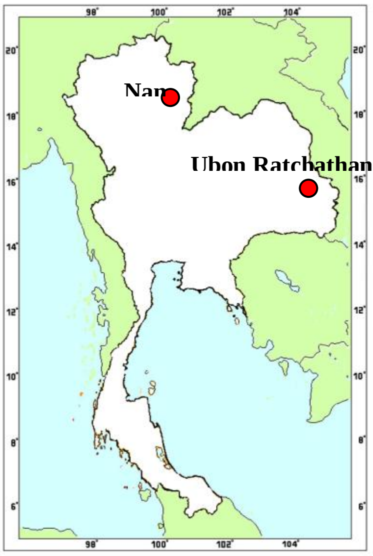


Figure 3.1 Map of Thailand, shows the location of sampling sites of freshwater edible macroalgae

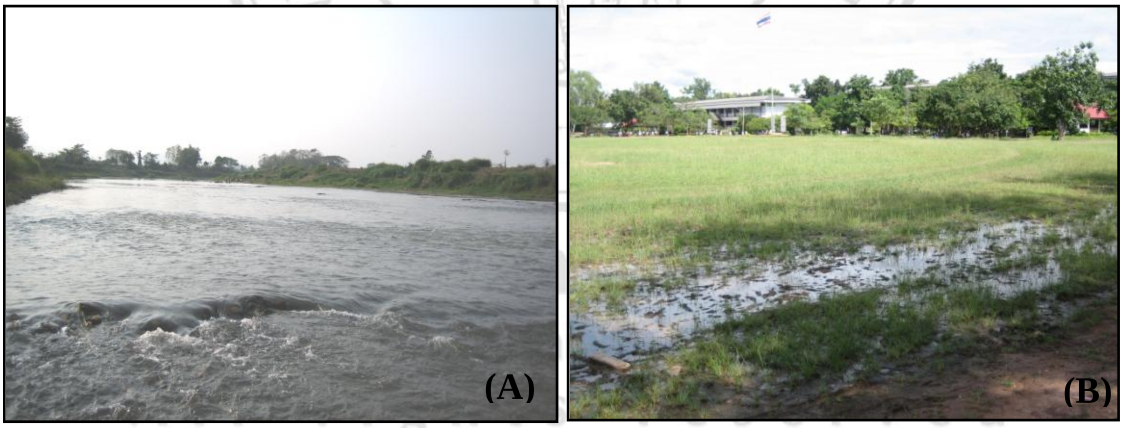


Figure 3.2 Photographs of sampling sites for freshwater edible macroalgae

(A) Nan River, Tha Khum Village, Nan Province
19°05'19" N 100°47'23" E

(B) Khemmaraj School, Ubon Ratchatani Province
16°02'11" N 105°13'14" E

3.2.2 Algal sampling

The algae was collected from the substrate and transferred into sterile plastic bags. All samples were kept at 4 °C until being subsequently processed in the laboratory.

3.2.3 Isolation of actinomycetes

1) Pre-treatment of algae for isolation of actinomycetes

Algal specimens (10g) were washed five times in sterile water to remove the loosely attached microorganisms, then grinded with a mortar and transferred into 100 ml of sterile water [Modified from: Bredholdt *et al*., 2007, 2008 and Hong *et al*., 2009]. The mixed solution was shacken at 25°C for 30 min, then dilutions of up to 10^{-3} were kept in sterile distilled water and incubated in a water bath at 55 °C for 6 min [Kim *et al*., 2005]. Aliquots (0.1ml) were spread onto plates of selective isolation media.

2) Selective isolation media

Twelve different types of media including Raffinose-Histidine (RH) agar, Gause No.2 (GNO2) agar, Starch casein (SC) agar, Hickey-Tresner (HT) agar, Proline-tap water (PTW) agar and Minimal medium (MM) agar were used and all media were supplemented with 10% algae water extract and were used as selective media for isolation (Table 3.1). Algal water extract was prepared by grinding 20 g of algae tissue in a mortar containing 20 ml of sterile water ; the homogenate was filtered through gauze, and centrifuged to collect the supernatant as an algae water extract [Zhang *et al*., 2006].

All media were supplemented with a final concentration of 50 µg/ml of potassium dichromate ($K_2Cr_2O_7$) and 25 µg/ml of nalidixic acid to facilitate the isolation of slow-growing actinobacteria. Potassium dichromate inhibits fungal growth [Yang *et al*. 1995], while nalidicix acid inhibited many types of fast-growing Gram-negative bacteria [Webster *et al*. 2001].

Table 3.1 Composition of 12 selective media for isolation of actinomycetes

Medium	**Composition**	**Reference**
RHA	10g raffinose, 1g L-histidine, 0.5g $MgSO_4.7H_2O$, 0.01g $FeSO_4.7H_2O$, 1g K_2HPO_4, 18g agar, 1 L distilled water	Vickers *et al.*, 1984
GNO2A	3g tryptone, 5g peptone, 10g glucose, 5g NaCl, 15g agar, 1 L distilled water	Tan *et al*., 2006
SCA	10g soluble starch, 2g KNO_3, 2g K_2HPO_4, 2g NaCl, 0.3g casein, 0.05g $MgSO_4.7H_2O$, 0.02g $CaCO_3$, 0.01g $FeSO_4.7H_2O$, 15g agar, 1 L distilled water	Küster and Williams, 1964
HTA	2g soluble starch, 0.4g peptone, 0.2g beef extract, 1.2g yeast extract, 12g agar, 1 L distilled water	Hickey and Tresner, 1952
PTWA	10g L-proline, 15g agar, 1L tap water	Xu *et al*, 2005
MMA	0.5g glucose, 0.5g yeast extract, 1g K_2HPO_4, 0.5g $MgSO_4.7H_2O$, 0.5g NaCl, 18g agar, 1 L distilled water, Microelement stock solution 1 ml	Hozzein *et al*., 2008
RHA algae extract	10g raffinose, 1g L-histidine, 0.5g $MgSO_4.7H_2O$, 0.01g $FeSO_4.7H_2O$, 1g K_2HPO_4, 18g agar, 900 mL distilled water, 100 ml algae extract	Modified from Vickers *et al.*, 1984 and Zhang *et al.*, 2006
GNO2A algae extract	3g tryptone, 5g peptone, 10g glucose, 5g NaCl, 15g agar, 900 mL distilled water, 100 ml algae extract	Modified from Tan *et al*., 2006 and Zhang *et al*., 2006
SCA algae extract	10g soluble starch, 2g KNO_3, 2g K_2HPO_4, 2g NaCl, 0.3g casein, 0.05g $MgSO_4.7H_2O$, 0.02g $CaCO_3$, 0.01g $FeSO_4.7H_2O$, 15g agar, 900 ml distilled water, 100 ml algae extract	Modified from Küster and Williams, 1964, and Zhang *et al*., 2006

Table 3.1 (continued)

Medium	**Composition**	**Reference**
HTA algae extract	2g soluble starch, 0.4g peptone, 0.2g beef extract, 1.2g yeast extract, 12g agar, 900 mL distilled water, 100 ml algae extract	Modified from Hickey and Tresner, 1952, and Zhang *et al.*, 2006
PTWA algae extract	10g L-proline, 15g agar, 900 mL distilled water, 100 ml algae extract	Modified from Xu *et al*, 2005 and Zhang *et al.*, 2006
MMA algae extract	0.5g glucose, 0.5g yeast extract, 1g K_2HPO_4, 0.5g $MgSO_4.7H_2O$, 0.5g NaCl, 18g agar, 900 mL distilled water, 100 ml algae extract Microelement stock solution 1 ml	Modified from Hozzein *et al.*, 2008 and Zhang *et al.*, 2006

Plates were incubated at 25 °C for 4-8 weeks and colonies of actinomycetes and other bacteria were recorded and expressed as CFU/g. Presumptive actinomycete colonies were purified and maintained in a Glucose-yeast extract (GYE) agar with 20% glycerol at -20 °C.

3) Statistical analyses

The effects of the medium on actinomycetes growth were analyzed using Duncan Multiple Range Test (SPSS software 14).

3.2.4 Identification and characterization of actinomycetes

1) Morphological identification

The actinomycete isolates were grown on Glucose Yeast Extract agar (GYEA) at 25 °C for 7 days, then checked for purity by the Gram stain method. The color of the spore masses and diffusible pigment production were visually estimated using the ISCC-NBS colour chart [Kenneth, 1958]. Spore chain morphology was observed on the isolates that were grown on ISP-2 medium at 25 °C for 7 days under light microscopy (400x and 1000x magnification). These were based on the groupings of actinomycete isolates into the Streptomycetes group and Non-Streptomycetes group.

2) Colour determination of presumptive streptomycetes group

Streptomycetes group of actinomycete isolates were grown on three kinds of International *Streptomyces* Project medium (ISP medium), which were ISP2, ISP3 and ISP6 [Shirling and Gottlieb, 1966; Williams *et al.*, 1983], and isolates were incubated at 25^{o}C for 7 days and then examined by eye. The substrate mycelium colours, aerial spore mass colour and diffusible pigment production were visually estimated using the National Bureau of Standards (NBS) Colour Name Charts [Kelly, 1958; Kelly, 1964]. These properties were recorded from mature heavily sporing cultures. Melanin pigment production was observed on peptone-yeast extract-iron agar (ISP6; Difco) plates after incubation for 7 days when the formation of brown to black soluble pigments indicated a positive result. The isolates were assigned to colour groups on the basis of these properties.

3) Sporulating characterization

Spore chain morphology of representative isolates selected from colour groups were observed by using a cross-hatched pattern streak plate on the surface of the agar [Holt *et al.*, 1994]. The isolates were grown on ISP-2 medium at 25^{o}C for 7 days then the mature mycelia displaying aerial spore mycelium growth (Figure 3.3) was viewed under light microscopy (400x and 1000x magnification)**.**

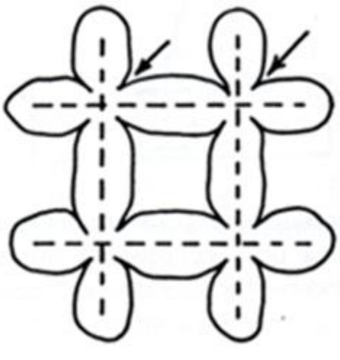


Figure 3.3 Cross – hatched streak plate method for the observative of mature mycelia with sporechain production in the angles of the streaks [Holt *et al.*, 1994 : page 607]

4) Chemotaxonomic analysis : Whole cell diaminopimelic acid analysis

Determination of the diaminopimelic acid isomers was done for the grouping of actinomycete isolates and these were analyzed as described by Hasegawa *et al.* [1983] with some modification [Boone and Pine, 1968; Hasegawa *et al.*, 1983]. Actinomycete isolates were cultured in GYEA broth and incubated at 25 ^{o}C with continuous shaking at 125 rpm for 7 days. The cells were harvested by centrifugation at 6,000 rpm for 15 minutes and washed twice with sterile distilled water, and then cells were frozen at -20°C overnight. The cells were transferred to a screw cap tube (12×70 mm, Kimax Co., Ltd.) and 0.1 ml of 6 N HC1 was added to the tubes which were then heated in an autoclave at 121°C for 15 minutes. Thin-layer-chromatography was carried out by applying 2 µl of each cell hydrolysate sample to a baseline 1.5 cm from the bottom of a 20 x 20 cm cellulose acetate sheet (Merck, Germany). A diaminopimelic acid standard that consisted of LL- and meso-DAP from the actinomycetes standard in each type was applied along side with the samples as a reference marker. The TLC plates were developed in a solvent system consisting of a mixture of methanol, water, 6N HCl and pyridine (80:26:4:10) until the ascending solvent front was about 5 cm from the top. After being dried at room temperature, amino acids in the chromatogram were detected by spraying the samples with ninhydrin in acetone (0.2%, w/v) and heating them at 100 °C for 2-5 min to reveal the spots. The isomeric forms of diaminopimelic acid, which appeared as a blue or violet spot, were identified by comparison with the LL- and the meso-DAP standard from *Streptomyces griseus* and *Micromonospora* sp TISTR 4545, respectively. (Members of the genus *Streptomyces* contain the LL isomer of diaminopimelic acid.)

3) Molecular identification

3.1) DNA extraction

Actinomycete isolates were cultured on non-spore production agar plates [Maldonado *et al.*, 2009] for 7-14 days and DNA was extracted according to a modification by Singka *et al.*, [2012]; Khamna and Lumyong, [2009] ; Zhou *et al.*, [1996] of the CTAB method [Murray and Thompson, 1980]. Actinomycete cells on non-spore production agar plate 0.1 g were transferred to 1.5 ml appendrof tube and 0.1

g of sterile glass bead were added. Then, the specimens were suspended with 0.5 ml of 10% CTAB DNA buffer and mixed by votex for 2-5 minutes. The breaking of the cells was done by mashing and the mixture was mixed by votex for 2-5 minutes. 500 µl of Phenol:Chloroform:Isoamylalcohol (25:24:1) were added and then specimens were vortexed and incubated in an ice-box for 1 minute (a repeat of the vortex and incubation in an ice-box was done for 1 minute 5 times). The suspension was centrifuged at 14,000 rpm for 15 min at 4 °C and then the upper layer was transferred to new tube. 500 µl of Phenol:Chloroform:Isoamylalcohol (25:24:1) were added and inverted for 1 min before being centrifuged at 14,000 rpm for 15 min at 4 °C. The upper layer was transferred to new tube. 500 µl of Chloroform:Isoamylalcohol (24:1) were added and the samples were inverted for 1 min before being centrifuged at 14,000 rpm for 15 min at 4 °C. The upper layer was transferred to a new tube. Cool isopropanol (0.6 times of supernatant) was added, the samples were mixed for 1 min and incubated at -20°C overnight, and then centrifuged at 14,000 rpm for 10 min at 4 °C. DNA was precipitated in the solution. The DNA was washed twice by adding 500 µl of 70% ethanol and the specimens were then dried. The DNA was suspended in 10-30 µl TE buffer and kept at –20°C until being used.

3.2) PCR amplification and sequencing

DNA preparations were used as the template DNA for Taq polymerase . All PCR amplifications were performed with a MyCycler^{TM} Thermal Cycler (Bio-Rad Laboratories, Irvine, CA, USA) using an Ex Taq reaction kit (Takara Shuzo, Shiga, Japan). Primers 11F (5’-AGTTTGATCATGGCTCAG-3’) and 1510R (5’-GGCTACCTTGTTACGA-3’) were used to amplify 16S rRNA gene regions [Tajima *et al*. 2001].. The reactions were performed in a final volume of 25 µl containing;

TaKaRa Ex Taq™ (250U)	0.15	µl
10X Ex Taq™ Buffer (contains 20 mM $MgCl_2$)	2.5	µl
dNTP Mixture (2.5 mM each dNTP)	2	µl
Primer 11F (20 nM)	1	µl
Primer 1510R (20 nM)	1	µl
extracted DNA (1:10 dilution)	5	µl
sterile deionized water	13.35	µl

Amplification reactions were performed in a MyCycler^{TM} Thermal Cycler, according to the following profile:

Initial denaturation	**Amplification**			**Final extension**
	Denaturation	**Annealing**	**Extension**	
94°C 5 min	94°C 30 sec	55°C 30 sec	72°C 30 sec	72°C 7 min
1 cycle	35 cycles			1 cycle

Amplification products were analyzed by electrophoresis in 1% (w/v) agarose gels stained with ethidium bromide. The PCR products were purified using High Pure PCR Product Purification Kit (Roche).

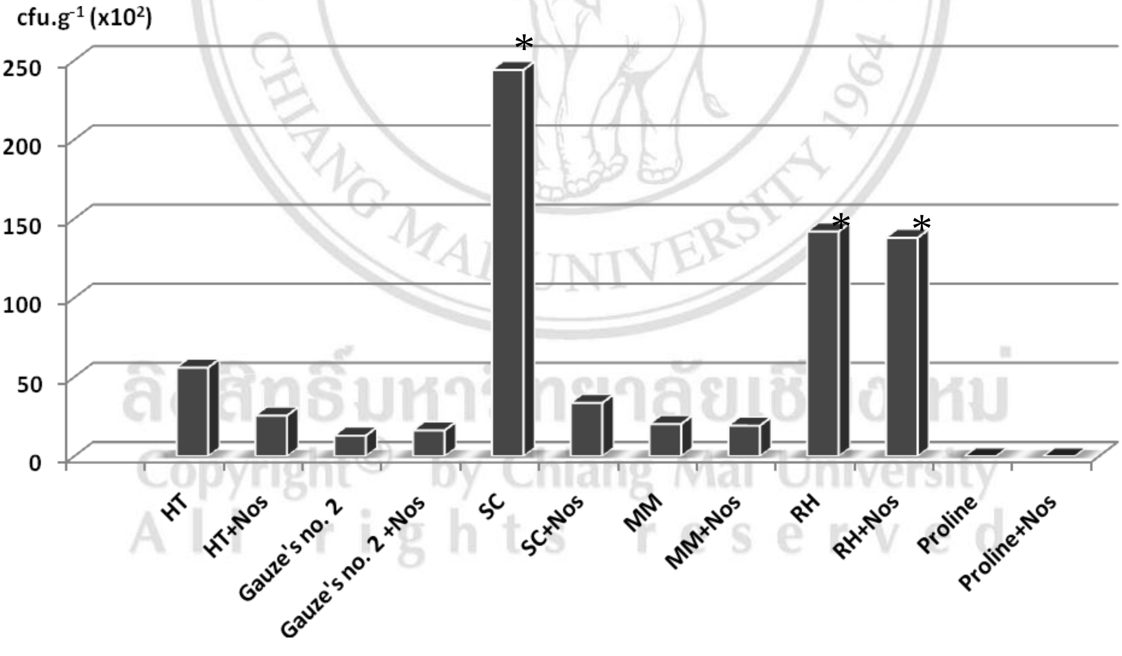
3.3) DNA Sequencing and phylogenetic analysis

The purified PCR products were sequenced using universal primers 11F and 1510R in an ABI PRISM DYE Terminator cycle sequencer (1stBase, Malaysia). The sequence data were proofread using Chromas, version 1.45 (Technelysium Pty Ltd.) and submitted to the BLAST function of GenBank. Phylogenetic analysis was carried out using CLUSTAL-W program in MEGA 4.0.2 (Molecular Evolutionary Genetics Analysis, version 4.0.2) [Tamura *et al*., 2007]. The tree topologies were evaluated by bootstrap analyses based on 1,000 replicates and phylogenetic trees were generated using the neighbor-joining method [Saitou and Nei, 1987]. Only bootstrap values of more than 50% were shown on the phylogenetic trees.

3.3 Results and Discussions

3.3.1 Effect of isolation media

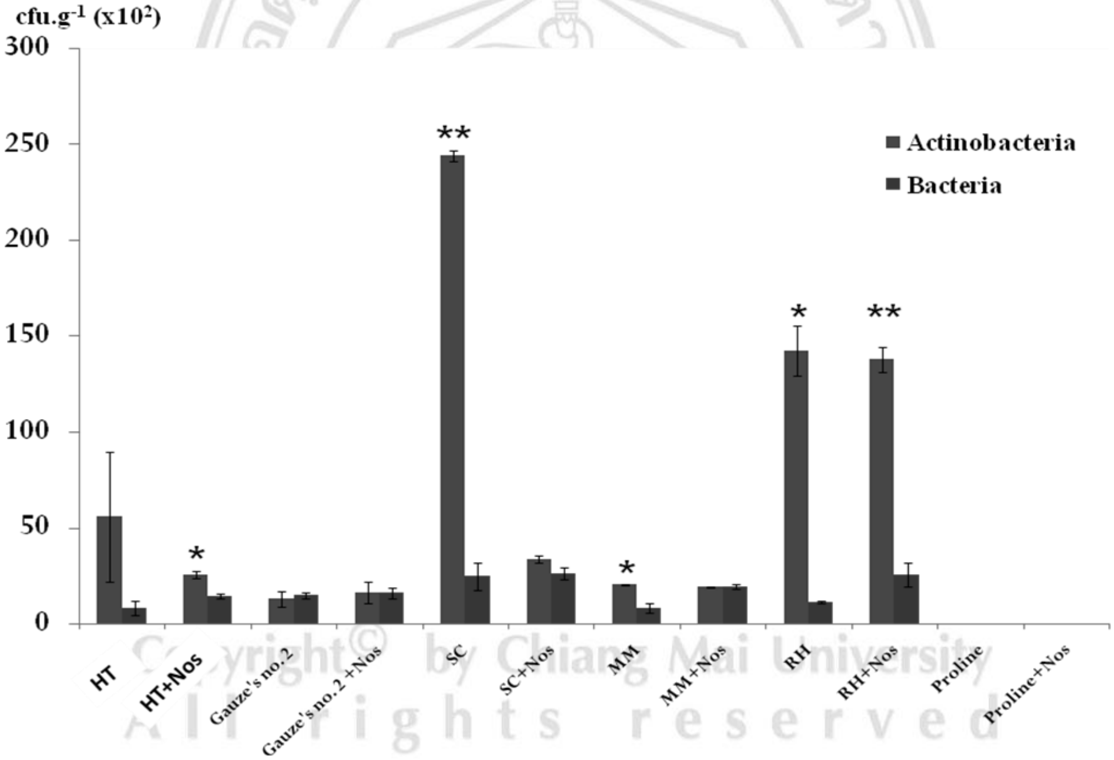
A number of actinomycetes and bacteria isolate ratios that isolated from the algae samples were pretreated by shaking at 25°C for 30 min and incubated in a water bath at 55 °C for 6 min. According to the SPSS for windows using the analysis of variance (ANOVA) with Turkey's-b test in the isolates from the selective media with $p \leq 0.05$, all selective media were used in this investigation and, Starch casein agar (SC) revealed the highest number of colony forming units (CFU) in a gram of *N. commune*; 2.5×10^4 cfu.g^{-1}, while other selective media gave much lower numbers than SC at, less than 6×10^3 cfu.g^{-1}, except for Raffinose-Histidine agar (RH) and Raffinose-Histidine agar with extracts (Figure 3.4).



Data shown are mean ± standard error (SE) of three replicates. a, b and c represent statistical comparison s between groups using ANOVA post hoc Tukey's b Test; ** and * are statistical comparison ats 99% and 95% confidence levels, respectively.

Figure 3.4 Effect of selective media to actinomycetes isolated from *Nostoc commune*

Hickey-Tresner agar with extracts *N. commune,* Starch casein agar, Minimal medium agar, Raffinose-Histidine agar and Raffinose-Histidine agar with extracts revealed significantly ($p \leq 0.05$) high ratios of actinobacteria to bacteria (Figure 3.5). Additionally, From the SPSS for windows using analysis of variance (ANOVA) with Turkey's-b test and the pair test, it was shown that isolates of actinomycetes were not affected by the extracts *N. commune*. On the contrary, Starch casein agar revealed significantly ($p \leq 0.05$) higher ratios of actinomycetes than Starch casein agar with extracts of *N. commune.*



Data shown are mean ± standard error (SE) of three replicates. a, b and c represent statistical comparisons between groups using ANOVA post hoc Tukey's b Test; ** and * are statistical c omparisons at 99% and 95% confidence levels, respectively.

Figure 3.5 Total counts of actinomycetes and viable bacteria isolated from *Nostoc commun*

From the SPSS for windows using analysis of variance (ANOVA) with Turkey's-b test on the isolates from selective media with $p \leq 0.05$, of all selective media used in this investigation, Minimal medium with extracts (MM+Nt) revealed the highest number of colony forming units (CFU) in a gram of *Nostochopsis* spp. ; 2.25×10^4 cfu.g^{-1}, while Proline tap water was not obtained any actinomycete isolates (Figure 3.6).

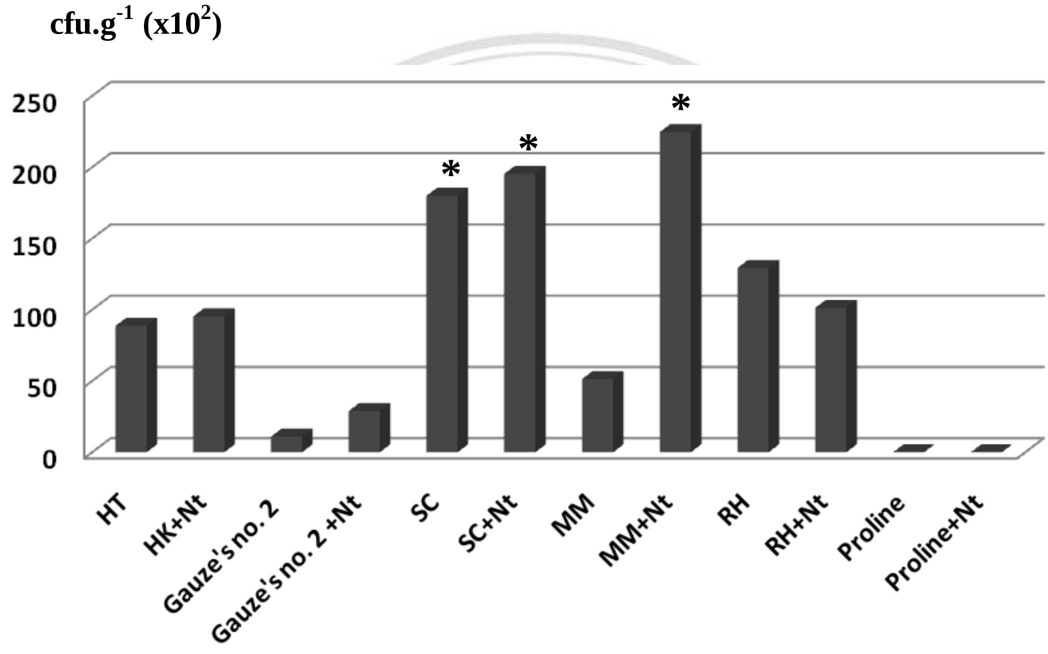


Figure 3.6 Effects of selective media on actinomycetes isolated from *Nostochopsis* spp.

Adding algal extract to the Minimal medium clearly affected the numbers of actinomycete isolates. Although, the number of actinomycetes isolated from the media, to which the algae extract was added were less than in the media without the algae extract, but the colonies of the isolates were found to be of bigger size (Figure 3.7).

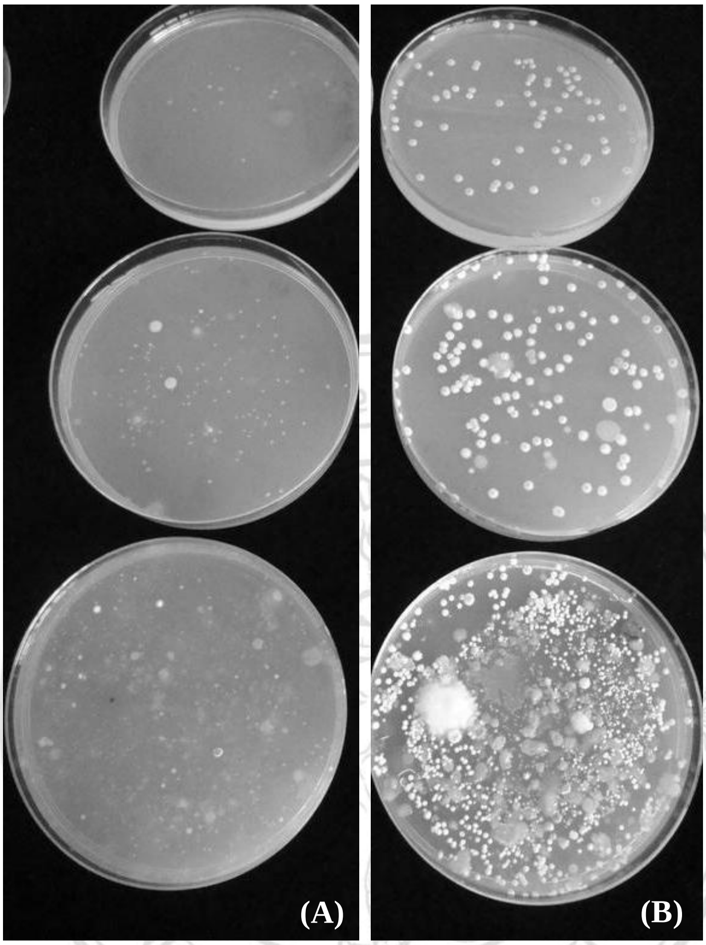
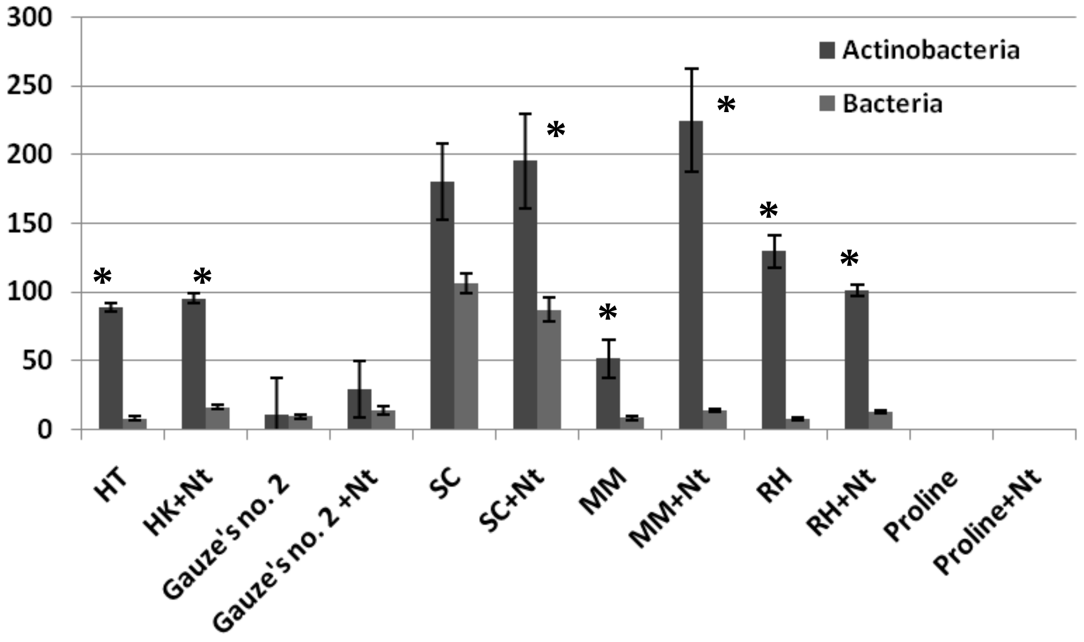


Figure 3.7 Comparison of quantity and size of actinomycete colonies in Raffinose-Histidine agar plate

(A) Raffinose-Histidine

(B) Raffinose-Histidine with algal extract

Hickey-Tresner agar, Hickey-Tresner agar with extracts *Nostochopsis* spp., Minimal medium agar, Minimal medium agar with extracts, Raffinose-Histidine agar and Raffinose-Histidine agar with extracts revealed significantly high ratios of actinobacteria to bacteria (Figure 3.8). Additionally, From the SPSS for windows using analysis of variance (ANOVA) with Turkey's-b test and the pair test revealed that the effect of the extract was clearly found. Minimal medium with algal extract can promote the growth of actinomycetes significantly.



Data shown are mean ± standard error (SE) of three replicates. a, b and c represent statistical comparisons between groups using ANOVA post hoc Tukey's b Test; ** and * are statistical comparisons at 99% and 95% confidence levels, respectively.

Figure 3.8 Total counts of actinomycetes and viable bacteria isolated from *Nostochopsis* spp

3.3.2 Diversity of actinomycete associated with algae

1) Morphology and chemotaxonomic analysis

A total of 80 isolates of actinomycetes obtained from *Nostoc commune* and *Nostochopsis* spp. were grown on 12 selected media. Fifty isolates were obtained from *Nostoc commune*. SCA+algae extract gave the highest number of isolates:, 10 isolates. In contrast with HTA, PTWA and PTWA media with algae extract did not show any isolates from *N. commune* (Table 3.2). Thirty isolates were obtained from *Nostochopsis* spp. HTA medium gave the highest number of isolates at, 7 isolates. But GNO2A, PTWA, and PTWA+algae extract did not show any isolates (Table 3.3). In terms of the basis of morphological observations, chemotaxonomic characterization for primary grouping revealed that the two major groups were streptomycetes and non-streptomycetes actinomycetes, and non-streptomycetes were split into two minor groups, as filamentous and non-filamentous groups. Most of the Actinomycete isolates belonged to the Streptomycetes group, (Tables 3.2-3.7). Figure 3.9 shows the chromatogram of the whole cell amino acid analysis. Actinomycete isolates minor grouping basis of morphology and chemotaxonomic analysis are, shown in detail in Tables 3.4-3.7.

With reference to the filamentous like Streptomycetes group, which was isolated from *Nostoc commune*, 43 isolates fall into this group. These Streptomycete group isolates were grown on 9 media. RHA with algae extract added and SCA with algae extract added were considered the best formulas in which 9 isolates grew of each type, and 8 isolates grew on GN02A (Table 3.2). This filamentous like-Streptomycetes group was separated into ten minor groups based on the morphological colour found in the ISP medium (Table 3.4). Group S8 showed the greatest number of members, at 27 isolates. S6 had 7 isolates, S5 had 2 isolates and the other groups had one isolate each (Tables 3.2 and 3.4). The second group consisted of the filamentous non-Streptomycetes group. Only one isolate was obtained in the RHA with algae extract added (Table 3.2) and the characteristics are shown in Table 3.5. The final group consisted of the non-filamentous Actinomycetes. This group was separated into 2 minor groups:, 2 isolates in B1 and 4 isolates in B2 (Tables 3.2 and 3.4).

The filamentous like Streptomycetes group, which was isolated from *Nostochopsis* spp., consisted of 19 isolates. These Streptomycetes-like isolates were grown on 7 media. HTA medium was considered the best formula for which 7 isolates were grown, and the runner up was GNO2A+algae extract (Table 3.3). This filamentous like Streptomycetes group was separated into nine minor groups based on the morphological colour found in the ISP medium. Group S3 showed the greatest number of members, at 6 isolates. S8 had 4 isolates, S9 and S6 had 2 isolates and the other groups had one isolate each (Tables 3.3 and 3.5). The second group consisted of the filamentous non-Streptomycetes group. Ten isolates were obtained in 4 media (Table 3.3) and this group was separated into 2 minor groups (A1 and A2). Eight isolates, which were considered the majority, were classified into the A1 group. The character in each minor group is shown in Table 3.7. The final group consists of non-filamentous Actinomycetes. Only one isolate was found and it was classified in the B1 group based on the characteristics on the ISP medium (Table 3.3).

Table 3.2 Actinomycete isolation cultured from *Nostoc commune* on various selective media and grouped by morphology and chemotaxonomic characterization.

Selective Media	**Actinomycetes isolates**													
	Streptomycetes												**Non -Streptomycetes**	**total**
	filamentous Actinomycetes										**Non - filamentous Actinomycetes**			
	S1	**S2**	**S3**	**S4**	**S5**	**S6**	**S7**	**S8**	**S9**	**S10**	**B1**	**B2**	**A1**	
RHA				1		4	1							6
GNO2A	1							7				2		10
SCA		1	1					2			2	2		8
HTA														0
PTWA														0
MMA						1								1
RHA+algae extract								9					1	10
GNO2A+algae extract								2						2
SCA+algae extract					2	2		5						9
HTA+algae extract								2		1				3
PTWA+algae extract														0
MMA+algae extract									1					1
total	1	1	1	1	2	7	1	27	1	1	2	4	1	**50**

Table 3.3 Actinomycete isolation cultured from *Nostochopsis* spp. on various selective media and grouped by morphology and chemotaxonomic characterization.

Selective Media	**Actinomycetes isolates**												
	Streptomycetes										**Non - Streptomycetes group**		**total**
	filamentous Actinomycetes									**Non-filamentous Actinomycetes**			
	S1	**S2**	**S3**	**S4**	**S5**	**S6**	**S7**	**S8**	**S9**	**B1**	**A1**	**A2**	
RHA	1								1	1			3
GNO2A													0
SCA					1		1						2
HTA			6			1							7
PTWA													0
MMA		1						1					2
RHA+algae extract								1	1				2
GNO2A+algae extract						1		2			3		6
SCA+algae extract												**1**	**1**
HTA+algae extract											**2**		**2**
PTWA+algae extract													**0**
MMA+algae extract				**1**							**3**	**1**	**5**
total	**1**	**1**	**6**	**1**	**1**	**2**	**1**	**4**	**2**	**1**	**8**	**2**	**30**

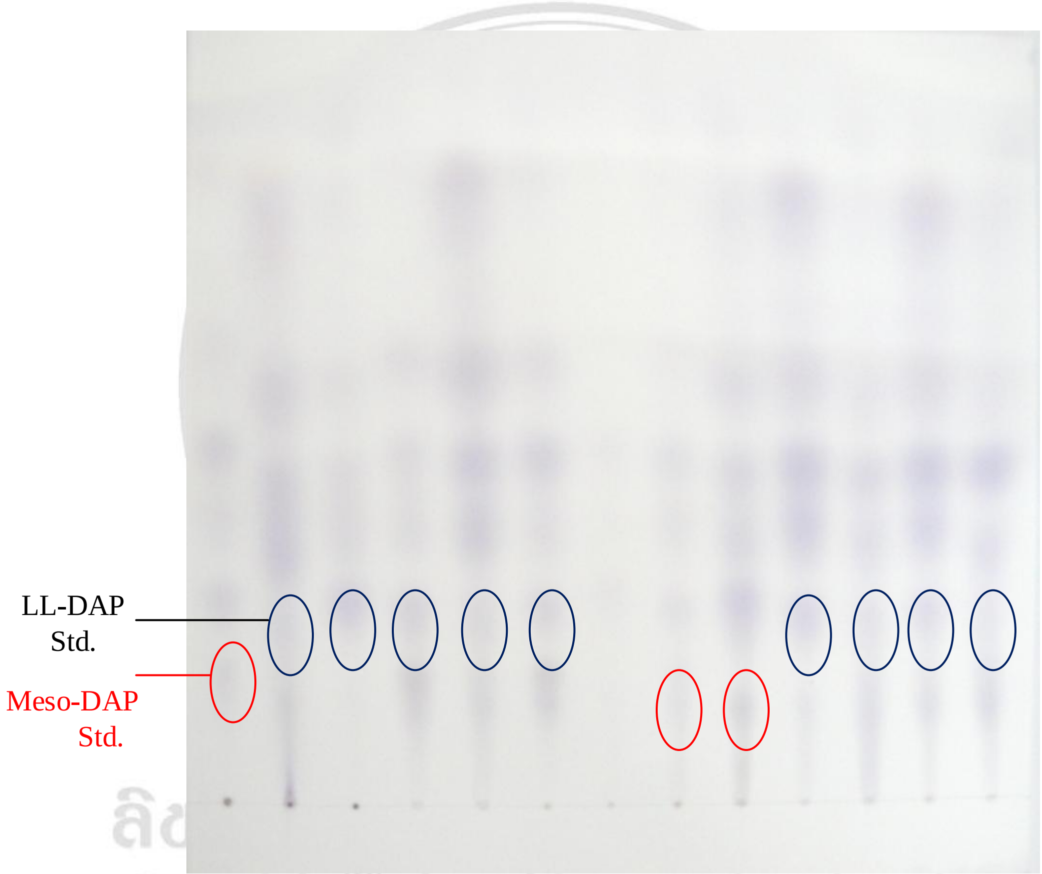


Figure 3.9 The chromatogram of whole cell amino acid analysis.The Streptomycetes isolate group contained LL-DAP and the non-Streptomycetes group contained *meso*-DAP.

Table 3.4 Morphological colour group characteristic on ISP medium of actinomycetes in the filamentous-like streptomycet group from *Nostoc commune*.

Group	**Morphological color groups**								
	ISP2			**ISP3**			**ISP6**	**Spore Morphology**	**Isolates**
	SM	**AM**	**Pig**	**SM**	**AM**	**Pig**			
S1	Light Yellow	White - Pale Yellowish Pink	-	Brilliant Yellow	White - Pale Pink	-	+	Spiral	NTG3 (1)
S2	Grayish Yellow	Gray	-	Olive Gray	Gray	-	-	Spiral	NTS9 (1)
S3	Dark Grayish Yellow	Gray	-	Olive Gray	Gray	-	-	Spiral	NTS10 (1)
S4	Brilliant Greenish Yellow	White - Yellowish White	-	Light Greenish Yellow	Pale Greenish Yellow	-	-	Spiral	NTRH7 (1)
S5	Light Greenish Yellow	White	-	Strong Greenish Yellow	Grayish Olive	-	-	Flexibilis	NTSn6, 7 (2)
S6	Moderate Greenish Yellow	Gray	-	Strong Greenish Yellow	Grayish Olive	-	-	Flexibilis	NTM3 NTRH1,2,6,19 NTSn4,5 (7)
S7	Light Olive	Gray – Light Bluish Green	-	Dark Yellow	Gray	-	-	Spiral	NTRH5 (1)

Table 3.4 (continued)

Group	**Morphological color groups**								
	ISP2			**ISP3**			**ISP6**	**Spore Morphology**	**Isolates**
	SM	**AM**	**Pig**	**SM**	**AM**	**Pig**			
S8	Moderate Yellow Green	White - Very Pale Green	-	Light Greenish Yellow	White - Light Yellow Green	-	-	Flexibilis	NTG1, 2, 4, 7, 8,10, 11 NTGn1, 5 NTHn1, 3 NTRHn1, 2, 6, 7, 10, 11, 12, 16, 18 NTS3, 4 NTSn8, 9, 10, 11, 12 (27)
S9	Moderate Yellow Green	White - Very Pale Green	-	Dark Yellow	Gray	-	-	Spiral	NTMn3 (1)
S10	Moderate Yellow Green	White - Very Pale Green	-	Strong Greenish Yellow	Grayish Olive	-	-	Flexibilis	NTHn2 (1) **Total 43**

SM = Substrate mycelium

AM = Aerial spore masses

Pig = Pigment

Table 3.5 Morphological colour group characteristics on ISP medium of actinomycetes in the filamentous-like streptomycete group from *Nostochopsis* spp.

Group	**Morphological color groups**								
	ISP2			**ISP3**			**ISP6**	**Spore Morphology**	**Isolates**
	SM	**AM**	**Pig**	**SM**	**AM**	**Pig**			
S1	Pale Yellowish Green	White	-	White	White	-	-	Flexibilis	NCRH1 (1)
S2	Vivid Yellow	White - Gray	-	Light Greenish Yellow	White - Light Yellow Green	-	-	Flexibilis	NCM1 (1)
S3	Grayish Yellow	Gray	-	Olive Gray	Gray	-	-	Spiral	NCH6 (1)
S4	Moderate Olive Brown	Gray	-	Strong Greenish Yellow	Gray	-	-	Spiral	NCMn9 (1)
S5	Brilliant Greenish Yellow	White - Yellowish White	-	Light Greenish Yellow	Pale Greenish Yellow	-	-	Spiral	NCS5 (1)
S6	Moderate Greenish Yellow	Gray	-	Strong Greenish Yellow	Grayish Olive	-	-		NCGn7 NCH5 (2)
S7	Moderate Olive	Gray	-	Strong Greenish Yellow	Gray	-	-	Spiral	NCS3 (1)
S8	Moderate Yellow Green	White - Very Pale Green	-	Light Greenish Yellow	White - Light Yellow Green	-	-	Flexibilis	NCGn3,6 NCM4 NCRHn8 (4)
S9	Very Dark Reddish Purple	Light Green	-	Deep Purplish Red	Grayish Green	-	-	Flexibilis	NCR3 NCRHn5 (2) **Total 14**

Table 3.6 Morphological grouping of non Streptomycete groups of filamentous and non filamentous actinomycetes isolated from *Nostoc commune*

Grouping		**Macroscopic Characterization**	**Microscopic Characterization**			**Isolate**
			Spore	**Cell**	**Spore**	
Filamentous Actinomycetes	A1	orange and moderately soft colony, convex and wrinkle	brown – black mucoid masses	filamentous	only one spore on mycelium	1
Non filamentous Actinomycetes	B1	orange – deep pink and soften colony, convex and smooth	-	cocci	-	1
	B2	Yellowish White, shiny and soften colony, convex and smooth	-	rod	-	4

Table 3.7 Morphological grouping of non Streptomycete groups of filamentous and non filamentous actinomycetes isolated from *Nostochopsis* spp.

Grouping		**Macroscopic Characterization**	**Microscopic Characterization**			**Isolate**
			Spore	**Cell**	**Spore**	
Filamentous Actinomycetes	A1	brownish orange colony, convex and filamentous	White - Pale Yellowish Pink	filamentous	long chain spore	8
	A2	orange and moderately soft colony, convex and wrinkle	brown – black mucoid masses	filamentous	only one spore on mycelium	2
Non filamentous Actinomycetes	B1	orange – deep pink and soften colony, convex and smooth	-	cocci	-	1

2) 16S rRNA gene sequencing analysis

All of actinomycetes isolates were grouped based on colony morphology and diaminopimelic acid characterization into Streptomycetes and non - Streptomycetes groups (filamentous and non - filamentous Actinomycetes). After DNA extraction, 16S rRNA gene sequences analysis revealed that the majority of the members in the Streptomycetes group fell into the genus *Streptomyces*.

Fifty isolates were obtained from *Nostoc commune*. The difference in number of isolates from each media was observed. Based on a visible examination of the growth characteristics, 43 representative isolates were selected for 16S rRNA genes sequence analysis. BLAST results indicated that the cultivable actinomycetes in *N. commune* were clustered with members of *Rhodococcus, Actinomadura , Arthrobacter, Gordonia, Sanguibacter* and *Streptomyces* (Table 3.8). The majority of isolates (36/43, 84%) belonged to *Streptomyces*. Ten isolates were closely related to *Streptomyces parvulus* NBRC13193^{T}, which is known for the production of actinomycin D [Kenneth Williams and Katz 1977]. Nine isolates were closely related to *Streptomyces tendae* ATCC 19812^{T} . The remaining isolates were members of the genus *Actinomadura* (1), *Arthrobacter* (2), *Gordonia* (1), *Rhodococcus* (2) and *Sanguibacter* (1). Two isolates of *Arthrobacter* were closely related to *Arthrobacter alpinus* S6-3^{T} . Two *Rhodococcus* isolates were closely related to *Rhodococcus corynebacterioides* DSM20151T and *Rhodococcus kyotonensis* DS472^{T}. One isolate each was closely related to *Gordonia terrae* NBRC100016^{T} *Sanguibacter inulinus* ST50^{T} and *Actinomadura namibiensis* DSM 44197 ^{T}. Only 8 isolates showed at least a 98% sequence similarity rate with other related type strains. The remaining isolates showed a 87-98% similarity rate with their closest type strains, values well below a 97% cut-off value were applied for delineation of novel species.

Table 3.8 Sequence analysis of the culture-dependent actinomycetes isolated from the freshwater macroalgae *Nostoc commune*, based on partial 16S rRNA gene sequencing

Isolates	**DDBJ Acc.no.**	**No.of base pairs sequenced**	**Most closely related hit**	**Accession NO.**	**Sequence similarity (%)**
NTS9	AB920566	1443	*Rhodococcus corynebacterioides* DSM 20151(T)	AF430066	99.10
NTS6	AB920567	1397	*Arthrobacter alpinus* S6-3(T)	GQ227413	98.93
NTS8	AB920568	1384	*Arthrobacter alpinus* S6-3(T)	GQ227413	98.99
NTS12	AB920569	1431	*Rhodococcus kyotonensis* DS472(T)	AB269261	98.25
NTS13	AB920570	1436	*Gordonia terrae* NBRC 100016(T)	BAFD0100 0032	99.72
NTS14	AB920571	1443	*Sanguibacter inulinus* ST50(T)	X79451	99.51
NTS10	AB920572	1393	*Streptomyces enissocaesilis* NRRL B-16365(T)	DQ026641	99.28
NTG2	AB920573	1279	*Streptomyces tendae* ATCC 19812(T)	D63873	95.78
NTG3	AB920574	1311	*Streptomyces toxytricini* NBRC 12823(T)	AB184173	94.56
NTG4	AB920575	1310	*Streptomyces parvulus* NBRC 13193(T)	AB184326	96.78
NTG5	AB920576	1323	*Streptomyces tendae* ATCC 19812(T)	D63873	94.02
NTGn4	AB920577	1309	*Streptomyces catenulae* ISP 5258(T)	AY999778	95.80
NTGn11	AB920578	1339	*Streptomyces parvulus* NBRC 13193(T)	AB184326	95.17
NTHn1	AB920579	1344	*Streptomyces tendae* ATCC 19812(T)	D63873	96.16
NTHn2	AB920580	1346	*Streptomyces tendae* ATCC 19812(T)	D63873	93.44
NTHn3	AB920581	1317	*Streptomyces parvulus* NBRC 13193(T)	AB184326	93.52
NTM1	AB920582	1310	*Streptomyces parvulus* NBRC 13193(T)	AB184326	93.64
NTM2	AB920583	1330	*Streptomyces tendae* ATCC 19812(T)	D63873	94.61
NTM3	AB920584	1310	*Streptomyces parvulus* NBRC 13193(T)	AB184326	97.02
NTMn4	AB920585	1311	*Streptomyces tendae* ATCC 19812(T)	D63873	96.41
NTRH1	AB920586	1324	*Streptomyces tendae* ATCC 19812(T)	D63873	94.78
NTRH2	AB920587	1310	*Streptomyces eurocidicus* NRRL B-1676(T)	AY999790	93.79
NTRH3	AB920588	1287	*Streptomyces griseocarneus* DSM 40004(T)	X99943	90.95
NTRH4	AB920589	1324	*Streptomyces parvulus* NBRC 13193(T)	AB184326	96.96
NTRH10	AB920590	1316	*Streptomyces tendae* ATCC 19812(T)	D63873	94.6
NTRH11	AB920591	1322	*Streptomyces parvulus* NBRC 13193(T)	AB184326	97.88
NTRHn1	AB920592	1322	*Streptomyces bungoensis* NBRC 15711(T)	AB184696	94.45
NTRHn3	AB920593	1350	*Streptomyces orinoci* NBRC 13566(T)	AB184866	92.35
NTRHn5	AB920594	1325	*Streptomyces eurocidicus* NRRL B-1676(T)	AY999790	92.55
NTRHn8	AB920595	1315	*Streptomyces griseoflavus* LMG 19344(T)	AJ781322	96.80

Table 3.8 (continued)

Isolates	**DDBJ Acc.no.**	**No.of base pairs sequenced**	**Most closely related hit**	**Accession NO.**	**Sequence similarity (%)**
NTRHn14	AB920596	1319	*Streptomyces tendae* ATCC 19812(T)	D63873	94.15
NTRHn16	AB920597	1308	*Streptomyces albidoflavus* DSM 40455(T)	Z76676	94.38
NTRHn17	AB920598	1327	*Streptomyces parvulus* NBRC 13193(T)	AB184326	93.56
NTS2	AB920599	1321	*Streptomyces parvulus* NBRC 13193(T)	AB184326	93.78
NTS4	AB920600	1302	*Streptomyces albidoflavus* DSM 40455(T)	Z76676	95.38
NTSn12	AB920601	1311	*Streptomyces hydrogenans* NBRC 13475(T)	AB184868	95.95
NTMn5	AB920602	1347	*Streptomyces parvulus* NBRC 13193(T)	AB184326	92.21
NTRHn4	AB920603	1379	*Actinomadura namibiensis* DSM 44197(T)	AJ420134	91.45
NTRHn18	AB920604	1363	*Streptomyces roseolus* NBRC 12816(T)	AB184168	94.36
NTS3	AB920605	1334	*Streptomyces violascens* ISP 5183(T)	AY999737	89.79
NTSn9	AB920606	1305	*Streptomyces pharetrae* CZA14(T)	AY699792	88.23
NTSn10	AB920607	1350	*Streptomyces djakartensis* NBRC 15409(T)	AB184657	88.83
NTSn11	AB920608	1391	*Streptomyces djakartensis* NBRC 15409(T)	AB184657	87.46

Thirty isolates were obtained from *Nostochopsis* spp. Based on visible examination of the growth characteristics, 26 representative isolates were selected for 16S rRNA genes sequence analysis. BLAST results indicated that the cultivable actinomycetes in *Nostochopsis* spp. were clustered with members of *Streptomyces*, *Pseudonocardia*, *Williamsia*, *Nocardiopsis* and *Nocardia* (Table 3.9). The majority of the isolates (19/26, 73%) belonged to *Streptomyces*. The remaining isolates were members of the genus *Pseudonocardia* (4), *Williamsia* (1), *Nocardiopsis* (1) and *Nocardia* (1). Four isolates of *Pseudonocardia* were closely related to *Pseudonocardia carboxydivorans* Y8$^{(T)}$ (2), *Pseudonocardia croxyli* D10$^{(T)}$ and *Pseudonocardia ammonioxydans* H9$^{(T)}$. One isolate each was closely related to *Williamsia phyllosphaerae* C7$^{(T)}$, *Nocardiopsis flavescens* SA6$^{(T)}$ and *Nocardia grenadensis* GW5-5797$^{(T)}$. All isolates revealed a 73-96% similarity rate with their closest type strains, while values well below a 97% cut-off value were applied for delineation of novel species.

Table 3.9 Sequence analysis of the culture-dependent actinomycetes isolated from the freshwater macroalgae *Nostochopsis* spp., based on partial 16S rRNA gene sequencing

Isolates	**No.of base pairs sequenced**	**Most closely related hit**	**Accession NO.**	**Sequence similarity (%)**
NCS1	1449	*Williamsia phyllosphaerae* C7$^{(T)}$	FR691321	87.30
NCS5	1313	*Pseudonocardia oroxyli* D10$^{(T)}$	DQ343154	91.19
NCSn1	1351	*Streptomyces coelicoflavus*NBRC15399$^{(T)}$	AB184650	89.51
NCRHn1	1426	*Nocardia grenadensis* GW5-5797$^{(T)}$	FR729900	90.93
NCRHn3	1368	*Streptomyces parvulus* NBRC 13193$^{(T)}$	AB184326	90.81
NCRHn5	1295	*Streptomyces puniceus* NBRC 12811$^{(T)}$	AB184163	95.90
NCRHn6	1389	*Nocardiopsis flavescens* SA6$^{(T)}$	GU997639	95.56
NCRHn7	1674	*Streptomyces scopuliridis* RB72$^{(T)}$	EF657884	72.50
NCRHn8	1362	*Streptomyces hydrogenans* NBRC13475$^{(T)}$	AB184868	91.95
NCRHn9	1311	*Streptomyces laurentii* LMG19959$^{(T)}$	AJ781342	89.28
NCRH1	1385	*Streptomyces globisporus* NBRC12867$^{(T)}$	AB184203	92.27
NCRH5	1348	*Streptomyces laculatispora* BK166$^{(T)}$	FR692106	91.50
NCRH6	1413	*Streptomyces pratensis* ch24$^{(T)}$	JQ806215	91.04
NCMn1	1335	*Pseudonocardia carboxydivorans* Y8$^{(T)}$	EF114314	93.12
NCMn3	1317	*Streptomyces sundarbansensis* MS1/7$^{(T)}$	AY550275	83.09
NCMn7	1283	*Streptomyces atrovirens*NRRL B-16357$^{(T)}$	DQ026672	80.89
NCM1	1411	*Streptomyces coelicoflavus*NBRC15399$^{(T)}$	AB184650	90.89
NCM3	163	*Streptomyces leeuwenhoekii* C34$^{(T)}$	AJ621602	96.32
NCM4	1353	*Streptomyces hydrogenans* NBRC13475$^{(T)}$	AB184868	89.96
NCH1	1282	*Streptomyces ambofaciens* ATOC23877$^{(T)}$	M27245	79.62
NCH3	1350	*Streptomyces parvus* NBRC3388$^{(T)}$	AB184756	93.89
NCGn1	1308	*Pseudonocardia ammonioxydans* H9$^{(T)}$	AY500143	88.97
NCGn3	1333	*Streptomyces gramineus* JR-43$^{(T)}$	HM748598	88.77
NCGn4	1040	*Pseudonocardia carboxydivorans* Y8$^{(T)}$	EF114314	83.63
NCGn7	133	*Streptomyces caeruleatus* GIMN4$^{(T)}$	GQ329712	76.92
NCG4	1350	*Streptomyces parvulus* NBRC 13193$^{(T)}$	AB184326	94.88